Impaired Follistatin Secretion in Cirrhosis

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Context: Follistatin is a liver-derived inhibitor of the muscle-growth inhibitor myostatin. Reduction in acute follistatin release may help explain muscle loss in liver cirrhosis.

Objective: The study aimed to investigate the capacity of acute follistatin release in patients with liver cirrhosis compared to healthy control participants.

Design, Setting, and Participants: To experimentally increase the glucagon-insulin ratio (mimicking the hormonal effect of exercise), we infused glucagon/somatostatin (to inhibit insulin secretion) and compared the acute follistatin increase in eight male cirrhosis patients with eight healthy control participants. Patients and controls received 1-hour glucagon/somatostatin and saline infusions on 2 separate days.

Main Outcome Measure: Follistatin was measured during and 5 hours after termination of infusions.

Results: The peak follistatin change was significantly decreased in patients with liver cirrhosis compared to healthy control participants (1.9 (interquartile range, 1.4–2.5) versus 3.6 (interquartile range, 3.0–4.0), respectively; $P = .003$). Patients with liver cirrhosis demonstrated significantly decreased amounts of appendicular lean mass compared to healthy controls (27.6 $\pm$ 3.8 vs 34.5 $\pm$ 2.9%, respectively; $P = .001$).

Conclusions: Patients with cirrhosis show impaired capacity to acutely secrete follistatin. The decrease in acute follistatin release may contribute to the loss of muscle mass in liver cirrhosis. (***J Clin Endocrinol Metab*** **101: 3395–3400, 2016)**

The protein follistatin exerts important functions with regard to muscle growth and differentiation. Follistatin knockout mice present with decreased muscle mass and die shortly after birth (1). Conversely, a muscle-specific overexpression of follistatin induces muscle hypertrophy (2, 3). Follistatin is a negative regulator of the muscle-growth inhibitor myostatin (2, 4) and other muscle regulators of the TGF-β family (5). Follistatin binds to myostatin and thereby inhibits myostatin effects (6). Follistatin is secreted by hepatocytes (7), the expression of follistatin mRNA in the liver increases with exercise (8), and the splanchnic bed releases follistatin (9), all suggesting a link between liver function and muscle growth.

Cirrhosis is accompanied by muscle loss, which is associated with morbidity and mortality (10, 11). The rodent cirrhosis model is associated with increased muscle myostatin expression, decreased muscle synthesis, and muscle atrophy (12, 13). Likewise, patients with liver cirrhosis exhibit increased plasma levels of myostatin (14) and increased muscle myostatin protein expression (15).

Two previous cross-sectional studies show elevated baseline levels of plasma follistatin in patients with liver

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Abbreviation: CI, confidence interval.

cirrhosis and other chronic and inflammatory diseases compared to healthy controls (16, 17), but results are conflicting, and not all studies present increased baseline follistatin levels in cirrhosis (18). The effect of chronic follistatin elevation is not clear, but acute follistatin injections inhibit myostatin function and increase muscle protein synthesis in rats (12, 19). In the rodent cirrhosis model, the inhibitory effect of myostatin is abolished by injections of follistatin, resulting in normalization of skeletal muscle synthetic rate (12).

During exercise, follistatin is produced by the liver, and plasma follistatin levels increase significantly (8). Glucagon levels increase (20) and insulin decreases (21) during exercise, resulting in an increased glucagon-insulin ratio. The in vitro production of follistatin by hepatocytes is partly mediated by glucagon stimulation (7), and circulating follistatin in humans is regulated by the glucagon-insulin ratio (9). In healthy subjects, exercise increases muscle protein synthesis (22). The increased synthesis may depend on a sufficient increase in follistatin. A reduced follistatin release in patients with liver cirrhosis may represent a mechanism linking cirrhosis with muscle loss.

We hypothesized that patients with cirrhosis have an impaired ability to acutely increase follistatin secretion. In order to mimic the hormonal changes of exercise, we applied a setup with an increase in the glucagon-insulin ratio by infusing glucagon and somatostatin (to inhibit insulin secretion) and saline, thereby testing the capacity of acute plasma follistatin increase in patients with liver cirrhosis compared to healthy control participants.

