

Role of IGF-I in follistatin-induced skeletal muscle hypertrophy

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Barbé C, Kalista S, Loumaye A, Ritvos O, Lause P, Ferracin B, Thissen JP. Role of IGF-I in follistatin-induced skeletal muscle hypertrophy. *Am J Physiol Endocrinol Metab* 309: E557–E567, 2015. First published July 28, 2015; doi:10.1152/ajpendo.00098.2015.— Follistatin, a physiological inhibitor of myostatin, induces a dramatic increase in skeletal muscle mass, requiring the type I IGF-I receptor/Akt/mTOR pathway. The aim of the present study was to investigate the role of IGF-I and insulin, two ligands of the IGF-I receptor, in the follistatin hypertrophic action on skeletal muscle. In a first step, we showed that follistatin increases muscle mass while being associated with a downregulation of muscle IGF-I expression. In addition, follistatin retained its full hypertrophic effect toward muscle in hypophysectomized animals despite very low concentrations of circulating and muscle IGF-I. Furthermore, follistatin did not increase muscle sensitivity to IGF-I in stimulating phosphorylation of Akt but, surprisingly, decreased it once hypertrophy was present. Taken together, these observations indicate that increased muscle IGF-I production or sensitivity does not contribute to the muscle hypertrophy caused by follistatin. Unlike low IGF-I, low insulin, as obtained by streptozotocin injection, attenuated the hypertrophic action of follistatin on skeletal muscle. Moreover, the full anabolic response to follistatin was restored in this condition by insulin but also by IGF-I infusion. Therefore, follistatin-induced muscle hypertrophy requires the activation of the insulin/IGF-I pathway by either insulin or IGF-I. When insulin or IGF-I alone is missing, follistatin retains its full anabolic effect, but when both are deficient, as in streptozotocin-treated animals, follistatin fails to stimulate muscle growth.

IGF-I; insulin; follistatin; myostatin; skeletal muscle hypertrophy

MYOSTATIN (Mstn), a member of the transforming growth factor (TGF)- β family expressed primarily in skeletal muscle, is a negative regulator of skeletal muscle mass, as shown by the increase in skeletal muscle mass and strength following Mstn inhibition or gene deletion (28, 44). Among Mstn inhibitors, follistatin (FS) and the soluble ligand-binding domain of activin receptor type IIB fused to the Fc domain IgG (sActRIIB-Fc) have been shown to bind and antagonize Mstn, leading to a dramatic increase in muscle mass (3, 30, 37). The muscle fiber hypertrophy induced by FS is due to increased protein synthesis and requires the activation of Smad1/5 (63) and the type I IGF-I receptor (IGF-IR)/Akt/mTOR pathway (34, 64).

IGF-I is known to be a major positive regulator of skeletal muscle mass. Indeed, IGF-I overexpression specifically in skeletal muscle increases skeletal muscle mass (13, 47), whereas IGF-I knockout mice exhibit significant muscle hyp-

oplasia (42). Increased muscle IGF-I expression has been reported in several models of muscle hypertrophy, such as exercise, overloading, GH, and testosterone treatment (1, 20, 27, 39, 40). The role of IGF-I in the muscle hypertrophy caused by Mstn inhibition is suggested by several pieces of evidence. First, muscle mRNA expression and circulating concentrations of IGF-I have been reported to be increased following Mstn inhibition (35, 62, 64). Moreover, Mstn inhibition causes muscle hypertrophy by increasing protein synthesis and satellite cell activation, as IGF-I does (5, 6, 24, 58, 64). Finally, muscle hypertrophy induced by both IGF-I overexpression and Mstn inhibition requires the IGF-IR/Akt/mTOR pathway (8, 34, 51, 64). Taking these observations together, we hypothesized that IGF-I might contribute to the muscle hypertrophy caused by Mstn inhibition. To answer this question, we overexpressed the hFS288 gene by electrotransfer in tibialis anterior (TA) muscle of animals deficient in IGF-I and/or insulin and treated or not by insulin or IGF-I. This work allowed us to delineate the role of IGF-I and insulin in the skeletal muscle hypertrophy caused by FS-induced Mstn inhibition.

