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# Upregulation of myostatin gene expression in streptozotocin-induced type 1 diabetes mice is attenuated by insulin

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ABSTRACT

Myostatin is a strong inhibitor of muscle growth, and its expression is increased in several types of muscle atrophy. However, whether or not myostatin expression is altered in muscle atrophy associated with type 1 diabetes (T1D) remains uncertain. In this study, we provided experimental evidence to show that myostatin mRNA increased in the early stage of T1D but came back to control levels later on. This expression pattern was closely correlated with the loss of body weight and atrogin-1 expression. Furthermore, induction of myostatin expression could be attenuated by insulin in T1D mice. Taken together, our findings indicate that the upregulation of myostatin expression most likely contributes to the muscle atrophy process during insulin deficiency.



## Introduction

Myostatin belongs to the transforming growth factor-β superfamily and plays an essential role in the regulation of skeletal muscle mass. As its name indicates, the major role of myostatin is to inhibit skeletal muscle growth. This is supported by evidence that inhibition of myostatin dramatically increases muscle mass in various animal species [1–4] and humans [5]. Conversely, administration of myostatin can induce profound muscle atrophy and a cachectic state as observed in transgenic mice overexpressing myostatin [6] or in mice transplanted with Chinese hamster ovary cells stably expressing myostatin [7]. Moreover, increased myostatin expression was observed in several types of muscle atrophy such as muscle inactivity [8–10], age [11], denervation [11–13], glucocorticoid treatment [14] and cancer cachexia [15]. However, whether myostatin expression is altered in muscle atrophy resulting from insulin deficiency remains uncertain.

In this report, the potential role of myostatin in the pathological progression of STZ-induced T1D in mice was investigated by examining its expression. We found that the level of myostatin mRNA increased in STZ-induced T1D mice and that this increased expression could be attenuated by insulin. Most interestingly, the expression pattern of myostatin was closely correlated with the severity of muscle wasting in T1D.

## Materials and methods

*Animals.* Seven-week-old C57BL/6J male mice were purchased from Beijing Vitalriver Laboratory Animal Inc. (Beijing, China). Animals were housed in a special pathogen-free environment with constant temperature at 22 °C, 50% humidity and a 12:12 h light-dark cycle in the Animal Facility of the Peking Union Medical College. All animals were fed a standard commercial chow diet and had free access to water *ad libitum*. All protocols of the animal experimental study were approved by the Animal Care Committee of Peking Union Medical College.

*Multiple low dose of STZ (mld-STZ)-induced T1D.* After one week of accommodation, mice were rendered diabetic by administration of an intraperitoneal injection of 45 mg/kg body weight STZ (Sigma, USA) for 5 days. STZ was dissolved in 0.1 M chilled sodium citrate buffer (pH 4.5) just before injection. In the control group, mice were intraperitoneally injected with an equal volume of sodium citrate buffer. Body weight and fasting plasma glucose were monitored after STZ injection. Fasting plasma glucose was measured by OneTouch Ultra Glucometer (LifeScan, USA). After onset of diabetes, mice were sacrificed by cervical dislocation. Gastrocnemius muscles from each mouse were removed and immediately frozen by liquid nitrogen. For examining myostatin expression patterns in the pathological progression of T1D, mice from both STZ and control groups were sacrificed at 1, 2, 3, 4 and 8 weeks after STZ injection. Body weight and fasting plasma glucose were measured at each time point. For the insulin therapy experiment, diabetic mice received a subcutaneous slow acting insulin (4 IU, Novolin N, Denmark) injection each day for 3 consecutive days. Five

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**Table 1**
Primer sequences for PCR.