Subjects and Methods

Participants

The study included eight male patients with liver cirrhosis and eight healthy male controls. Patients were recruited from the outpatient clinic at the Gastrounit, Hvidovre Hospital, and healthy control participants were recruited via a local newspaper. We included patients irrespective of the etiology of the underlying liver disease. Exclusion criteria were: lack of informed consent, Child-Pugh C cirrhosis, grade 3 ascites, overt hepatic encephalopathy, malignant disease, clinically significant heart disease, renal disease, diabetes, ongoing infections, ongoing alcohol abuse, and ongoing drug abuse. All participants underwent a clinical examination including electrocardiography, blood samples, and a dual-energy x-ray absorptiometry scan.

Infusions

All participants underwent 2 test days (days A and B) separated by at least 1 week. On both days, participants reported to the laboratory at 8 AM after an overnight fast. The order of the test days was randomly assigned. Medication was paused before the test. On test day A, participants rested in the supine position while having two catheters placed in antecubital veins on both arms. After initial blood samples were collected, an infusion consisting of glucagon (6.0 ng/kg/min) (GlucaGen; Novo Nordisk) and somatostatin (100 ng/kg/min) (Octreotide; Hospira) was commenced and continued for 1 hour. On test day B, participants rested in the supine position while having two catheters placed in the antecubital veins on both arms. After initial blood samples were collected, infusion of saline was commenced and continued for 1 hour. On both test days, blood samples were collected as follows. For the measurement of plasma glucose, blood samples were collected every 15 minutes during the first 2 hours and then every 30 minutes for 4 hours. For measurement of glucagon, insulin, and follistatin, blood samples were collected every 30 minutes during the first 2 hours then every 60 minutes for 4 hours.

Blood samples and measurements

Blood samples for glucose measurement were collected into Pico syringes (Radiometer Medical) and measured bedside using an ABL 800 flex (Radiometer). Blood samples for glucagon were obtained in tubes containing aprotinin, and samples for insulin and follistatin were obtained in tubes containing EDTA. All blood samples were immediately spun at 4°C at 3500 *g* for 15 minutes, and the plasma fractions were stored at −80°C until analysis. Glucagon was measured using a RIA kit (Millipore). Insulin was measured using a Mercodia Insulin ELISA (Mercodia), and plasma follistatin was measured by ELISA (R&D systems).

Ethical approval

The study was approved by the local Ethical Committee of the capital region of Denmark (H-4–2012-162). The study was executed in accordance with the Declaration of Helsinki. All participants received oral and written information about the experimental procedures before providing their written informed consent.

Statistics

All analyses were conducted in STATA, version 14 (StataCorp) and Graphpad Prism, version 6 (GraphPad Software, Inc).

Quantitative data were summarized using medians and interquartile range, and range and frequencies using proportions. Based on the distribution of data, both nonparametric and parametric analyses were applied. Dynamic changes in levels of glucose, glucagon, insulin, and follistatin were calculated using repeated measures ANOVA calculating the area under the curve based on geometric mean values. Parametric analyses were conducted with and without log transformation. Both analyses reached the same conclusion.

The peak increase of follistatin levels during glucagon/somatostatin and saline infusions was calculated. Groups were compared using the Wilcoxon rank sum test, and the correlation between peak follistatin and lean body mass was assessed using the Spearman correlation coefficient. Two-sided *P* values were used.