MATERIALS AND METHODS

Animals

To assess the effect of Mstn inhibition on skeletal muscle mass and IGF-I expression, we used C57BL/6 transgenic mice (mTr-FS) overexpressing a human FS288 (hFS288) short form specifically in skeletal muscle (37) and their control wild-type (WT) littermates; FVB Mstn knockout (Mstn KO) mice harboring a constitutive deletion of the third Mstn exon (28) and their control WT littermates; and FVB mice (Janvier Breeding, Le-Genest-Saint-Isle, France) treated by intraperitoneal (ip) injection with sActRIIB-Fc (31) at a dose of 10 mg/kg twice a week for 14 days or with PBS (CTRL). The sActRIIB-Fc was prepared as described by Hoogaars et al. (30). All the mice used were male, between 6 and 8 wk old, unless otherwise stated. To assess the role of IGF-I in the muscle hypertrophy induced by FS, we used hypophysectomized female Wistar rats purchased from Charles River (Charles River Laboratories, France). Hypophysectomy was performed at 5 wk of age and hypophysectomized (HYPOX) animals were delivered to our animal quarters 7 days after surgery with their control intact littermates (CTRL). To assess the effect of FS on IGF-I sensitivity, we used 8-wk-old male mTr-FS mice and their control WT littermates. To assess the effect of sActRIIB-Fc on IGF-I sensitivity, we treated 8-wk-old C57Bl/6 male mice with sActRIIB-Fc for 5 days. To explore the role of insulin in the muscle hypertrophy induced by FS, we used streptozotocin (STZ)-injected male Wistar rats. Animals (100–124 g) were provided by Janvier Breeding at 4 wk of age and treated by STZ injection a week later. All the animals were housed individually under controlled conditions of lighting (12:12-h light-cycle) and temperature ($22 \pm 2^\circ\text{C}$). They received standard chow pellets and water ad libitum. The study was conducted in accordance with the directives of and approved by the

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Animal Ethics Committee of the Catholic University of Louvain (Brussels, Belgium).

Experimental Design

Experimental design 1: role of IGF-I in FS-induced muscle hypertrophy. To assess the role of IGF-I in FS-induced muscle hypertrophy, we used HYPOX rats. The experiment was started after a 9-day adaptation period in our animal quarters. To attest the success of hypophysectomy, changes in body weight were recorded during this period. As expected, the body weight gain of HYPOX rats was severely blunted compared with intact animals ($+18.0 \pm 2.1$ vs. $+94.9 \pm 7.4$ g, $n = 6/\text{group}$, $P < 0.001$). During the adaptation period, water was supplemented with 5% glucose and 0.9% NaCl. Throughout the remainder of the experiment, the rats received water containing 0.9% NaCl only and a hormonal replacement. HYPOX rats ($n = 6$) were given replacement therapy with L-thyroxine ($10 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$; Sigma-Aldrich, Diegem, Belgium) and hydrocortisone hemisuccinate ($500 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$; Solu-Cortef, Pfizer, Oslo, Norway) diluted in saline by daily subcutaneous injection (8:00 AM), while CTRL rats ($n = 6$) were injected with saline solution. One week after the beginning of the hormonal treatment, TA muscles of HYPOX and CTRL rats were transfected with the plasmid pM1-hFS288-c-myc (left leg) and the control plasmid pM1 (right leg). TA muscles and serum were collected 17 days after electroporation for assessment of muscle hypertrophy and IGF-I concentrations.

Experimental design 2: effect of FS and sActRIIB-fc on skeletal muscle IGF-I sensitivity. To investigate the effect of FS on IGF-I sensitivity, mTr-FS and WT mice were fasted overnight, and muscle and liver Akt phosphorylation (pAkt) was assessed after ip injection of recombinant human IGF-I (Genentech, South San Francisco, CA). One hundred microliters of a solution of IGF-I (200 or 400 $\mu\text{g}/\text{kg}$ body wt) or 100 μl of saline solution (vehicle, 0.9% NaCl) was injected 15 min before the animals were euthanized. To investigate the effect of the sActRIIB-Fc on IGF-I sensitivity, mice were treated with two ip injections of 10 mg/kg sActRIIB-Fc (or same volume of PBS for control animals) for 5 days. Animals were fasted overnight and muscle pAkt was assessed after ip injection of recombinant human IGF-I (Genentech). One hundred microliters of a solution of IGF-I (400 $\mu\text{g}/\text{kg}$ body wt) or 100 μl of saline solution (vehicle, 0.9% NaCl) was injected 15 min before the mice were euthanized. Liver and gastrocnemius (GC) muscles were collected for assessment of pAkt by Western blotting analysis. Therefore, muscle IGF-I sensitivity was explored in a transgenic model characterized by a marked hypertrophy and in a nontransgenic model at the early stages of hypertrophy.