| Gene name | Forward primer | Reverse primer |
|---|---|---|
| *For semi-quantitative RT-PCR* | | |
| Myostatin | 5′-TGTTGCAAAATTGGCTCAAA-3′ | 5′-GCACAAGATGAGTATGCGGA-3′ |
| GAPDH | 5′-GTCTTCACCACCATGGAGAAG GC-3′ | 5′-ATTCATTGTCATACCAGGAAA-3′ |
| *For real time PCR* | | |
| Myostatin | 5′-AACCTTCCCAGGACCAGGAG-3′ | 5′-CGCAGTCAAGCCCAAAGTCT-3′ |
| Atrogin-1 | 5′-AAGCTTGTGCGATGTTACCCA-3′ | 5′-CATGGATGGTCAGTGCCCTT-3′ |
| GAPDH | 5′-TGGAGAAACCTGCCAAGTATGA-3′ | 5′-CTGTTGAAGTCGCAGGAGA CA-3′ |

animals were randomly allocated in each group in all the experiments as indicated.

*High dose STZ (HD-STZ)-induced T1D.* To generate an acute T1D model, mice were intraperitoneally injected with a single high dose of STZ (180 mg/kg body weight). In the control group, mice received an equal volume of citrate buffer. Four days after STZ injection, body weight and fasting plasma glucose were determined before the mice were sacrificed. Gastrocnemius muscles were collected according to the protocol mentioned above.

*Semi-quantitative RT-PCR and real time PCR for gene expression assays.* Total RNA extraction from gastrocnemius was performed using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. The integrity of RNA was checked on 2% agarose gels, and total RNA concentration was determined by a spectrophotometer (Eppendorf, Germany). Reverse transcription was carried out with 2 μg of total RNA using M-MLV reverse transcriptase (Promega, USA). Semi-quantitative RT-PCR was performed as described previously [16]. Briefly, 100 ng of cDNA templates were used in

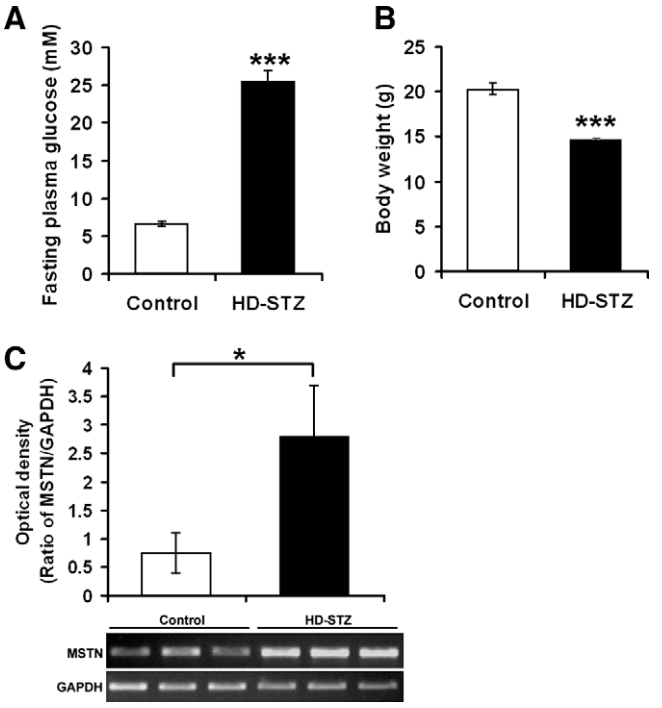


**Fig. 2.** Myostatin expression in skeletal muscle of the HD-STZ-induced T1D model. (A and B) Fasting plasma glucose and body weight were measured at 4 days after STZ injection, respectively. (C) Four days after STZ injection, total RNA from gastrocnemius muscles of each mouse was extracted, and myostatin expression was determined by semi-quantitative RT-PCR and normalized to GAPDH expression. Quantitative results were calculated by optical density. Values are means ± SEM ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group).

