

Exercise alters myostatin protein expression in sedentary and exercised streptozotocin-diabetic rats

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ABSTRACT

Objective: The aim of this study was to analyze the effect of exercise on the pattern of muscle myostatin (MSTN) protein expression in two important metabolic disorders, *i.e.*, obesity and *diabetes mellitus*. **Materials and methods:** MSTN, is a negative regulator of skeletal muscle mass. We evaluated the effect of exercise on MSTN protein expression in diabetes mellitus and high fat diet-induced obesity. MSTN protein expression in gastrocnemius muscle was analyzed by Western Blot. $P < 0.05$ was assumed. Exercise induced a significant decrease in glycemia in both diabetic and obese animals. **Results:** The expression of precursor and processed protein forms of MSTN and the weight of gastrocnemius muscle did not vary in sedentary or exercised obese animals. Diabetes reduced gastrocnemius muscle weight in sedentary animals. However, gastrocnemius muscle weight increased in diabetic exercised animals. Both the precursor and processed forms of muscle MSTN protein were significantly higher in sedentary diabetic rats than in control rats. The precursor form was significantly lower in diabetic exercised animals than in diabetic sedentary animals. However, the processed form did not change. **Conclusion:** These results demonstrate that exercise can modulate the muscle expression of MSTN protein in diabetic rats and suggest that MSTN may be involved in energy homeostasis. Arch Endocrinol Metab. 2015;59(2):148-53

Keywords

Myostatin; exercise; diabetes; obesity; western blot

INTRODUCTION

Myostatin (MSTN) or growth and differentiation factor 8 (GDF8) is a member of the transforming growth factor- β (TGF β) superfamily and has been characterized as a negative regulator of skeletal muscle mass in different (1). It is expressed mainly in muscle but also in adipose tissue (2). Following synthesis as a precursor protein, MSTN is processed by proteolysis by the furin family enzymes, with removal of the 24-amino acid signal peptide and generation of an N-terminal propeptide domain and a C-terminal domain. These peptides dimerize through disulfide bonds (latent MSTN) and are then cleaved again by members of the BMP-1/tolloid family of metalloproteinases. This second cleavage results in MSTN propeptide and mature MSTN. The mature MSTN then binds to its cell surface receptors, activin receptor type II or IIB, and activates intracellular members of the SMAD family of signaling proteins, which then translocate to the nucleus and regulate transcription

of specific genes in association with diverse transcription factors (3,4).

In addition to the roles of MSTN in skeletal muscle growth, both *in vivo* and *in vitro* studies have suggested that MSTN also regulates metabolism by effects on muscle as well as on other metabolic sites such as adipose tissue (5-10). Introduction of MSTN null mutation into different obese strains or deletion of MSTN in mice results in skeletal muscle hypertrophy and, in contrast, reduction in fat accumulation and prevention of insulin resistance (2-5,7,8,10-13). In addition, MSTN gene expression in muscle was upregulated and attenuated by insulin in streptozotocin-induced type 1 diabetic mice (14).

There is also evidence that MSTN is regulated by physical exercise (15-18) which is linked to improved glucose homeostasis and enhanced insulin sensitivity.

In the present study, we evaluated the protein expression of MSTN in two animal models of metabolic diseases, diet-induced obesity and streptozotocin-induced diabetes, in sedentary and exercised rats.

MATERIAL AND METHODS

Experimental models

All procedures were approved by the Ethics Committee of the Federal University of São Carlos (UFSCar, 035/2007). Thirty days old male *Wistar* rats, were initially acclimated in individual polypropylene cages (30 cm×20 cm×13 cm) under controlled temperature, humidity and lighting (12-h dark/light cycle), with free access to water and standard rodent chow (PRIMOR – São Paulo, Brazil) for 7 days.