Results

As shown in Table 1, three patients had Child-Pugh A (37%), and five patients had Child-Pugh B (63%). The

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Table 1. Participant Characteristics

	Healthy Controls	Patients With Liver Cirrhosis	*P*
n	8	8	
Age, y	52.8 ± 5.1	56.0 ± 7.3	.320
Weight, kg	83.6 ± 7.6	74.9 ± 17.5	.231
Height, cm	178.9 ± 7.6	172.8 ± 7.5	.130
Body mass index, kg/m^2	26.1 ± 2.1	25.0 ± 4.9	.551
Appendicular lean mass, %	34.5 ± 2.9	27.6 ± 3.8	.001
Total fat mass, %	23.5 ± 5.5	29.4 ± 8.6	.508
ALT, U/L	25.9 ± 8.7	18.6 ± 7.7	.099
Bilirubin, μmol/L	11.3 ± 5.8	16.4 ± 10.9	.266
Creatinine, μmol/L	84.0 ± 7.1	92.5 ± 41.7	.587
INR	1.1 ± 0.1	1.3 ± 0.3	.186
Albumin, g/L	37.4 ± 1.7	33.0 ± 2.9	.003
Child-Pugh score (A/B)		3/5	

Abbreviations: ALT, Alanine transaminase; INR, international normalized ratio. Data are presented as mean ± SD.

etiology was alcohol for all participants. The groups showed no difference in mean age, height, weight, body mass index, or total fat mass. Participants with cirrhosis had lower appendicular lean mass ($P = .001$; Table 1). Baseline levels of glucose and follistatin were not different between groups (Table 2). Glucagon levels were slightly higher in the cirrhosis group, but the difference did not reach statistical significance. Insulin levels were significantly higher in the cirrhosis group compared to healthy control participants (Table 2).

Infusion of glucagon/somatostatin resulted in a significant increase in plasma glucose in both healthy control participants (5.4 mmol/L [95% confidence interval (CI), 5.1 to 5.7] to 11.7 mmol/L [95% CI, 10.8 to 12.5]) and cirrhosis patients (5.6 mmol/L [95% CI, 5.1 to 6.2] to 8.1 mmol/L [95% CI, 6.4 to 9.9]). No changes in plasma glucose were seen after saline infusion in either group (Figure 1, A and B).

Glucagon levels increased significantly in both healthy controls and liver cirrhosis patients during glucagon/somatostatin infusion (19.4 pmol/L [95% CI, 15.9 to 23.0] to 99.6 pmol/L [95% CI, 94.7 to 105.1] and 32.8 pmol/L [95% CI, 16.4 to 49.2] to 106.3 pmol/L [95% CI, 93.5 to 119.1], respectively). Saline infusion did not change glucagon levels in either group (Figure 1, C and D). After termination of glucagon/somatostatin infusion, glucagon levels decreased to levels that were lower than baseline in both groups (6.4 pmol/L [95% CI, 2.0 to 10.7] and 22.0 pmol/L [95% CI, 11.2 to 32.7], respectively).

Insulin levels decreased during glucagon/somatostatin infusion in both groups (40.9 pmol/L [95% CI, 14.2 to 67.7] to 5.7 pmol/L [95% CI, 2.6 to 8.8] and 97.8 pmol/L [95% CI, 61.9 to 134.4] to 12.7 pmol/L [95% CI, 0.1 to 25.3], respectively). During saline infusion, insulin levels showed a slight decline during the 360 minutes (Figure 1, E and F). During the last 30 minutes of the glucagon/somatostatin infusion, insulin levels increased in both groups despite continuous infusion. From 60 to 360 minutes, insulin levels continued to decrease slightly.

Glucagon/somatostatin infusion caused a significant increase in plasma follistatin levels in healthy control participants (1600 pg/mL [95% CI, 1257 to 1945] to 5531 pg/mL [95% CI, 4533 to 6529]) and cirrhosis patients (2149 pg/mL [95% CI, 1575 to 2722] to 4043 pg/mL [95% CI, 2512 to 5576]). In both groups, the increase in follistatin peaked 120 minutes after termination of the glucagon/somatostatin infusion (Figure 1, G and H). After 360 minutes, follistatin concentration had almost returned to baseline levels in both groups. The peak follistatin change in the healthy control participants was significantly higher than in the cirrhosis group ($P = .003$; Figure 2). There was a significant positive correlation between peak follistatin change and appendicular lean mass when including all participants ($P = .044$; $r = 0.51$; Figure 3), but not when adjusting for group (cirrhosis patients and healthy controls).