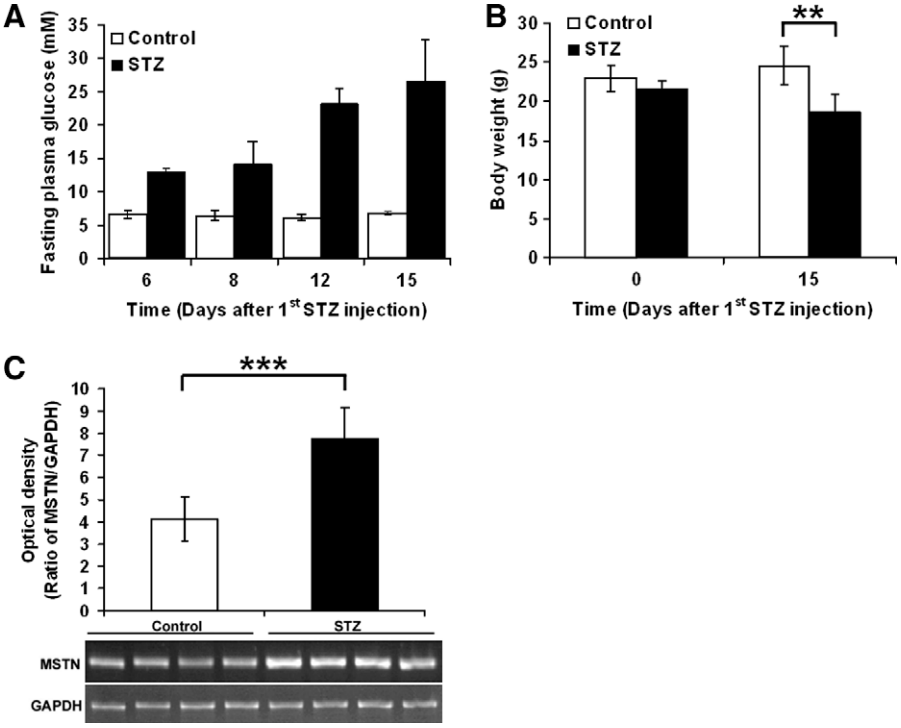


**Fig. 1.** Expression pattern of myostatin (MSTN) in skeletal muscle of the mld-STZ-induced T1D model. (A) Fasting plasma glucose was measured on the indicated days after first STZ injection. (B) Body weight was measured before and 15 days after STZ injection. (C) Total RNA from gastrocnemius muscles at day 15 after STZ injection was extracted, and myostatin mRNA level was determined by semi-quantitative RT-PCR and normalized to GAPDH expression. Quantitative results were calculated by optical density. Values are means ± SEM ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group).

PCR under the same conditions to amplify myostatin and GAPDH, respectively. The expression of GAPDH was measured as an internal control. For real time PCR, 25 ng of cDNA and 0.5 μM of each primer were used in a 20 μl volume system containing iQ SYBR Green Supermix (Bio-Rad, USA). The expression of all genes was normalized to GAPDH expression. Each PCR was performed in triplicate. The primer sequences are listed in Table 1.

*Statistical analysis.* All data are expressed as means ± SEM from different individuals. Statistical analyses were performed with unpaired Student's *t*-test. Group differences at the level of $P < 0.05$ were considered statistically significant.

## Results

### *Muscle myostatin expression was increased in STZ-induced T1D mice*

To investigate whether myostatin expression is altered under an insulin-deficient state, we first established a T1D mouse model by injecting multiple low dose of STZ. As shown in Fig. 1A, fasting plasma glucose was continuously elevated in diabetic mice. By examining body weight and myostatin expression of this mouse model, we found that the body weight of the STZ group was reduced by 13% compared with the control group (Fig. 1B) and that the level of myostatin mRNA was about 1.88-fold increased in STZ-induced diabetic mice (Fig. 1C).