Streptozotocin-induced diabetes

After 7 days of acclimation, animals (156.7 ± 3.7 g) were randomly assigned to 2 groups, control rats (C) (n = 12) and diabetic rats (D) (n = 12). Diabetic rats received streptozotocin (STZ, Sigma-Aldrich, St. Louis, USA, 60 mg/kg, intraperitoneally) and control rats received vehicle (sodium citrate, intraperitoneally). Seven days after diabetes induction, control (C) and diabetic (D) animals were randomly assigned to 2 groups: sedentary control (SC) (n = 5) or exercised control (EC) (n = 6) and sedentary diabetic (SD) (n = 6) or exercised diabetic (ED) (n = 6). Glycemia was determined in tail blood using a portable glucometer (Accu-Check glucose meter (Roche Diagnostic, Indianapolis, USA). Soon after diabetes induction, all diabetic animals received 1 U of insulin Lantus® (Sanofi Aventis, Deutschland) intraperitoneally on alternate days to avoid very high glycemia.

Diet-induced obesity

After 7 days of acclimation, animals (190 ± 18.4 g) were randomly assigned to 2 groups, control rats (CG) (n = 12) fed standard rodent chow and rats fed a high-fat diet (HF) (n = 12) containing 60% of kcal as fat (Research Diets, New Brunswick, NJ, USA). Animals and food were weighed daily. After 12 weeks, animals of both groups were randomly divided into 2 groups: sedentary control (SC) (n = 5) or exercised control (EC) (n = 6) and sedentary high-fat diet (SHF) (n = 5) or exercised HF (EHF) (n = 6).

The glucose tolerance test (GTT) was performed before and after exercise training. After an overnight fast, unanesthetized rats were injected intraperitoneally with 1.5 of a 50% glucose solution per kg body weight (BW). Blood samples were obtained from the tail vein before injection and at 30, 60, 90, and 120 min after glucose injection. The insulin tolerance test

(ITT) was also performed before exercise training. After an overnight fast, unanesthetized rats were injected intraperitoneally with human insulin, 1 U/kg BW. Blood samples were obtained from the tail vein before injection and at 15, 30, 60, and 90 min after the insulin challenge. Blood glucose concentrations were measured with an Accu-Check glucose meter (Roche Diagnostic, Indianapolis, USA).

Exercise protocol

In both models, rats in the exercise groups were exercised by swimming for 1.5 h/day, 45 min at 9:00 AM and 45 min at 5:00 PM, for 4 weeks. The swimming exercise was performed in plastic barrels (30 cm in diameter) filled with water (50 cm deep) maintained at 32–36°C and with a weight corresponding to 5% BW attached to the tail of each rat. The protocol started with the animals swimming 30 min on the first day, with a gradual increase so that on the fourth day they could swim for 90 minutes. All animals were towel dried before being returned to their cages. The sedentary groups were placed in shallow water for 5 minutes, towel dried and then returned to their cages at the same time as the exercised groups.

After the training period, rats were sacrificed by decapitation. White gastrocnemius muscle was dissected, weighed, immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

Western blotting analysis

MSTN protein levels in gastrocnemius muscle were analyzed by Western Blot according to a method previously described (15). Muscle (50 mg) protein was extracted using Tris-Triton buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate) and the protein content of the supernatant was quantified by the BCA method (BCA™ Protein Assay Kit, Thermo Scientific, USA). Samples (250 µg of total protein) were separated on 12% polyacrylamide gel and then transferred to a nitrocellulose membrane. Coomassie Blue staining of the gels confirmed optimal protein transfer. Nitrocellulose membranes were incubated overnight at 4°C with antimyostatin primary antibodies (GDF-8 N-19-R sc-6885-R, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200 with Tris buffered saline with Tween added (TBST). Membranes were then washed with TBST and incubated with a

peroxidase-conjugated secondary antibody (goat anti-rabbit IgG-HRP: sc-2004). Protein levels were normalized to those of actin (I-19 – sc 1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL) Plus Western Blotting Detection Reagents (GE Healthcare Chalfont St. Giles, UK) was used to detect bound antibody.