Experimental design 3: role of insulin in FS-induced muscle hypertrophy. To explore the role of insulin in FS-induced muscle hypertrophy, we used a model of STZ-induced diabetes. After a 7-day adaptation period in our animal quarters, TA muscles of rats were transfected with the plasmid pM1-hFS288-c-myc (left leg) and the control plasmid pM1 (right leg). Three days after electroporation, diabetes was induced by injecting STZ (Sigma-Aldrich, St. Louis, MO) freshly prepared in 0.01 M citrate buffer, pH 4.5, at the dose of 60 mg/kg body wt into the tail vein. The CTRL group was injected with an equivalent volume of 0.01 M citrate buffer, pH 4.5. Only rats showing polydipsia, polyuria, glycosuria, and glycemia over 400 mg/dl were included in the experiment. Two days after STZ injection, diabetic animals were randomized in two groups: one was infused with insulin (STZ+INS group); the second group was not treated (STZ group). Insulin treatment was achieved with two insulin implants of Linplant (Linshin Canada, ON, Canada) placed subcutaneously in the interscapular region of STZ+INS rats. For implantation, the three groups of rats (CTRL, STZ, STZ+INS) were anesthetized with a mixture of 100 mg/kg ketamine (Anesketin; Pfizer, Oslo, Norway) and 10 mg/kg xylazine hydrochloride (Rompun; Bayer, Fernwald, Germany) administered by ip injection. Two implants were placed in STZ+INS animals, while CTRL and STZ animals were

sham operated. Each implant delivered an average daily dose of 2 U of insulin. Growth was assessed by body weight measurement. TA muscles and serum were collected 14 days after electroporation for assessment of muscle hypertrophy and IGF-I concentrations.

Experimental design 4: role of low IGF-I concentrations in the attenuation of FS-induced muscle hypertrophy by insulinopenia. To explore the role of low IGF-I concentrations in the attenuation of FS-induced muscle hypertrophy by insulinopenia, we used the model of STZ-induced diabetes described in *experimental design 3*. In this experiment, IGF-I was infused in place of insulin. Two days after STZ injection, diabetic animals were randomized in two groups: one was infused with IGF-I (STZ+IGF-I group) and the second group was not treated (STZ group). Recombinant human IGF-I Increlex, generously given by Ipsen (Ipsen Biopharmaceuticals, Merelbeke, Belgium) was administrated at a dose of 3 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ with an Alzet osmotic pump (model 2M2L; Durect, Cupertino, CA) implanted subcutaneously in the interscapular region of STZ+IGF-I rats. For implantation, the three groups of rats (CTRL, STZ, STZ+IGF-I) were anesthetized with a mixture of 100 mg/kg ketamine (Anesketin; Pfizer, Oslo, Norway) and 10 mg/kg xylazine hydrochloride (Rompun; Bayer, Fernwald, Germany) administered by ip injection. One pump filled with IGF-I was placed in STZ+IGF-I animals, while CTRL and STZ animals received a pump filled with vehicle (0.05 M acetate buffer, pH 5.4). Growth was assessed by body weight measurement. TA muscles were collected 14 days after electroporation for assessment of muscle hypertrophy.

Expression Plasmids and DNA Preparation

A pM1-hFS288 c-myc plasmid coding for the human FS containing 288 amino acids was constructed as previously described (24, 34). Empty pM1 was used as a control plasmid. Plasmids were amplified in *Escherichia coli* top 10 F' (Invitrogen, Carlsbad, CA) and purified with an EndoFree plasmid giga kit (Qiagen, Valencia, CA). Plasmids were stocked at -80°C . The day before injection, 100 μg of plasmid was lyophilized and resuspended in 100 μl of 0.9% NaCl solution.

DNA Electrotransfer

Each animal was anesthetized with a mixture of 100 mg/kg ketamine (Anesketin) and 10 mg/kg xylazine hydrochloride (Rompun) administered by ip injection. Ten microliters of plasmid solution (1 $\mu\text{g}/\mu\text{l}$) was injected into 10 different sites (total volume per muscle = 100 μl) in each TA muscle, and the muscles were then electroporated using the electroporation conditions described previously (53). The animals were euthanized 14–17 days after electroporation.