To further study whether the increased myostatin expression was associated with the severity of muscle atrophy, we generated an acute T1D mouse model by injecting a high dose of STZ. In this diabetic model, an even more significant loss of body weight was observed than that in the mld-STZ-induced T1D model (27% in HD-STZ versus 13% in mld-STZ, Fig. 2B). Myostatin expression in this acute diabetic model was also significantly increased by 3.7-fold compared with the control group (Fig. 2C). These results showed that the upregulation of myostatin expression in HD-STZ-induced T1D mice was more dramatic than that in mld-STZ-induced T1D mice (3.7-fold in HD-STZ versus 1.8-fold in mld-STZ), suggesting that the induction of myostatin expression is correlated with the severity of muscle wasting in STZ-induced T1D.

### *Expression pattern of myostatin during the pathological progression of the STZ-induced T1D model*

To further examine the expression pattern of myostatin during the pathological progression of STZ-induced T1D, we examined the level of myostatin mRNA at 1, 2, 3, 4 and 8 weeks after STZ injection. The fasting plasma glucose level in the diabetic group continued to elevate, while the body weight of this group decreased in the first 3 weeks but was restored during the chronic period (Fig. 3A and B). Real time PCR analysis showed that myostatin expression was gradually elevated in the first two weeks and reached a peak at the second week (2.1-fold). Then, the level of myostatin mRNA started to decrease at the third week and almost came back to the control level at the end of this experiment