Data analysis

Data were analyzed statistically by analysis of variance (ANOVA). Tukey's multiple comparisons test was used for post hoc analysis of between-group comparisons. Data are reported as means \pm SEM. P values < 0.05 were considered to be statistically significant.

RESULTS

STZ-induced diabetes

By the third week of the experiment, the BW of SD animals was significantly lower than that of SC animals ($P < 0.05$) and this difference was maintained till the end of the experiment. There was no difference in BW between sedentary and exercised animals (data not shown). Before training, diabetic rats had significantly higher glycemia than control rats ($P < 0.05$). After training, ED animals had significantly lower glycemia than SD animals ($P < 0.05$). No difference was observed between sedentary and exercised control animals after training (Figure 1).

Diabetes induced a significant decrease of gastrocnemius muscle weight in sedentary diabetic animals compared to sedentary control animals ($P < 0.05$). However, exercise induced an increase in gastrocne-

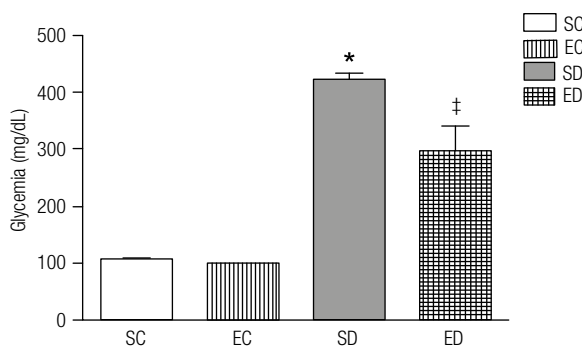


Figure 1. Glycemia of STZ-induced diabetic animals.

Glycemia (mg/dL) at the end of the training period in the sedentary control group (SC), exercised control group (EC), sedentary diabetic group (SD) and exercised diabetic group (ED). Values are means \pm SEM for $n = 5-6$ per group. $P < 0.05$, *SC vs. SD; †SD vs. ED.

mus muscle weight in diabetic animals compared to sedentary diabetic animals ($P < 0.05$) (Figure 2).

The precursor and processed forms of MSTN in gastrocnemius muscle were significantly higher in sedentary diabetic rats than in sedentary control rats ($P < 0.05$). However, the precursor form was significantly lower in diabetic exercised animals compared to diabetic sedentary animals ($P < 0.05$) and the processed form did not change in these two groups (Figures 3A and 3B).

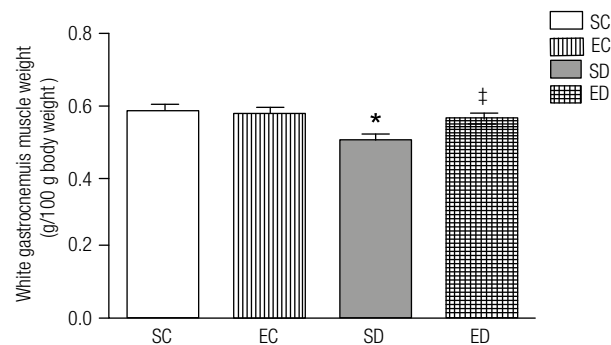


Figure 2. Muscle weight of STZ-induced diabetic animals: weight of white gastrocnemius muscle (g/100 g body weight) of the sedentary control group (SC), exercised control group (EC), sedentary diabetic group (SD) and exercised diabetic group (ED). Values are means \pm SEM for $n = 5-6$ per group. $P < 0.05$, *SC vs. SD; †SD vs. ED.