Discussion

Here we show that the acute plasma follistatin induction upon an increase in the glucagon-insulin ratio is significantly decreased in patients with liver cirrhosis compared to healthy control participants. Moreover, individuals with the lowest peak follistatin change had the lowest amount of lean body mass. Given that follistatin is a potent regulator of muscle mass, our findings suggest a liver-mus-

Table 2. Baseline Levels of Glucose, Glucagon, Insulin, and Follistatin

	Healthy Controls	Patients With Liver Cirrhosis	*P*
Glucose, mmol/L	5.4 (5.1 to 5.7)	5,6 (5.1 to 6.2)	.67
Glucagon, pmol/L	19.4 (15.9 to 23.0)	32.8 (20.9 to 52.4)	.09
Insulin, pmol/L	40.9 (14.2 to 67.7)	97.8 (61.9 to 134.4)	.02
Follistatin, pg/mL	1600 (1257 to 1945)	2149 (1575 to 2722)	.12

Data are expressed as geometric mean (95% CI).

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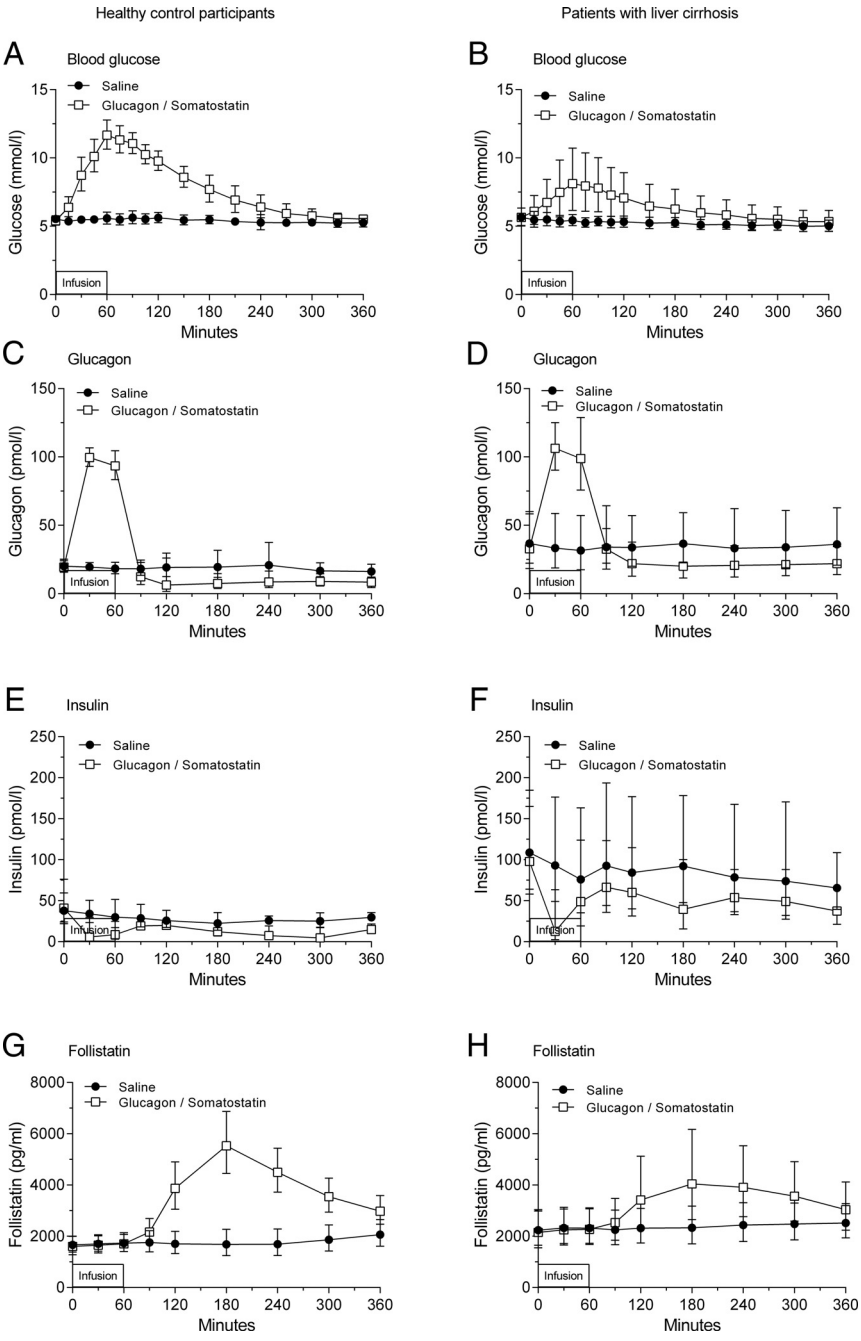