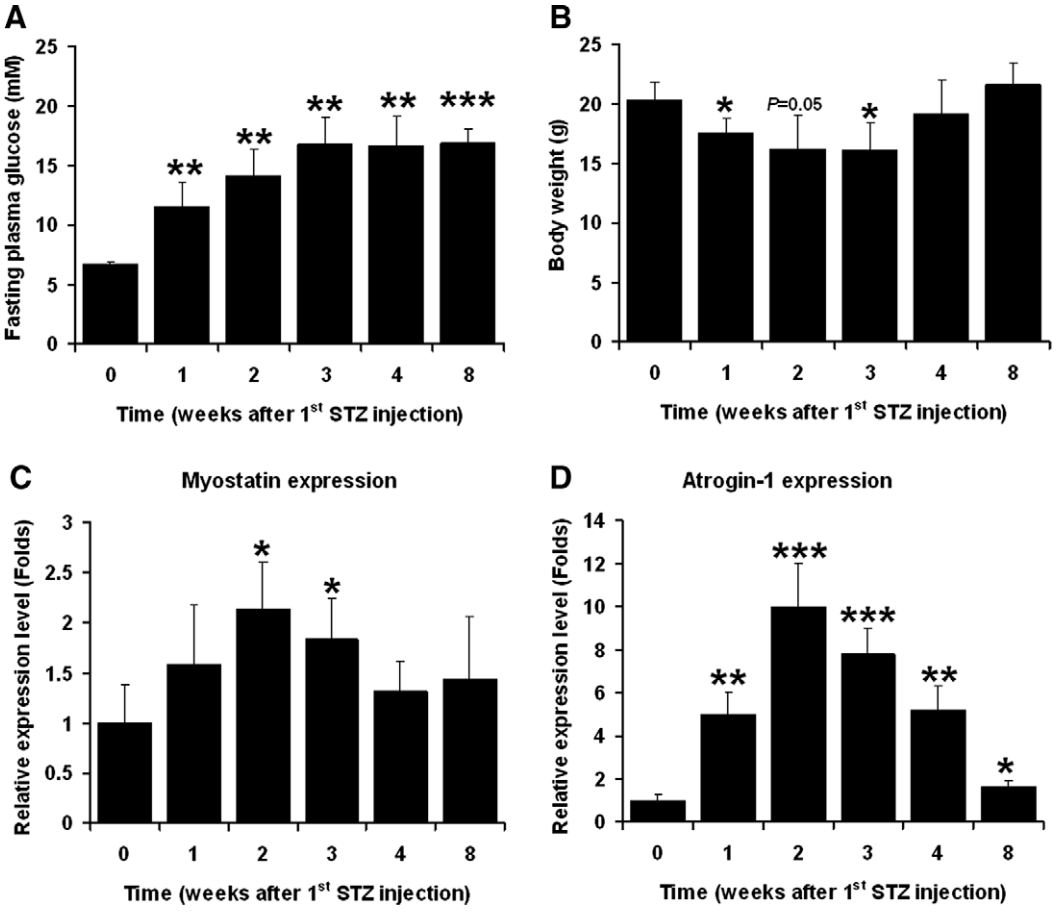


**Fig. 3.** Expression pattern of myostatin during the pathological progression of mld-STZ-induced T1D mice. (A and B) Fasting plasma glucose level and body weight were measured at weeks 0, 1, 2, 3, 4, and 8 after STZ injection, respectively. (C and D) Myostatin and atrogin-1 mRNAs were determined by real time PCR at the indicated time points, respectively. All the gene expressions were normalized to GAPDH expression. Values are means ± SEM ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group).

(Fig. 3C). Most interestingly, the muscle atrophy marker gene atrogin-1 had a similar expression pattern to myostatin during the pathological progression of STZ-induced T1D (Fig. 4D). Taken together, our results indicate that the expression pattern of myostatin is associated with muscle wasting in the STZ-induced T1D mouse model.

*Induction of myostatin in STZ-induced T1D mice can be attenuated by insulin treatment*

Insulin deficiency is characteristic of the STZ-induced T1D model. All symptoms presented in this model could be ameliorated by insulin therapy. Two weeks after onset of diabetes, the mice were treated with insulin for 3 days. This treatment was able to reverse the hyperglycemia and body weight loss in diabetic mice, as shown in Fig. 4A and B. Interestingly, the increase in myostatin expression in the T1D mice was completely attenuated by insulin (Fig. 4C). Simultaneously, atrogin-1 expression was also reversed by insulin (Fig. 4D). Altogether, these results suggest that insulin might act as a potential regulator of myostatin expression *in vivo*.