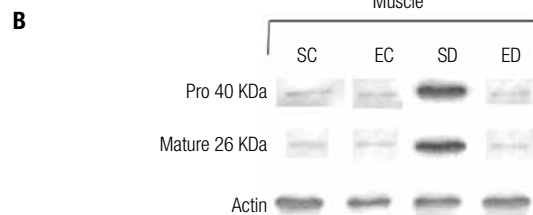
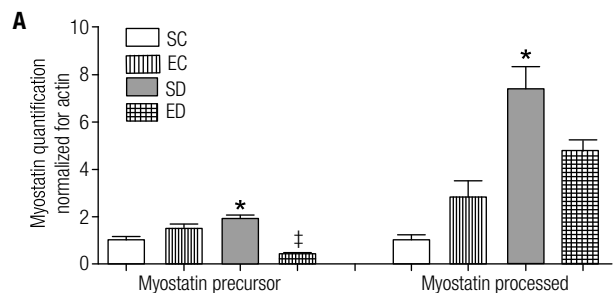


Figure 3 (A) MSTN protein expression in STZ-induced diabetic animals: expression of MSTN protein precursor (A) and processed (B) forms in white gastrocnemius muscle of the sedentary control group (SC), exercised control group (EC), sedentary diabetic group (SD) and exercised diabetic group (ED). Values are means \pm SEM for $n = 5-6$ rats per group. * $P < 0.05$, sedentary diabetic group (SD) vs. sedentary control group (SC); † $P < 0.05$, exercised diabetic group (ED) vs. sedentary diabetic group (SD). **(B)**. Representative Western blotting of MSTN protein forms in white gastrocnemius muscle of sedentary control group (SC), exercised control group (EC), sedentary diabetic group (SD) and exercised diabetic group (ED).

Diet-induced obesity

A high-fat diet induced a significant increase in BW in the rats fed a high-fat diet compared to controls ($P < 0.05$). After training, both exercised high-fat diet-fed and control animals had significantly lower body weight compared to sedentary high-fat diet-fed and control animals, respectively ($P < 0.05$) (Figure 4). There was no difference in gastrocnemius muscle weight between high-fat diet-fed and control animals, sedentary or exercised. High-fat diet induced significantly increase in mesenteric, retroperitoneal and epididymal adipose tissue mass in sedentary high-fat fed diet animals compared to sedentary control animals.

Before training, rats fed a high-fat diet showed a significantly higher glucose response to both insulin and glucose injections than control rats (Figure 5). After exercise, the glucose response to glucose injection was

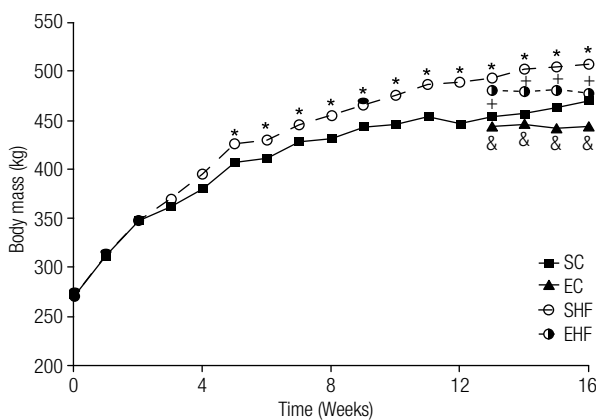


Figure 4. Body mass in animals fed a high-fat diet: body mass of the sedentary control group (SC), sedentary high-fat diet group (SHF), exercised control group (EC) and exercised high-fat diet group (EHF). Values are means \pm SEM for 5-6 rats per group. $p < 0.05$, * SC vs SHF; + SHF vs. EHF; & SHF vs. EHF.