Figure 1. Blood glucose (A and B), glucagon (C and D), insulin (E and F), and follistatin (G and H) levels before, during, and after glucagon/somatostatin and saline infusion in healthy control participants and patients with liver cirrhosis. Data are presented as geometric mean with 95% CI. ●, Saline infusion; □, glucagon/somatostatin infusion. A significant difference is shown between the increase in blood glucose, glucagon, and follistatin levels and the decrease in insulin levels during glucagon/somatostatin and saline infusion in both groups.

cle interaction that may help to explain cirrhosis-associated muscle loss.

Exercise increases plasma follistatin levels (8). Follistatin mRNA is expressed in both muscle and liver tissue (23). The expression of follistatin mRNA does not increase in muscle tissue after exercise (8, 24) but is increased at mRNA and protein level in the liver (8). The concept of follistatin as a liver-secreted protein is supported by arterial-venous differences over the splanchnic bed in exercising male subjects (9), whereas follistatin is not released by the exercising leg (8).

Follistatin is able to form complexes with and antagonize the effect of the muscle growth inhibitor myostatin, and follistatin serves as an endogenous antagonist for myostatin (6). However, overexpression of follistatin results in muscle growth that exceeds the hypertrophy seen in myostatin knockout models, indicating a more extensive effect of follistatin as a muscle growth regulator (2). In the liver, follistatin attenuates hepatic fibrosis by inhibiting the TGF-β family member activin (25). Production of follistatin in the muscle overlaps with activin and may take part in mediating muscle growth and development (26). These results emphasizes regulatory effects of follistatin on other TGF-β members with regard to muscle growth (26).

The liver is important with regard to growth and development (27). Production of follistatin in the hepatocytes is stimulated by glucagon (7). Exercise causes glucagon release (20) and a decrease in insulin (21) and thereby a change in the glucagon-insulin ratio. When mimicking the hormonal changes seen during exercise, we found a reduced capacity of acute follistatin release in patients with cirrhosis compared to healthy control participants. The result may reflect a decrease in production or a consequence of portosystemic shunting. Accordingly, in the rodent portacaval anastomosis cirrhosis model, myostatin expression is increased and muscle protein synthesis is decreased, and the changes are reversed by follistatin injections (12).

Previous cross-sectional studies found that resting follistatin levels are either increased in cirrhosis (16, 17) or similar in patients with cirrhosis and healthy controls (18). We found no difference in resting follistatin levels between groups. The discrepancy may reflect differences in partic-

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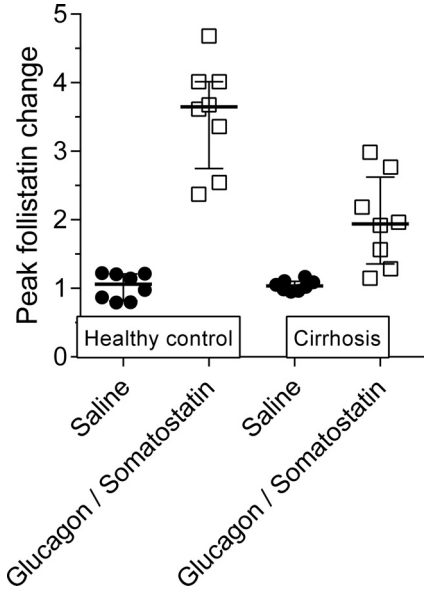