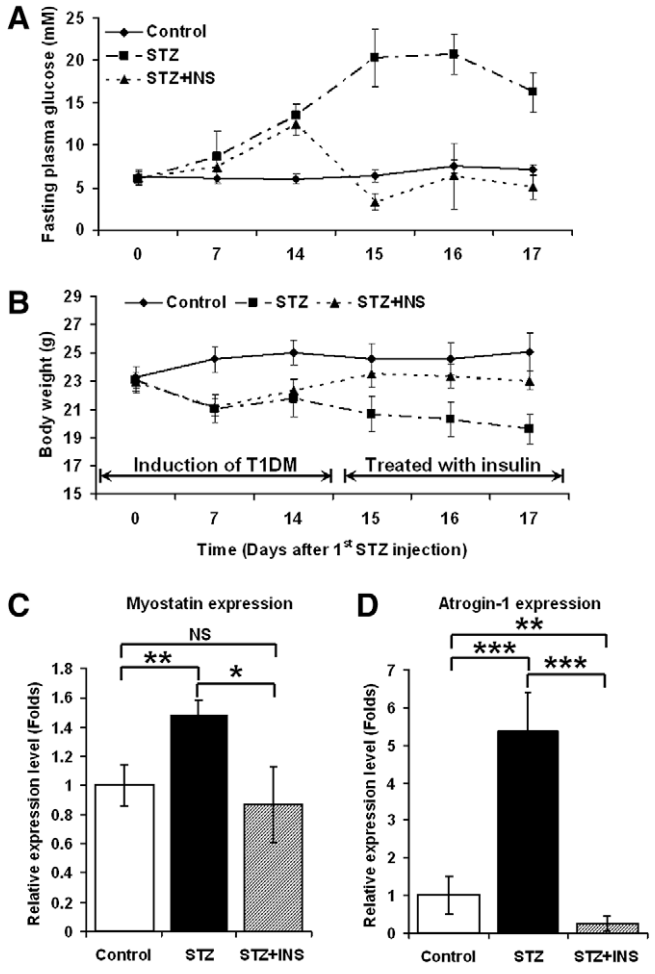


**Fig. 4.** Effect of insulin (INS) treatment on myostatin expression in the STZ-induced T1D mice. Fourteen days after STZ injection, diabetic mice were treated with insulin from day 15 to day 17. Then, gastrocnemius muscles were collected from each group. (A and B) Fasting plasma glucose and body weight were measured at the indicated time points, respectively. (C and D) The mRNA levels of myostatin and atrogin-1 were determined by real time PCR after 3 days of insulin treatment, respectively. All the gene expressions were normalized to GAPDH expression. Values are means ± SEM ($n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group).

## Discussion

Myostatin is considered to be one of the most powerful muscle growth inhibitors [1–5]. The increase of myostatin expression in several muscle atrophy models has already been well studied. However, the expression pattern of myostatin during the pathological progression of T1D was still uncertain. Barazzoni et al. have reported that the mRNA level of myostatin is not altered in chronic insulin-deficient rats [17]. In this study, we presented evidence that myostatin expression levels increase in the early stage of STZ-induced T1D in mice by observing several time points during the pathological progression of T1D. Our data show that the change in myostatin expression is dynamic during the pathological progression of STZ-induced T1D. Thus, the discrepancy between our results and the data reported by Barazzoni et al. may be due to the different time points used in the experiments. In addition, the different animals used for those experiments might also contribute to the inconsistent observations.

Published results indicate that the increase in myostatin expression during the progression of muscle atrophy is not always constant. For example, myostatin mRNA level only increased in the early stage after denervation, but not in the chronic period [11,13]. In our work, an increase in myostatin levels was also found in the early stage but not in the chronic stage of T1D mice. This parabola-like expression pattern is well correlated with the loss of body weight and the expression pattern of muscle atrophy marker atrogin-1, suggesting that induction of myostatin expression is possibly linked to the progression of muscle wasting during insulin deficiency. High levels of myostatin mRNA in T1D could be an indication of a high rate of muscle protein catabolism.

Atrogin-1, which was first identified as a muscle-specific ubiquitin E3 ligase, plays an important role in skeletal muscle atrophy [18,19]. An increase in atrogin-1 expression was found in muscle atrophy conditions such as starvation, diabetes, denervation and cancer cachexia [19,20]. It has been reported that myostatin can regulate atrogin-1 expression though FOXO1 in C2C12 myotubes [21]. Without myostatin, atrogin-1 expression could not be induced in muscle atrophy due to glucocorticoid treatment [22]. In the present study, our results indicate that myostatin exhibits a similar expression pattern as atrogin-1 during the pathological progression of STZ-induced T1D in mice. Whether the increased expression of myostatin contributes to the induction of atrogin-1 expression during this process needs to be taken into consideration. Establishing an STZ-induced T1D model with myostatin null mice may help to address this question in the future.

Myostatin and insulin have opposite effects on muscle protein anabolism [23,24]. Whether myostatin expression can be regulated by insulin is still unknown. Based on our observation in this study that the increased level of myostatin mRNA can be attenuated by insulin in T1D mice, we suspect that insulin could be a potential hormone to regulate myostatin expression *in vivo*. FOXO1, a downstream target of insulin, is phosphorylated and loses its transcriptional activity due to insulin signaling. It has been reported that FOXO1 can bind to the myostatin promoter region and enhance myostatin expression [25]. In addition, myostatin expression was suppressed by knockdown of FOXO1 in skeletal muscle [26]. Therefore, it is conceivable that the expression of myostatin could potentially be regulated by insulin through FOXO1 in muscle cells. Taken together, our findings suggest that myostatin may function in concert with insulin to regulate muscle catabolism and anabolism during pathological progression of T1D.

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