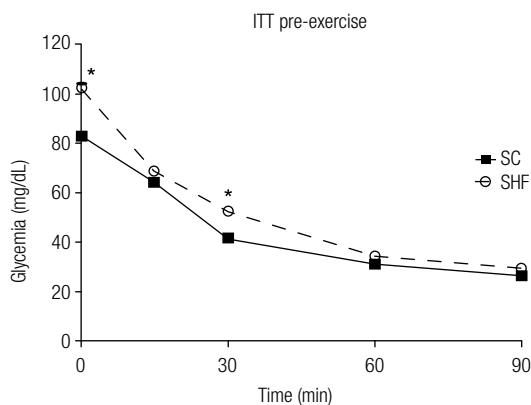


Figure 5. Insulin test tolerance in animals fed a high-fat diet (ITT) pre-exercise: sedentary control group (SC), sedentary high-fat diet group (SHF). Values are means \pm SEM for 5-6 rats per group. $p < 0.05$, * SC vs. SHF.

significantly lower than the previous test response in rats fed a high-fat diet ($P < 0.05$) (Figure 6). No difference was observed between the pre- and post-exercise glucose response to glucose injection in control rats.

No difference in the expression of MSTN protein precursor and processed forms was observed between the various groups (Figures 7A and 7B).

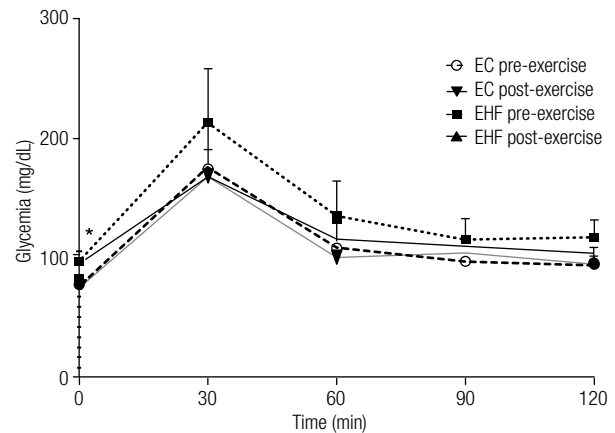


Figure 6. Glucose tolerance test in animals fed a high-fat diet: glycemia profile in the glucose tolerance test (GTT) pre- and post-exercise training. Values are means \pm SEM for $n = 5-6$ rats per group. * $P < 0.05$, exercised control group (EC) pre-exercise vs. exercised high-fat diet group (EHF) pre-exercise; & $P < 0.05$, exercised high-fat diet group (EHF) pre-exercise vs. exercised high-fat diet group (EHF) post-exercise.

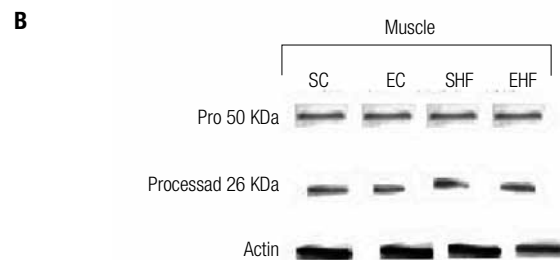
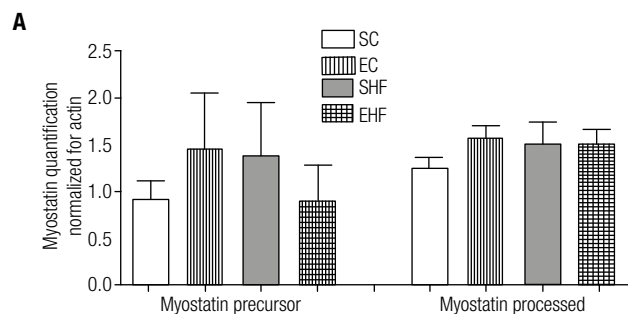


Figure 7 (A) MSTN protein expression in animals fed a high-fat diet: Expression of MSTN protein precursor (A) and processed (B) forms in white gastrocnemius muscle of the sedentary control group (SC), sedentary high-fat diet group (SHF), exercised control group (EC) and exercised high-fat diet group (EHF). Values are means \pm SEM for $n = 5-6$ rats per group. **(B)**. Representative Western blotting of MSTN protein forms in white gastrocnemius muscle in the sedentary control group (SC), sedentary high-fat diet group (SHF), exercised control group (EC) and exercised high-fat diet group (EHF).