Figure 2. Peak follistatin changes at 120 minutes after termination of glucagon/somatostatin and saline infusions. Data are presented as median mean with interquartile range. ●, Saline infusion; □, glucagon/somatostatin infusion. Peak follistatin change is significantly higher in healthy control participants than patients with liver cirrhosis after glucagon/somatostatin infusion. $P = 0.003$.

ipant characteristics between studies. One study included participants with relatively high Child-Pugh scores (17). The study did not consider the renal status (17), which may be important because renal disease contributes to the elevation of follistatin (16), but the studies are relatively small, and the lack of differences between cirrhosis patients and controls may reflect low statistical power.

Given the link between follistatin and muscle growth, chronically elevated plasma follistatin levels would expectedly correlate with muscle hypertrophy. However, previous studies show no relation between resting levels of follistatin and stages of muscle loss (28, 29). This is in accordance with the present study, where resting levels of follistatin did not correlate with lean mass as measured by dual-energy x-ray absorptiometry scan (results not shown). Chronic levels of plasma follistatin and acute increases in plasma follistatin may signify two different situations, emphasized by the correlation between acute follistatin changes and lean mass. We hypothesize that acute elevation of plasma follistatin as observed after exercise may take part in the regulation of myostatin and other TGF-β members, and that repeated bouts of increased plasma follistatin exert positive effects on muscle synthesis and growth. The positive effect of circulating follistatin (an endogenous myostatin inhibitor) on muscle growth is supported by a study using injections of a myostatin antibody, presenting an increase in muscle mass and an increase in physical performance in humans (30).

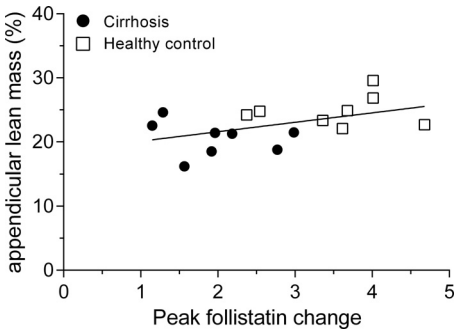

Figure 3. Correlation between peak follistatin change and appendicular lean mass (%). ●, Patients with liver cirrhosis; □, healthy control participants. Significant correlation between peak follistatin change and appendicular lean mass (%), $P = .044$; $r = 0.51$.

Several factors may affect follistatin release in liver cirrhosis. Most patients with cirrhosis are glucose intolerant, with hyperinsulinemia and insulin resistance, and 30% develop diabetes (31, 32). Hyperglucagonemia is also common (33). Infusion of glucagon results in a smaller increase in insulin in cirrhosis patients than healthy controls. The initial response is characterized by a reduced hepatic glucose production as reflected by diminished glycogenolysis. The changes are likely to reflect glucagon resistance in cirrhosis (34). During glucagon/octreotide infusion, patients with cirrhosis show decreased glucose production compared to healthy controls, due to decreased glycogenolysis rather than gluconeogenesis (35). Decreased glucose production may be associated with impaired glucagon sensitivity or decreased glycogen stores. The decrease in glucose production is similar before and after glycogen repletion, emphasizing impaired hepatic glucagon sensitivity as an important potential mechanism (35). Given a connection between liver-secreted follistatin and muscle synthesis, the possible reasons for decreased follistatin release need further examination. It is important to note that regardless of the explanation, we show that the same relative stimulus results in different acute follistatin release in patients with liver cirrhosis and healthy control participants.

In summary, we show decreased acute follistatin release during glucagon/somatostatin infusions in patients with liver cirrhosis compared to healthy controls. The inhibitory effect of follistatin on myostatin and other TGF-β members may be diminished in liver cirrhosis, and this may help to explain the loss of muscle mass experienced by these patients.

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