DISCUSSION

The present results show that the expression of MSTN protein varies in muscle of both sedentary and exercised rats with streptozotocin-induced diabetes, but not in animals with high fat diet-induced obesity.

Training improved postprandial glycemia in diabetic animals and glucose tolerance in rats fed a high-fat diet. It has been demonstrated that exercise improves insulin sensitivity in diet-induced obese rats by improving insulin and AMP-activated protein kinase (AMPK) signaling pathways (19,20). In addition, there is evidence suggesting that exercise increases insulin secretion and has a protective effect in STZ-induced diabetes by decreasing oxidative stress and preserving pancreatic beta cell integrity (21-23) observed was increasing muscle mass by myostatin inhibition elevates basal metabolic rate dystrophic muscle by myostatin blockade. Thus clinical applications; for example, treatment of type II diabetics by myostatin inhibition to altering muscle mass would hypothetically result in increased whole body energy consumption, reduce peripheral fat mass and potentially lower blood glucose (24).

In STZ-induced diabetic rats, the expression of both MSTN protein precursor and mature forms was increased in skeletal muscle. The expression of MSTN protein precursor was decreased in exercised diabetic rats, but the expression of mature protein did not change. It has been previously demonstrated that MSTN mRNA expression (14,25) in gastrocnemius muscle is increased in STZ-induced diabetes, suggesting that upregulation of MSTN expression contributes to muscle atrophy in insulin deficiency. However, Barazzoni and cols., (26) have reported that MSTN mRNA expression is not altered in the lateral gastrocnemius and soleus muscles of STZ-induced diabetic rats with or without insulin replacement. The discrepancy between these studies may be due to different experimental designs.

The different profile of expression of MSTN precursor and mature forms in muscle of rats with streptozotocin-induced diabetes could possibly be explained by the modulation of proteolysis processing of MSTN. In fact, it has been demonstrated that BMP-1/metalloproteases are involved in the regulation of MSTN maturation (27-29). Recent data have shown that the increased insulin sensitivity and adipose tissue reduction observed in MSTN null mice is an indirect result of metabolic changes in skeletal muscle (6). Previous studies

have shown that MSTN may signal through Akt phosphorylation and thus affect insulin sensitivity in muscle. Previous reports suggest that myostatin protein concentrations are regulated not only at the transcriptional level but by posttranscriptional mechanisms as well (27-29). In muscle and adipose tissue from MSTN null mice and transgenic mice overexpressing MSTN propeptide, insulin signaling was increased by a higher level of Akt phosphorylation (6,30,31). One mechanism by which myostatin stimulation induces hypoglycaemia is by increasing mRNA activity of several glucose-regulating proteins, including GLUT1 and GLUT4, IL6, hexokinase and phosphorylated adenosine triphosphate kinase – AMPK, thereby increasing cellular glucose uptake (32).

In the present study, MSTN protein expression did not vary in muscle of obese rats. However, an increase of MSTN has been shown in muscle of extremely obese women (33). Although the expression of MSTN protein did not vary in the gastrocnemius muscle of control and obese animals, sedentary or exercised groups, the presence of both precursor and mature forms of MSTN in muscle suggests the occurrence of proteolysis processing of MSTN in this tissue.

We have previously shown the profile of MSTN mRNA expression in obese and exercised animals (3) which differs from the MSTN protein expression profile shown in the present study. The discrepancy observed in MSTN mRNA and protein expression has been observed in other studies and suggests that MSTN protein concentrations are regulated not only at the transcriptional level but by post-transcriptional mechanisms as well (4,34).

In conclusion, the results demonstrate that exercise can modulate the increased muscle expression of MSTN protein in diabetic rats and suggest that MSTN may participate in energy homeostasis. However, future studies are needed to elucidate the role and mechanisms of action of myostatin.

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