Animal Euthanasia and Biological Sample Collection

All the animals were euthanized by decapitation after CO_2 administration, and blood was collected from the trunk vessels. TA muscles were dissected, and a transverse slice of 0.5-cm thickness was fixed with buffered formalin for 48 h and embedded in paraffin for morphological analysis. The remaining ends of the TA muscles were frozen in liquid nitrogen for ELISA and mRNA analysis. GC muscles were removed and frozen in liquid nitrogen for ELISA, mRNA, and Western blot analysis.

mRNA Analysis by Real-Time Quantitative PCR

Total RNA was isolated from the TA muscle using Tri Reagent as described by the manufacturer. Recovery was 1 $\mu\text{g}/\text{mg}$ of TA muscle. Reverse transcription and real-time quantitative PCR were done as previously described (18). Accession numbers for the sequences and primers used were as follows: IGF-I: AH002176 (CAGGCTATG-GCTCCAGCAT-GGAAGCAACACTCATCCACA) IGF-IR: X044434.1 (TCATGCCCTTGGTCTCCTTGTCTT-GTCACTTCTCCATGCGGTAAATTTTCG), IGFBP-5: NM_010518.2 (TCGAACAAGGCCCTGCCG-GCTGTCTGAAGGCGTGGCACT),

MAFbx/atrogen-1: NM_026346.3 (CCATCAGGAGAAGTGGATC-TATGTT-GCTTCCCCCAAAGTGCAGTA), and REDD1: NM_029083.2 (AGACTCCTCATACCTGGATGGG-AGCTGCAT-CAGGTTGGCAC), and GAPDH, AF106860 (TGCACCACCAACT-GCTTA-GGATGCAGGGATGATGTTC), used as the reporter gene. Primers were tested to avoid primer dimers, self-priming formation, or unspecific amplification. The primers were designed to have standardized optimal PCR conditions. We confirmed that the expression values of GAPDH normalized to RNA were not affected by Mstn inhibition in our different animal models. This was confirmed at the protein level, as illustrated on the blots presented in Fig. 3A, showing that the abundance of GAPDH protein was not affected by FS overexpression, making GAPDH a valuable control.

Muscle and Circulating IGF-I Concentrations

One hundred milligrams of TA muscle, previously pestled in liquid nitrogen, was homogenized with Ultraturrax (IKALabortechnik, Staufen, Germany) in 1 ml of pH 7.4 $1 \times$ PBS (130 mM NaCl, 17 mM Na_2HPO_4 , and 3 mM NaH_2PO_4) and stored overnight at -20°C . After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 min at 5,000 g. The supernatant was removed and stored at -80°C until the assay. Blood was allowed to clot for 15 min at room temperature before centrifuging for 10 min at 2,000 g. Serum was removed and stored at -80°C until the assay. Muscle and circulating levels of IGF-I peptide were determined using Quantikine ELISA Mouse/Rat according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Western Blots

Muscle proteins of GC muscle were homogenized in ice-cold pH 7.0 buffer containing 20 mM Tris, 270 mM sucrose, 5 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM β -glycerophosphate, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM DTT, 1% (vol/vol) Triton X-100, and 10% protease inhibitor cocktail (Roche Applied Science, Belgium). Homogenates were centrifuged at 10,000 g for 10 min at 4°C , and supernatants were immediately stored at -80°C . Equal amounts of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel 10% electrophoresis and transferred to PVDF membranes. Membranes were probed with the following primary antibodies: anti-phospho-Akt (Ser⁴⁷³, 1:2,000; Cell Signaling Technology, Leiden, Netherlands), anti-Akt (1:2,000; Millipore, Overijse, Belgium), and anti-GAPDH (1:40,000; Cell Signaling Technology). Membranes were then incubated with a horseradish peroxidase-coupled secondary antibody (Cell Signaling Technology) and developed using Enhanced Chemiluminescence (ECL) Western Blotting Detection System Plus (GE Healthcare, Belgium). Developed film was scanned and analyzed as previously described (54). All results were normalized for the signal to GAPDH protein.

Histological Analysis of Muscle

Serial sections (5 μm thick) were cut and mounted on glass slides (Superfrost Plus; Menzel-Glaser, Braunschweig, Germany). FS-c-myc was detected by immunohistochemistry with rabbit polyclonal anti-c-myc as previously described (24). Fiber cross-sectional areas (CSA) were measured with a microscope Axio-Star (Carl Zeiss, Oberkochen, Germany) coupled to a Zeiss Axiocam digital camera MRc and to image analyzer software (Axiovision software v. 4.7, Carl Zeiss). To evaluate the effect of FS on muscle fiber CSA, all the positive muscle fibers in the TA transfected with the FS gene were measured. Two hundred negative fibers, randomly chosen in the contralateral TA transfected with insertless plasmid (pM1), were measured and considered as controls.

Statistical Analysis

Results are presented as means \pm SE. Statistical analyses were performed using a one-way ANOVA followed by a Bonferroni

multiple comparison test or unpaired *t*-test to compare muscles from different animals undergoing different experimental conditions. Interactions between FS and hypophysectomy, as well as between FS/sActRIIB and IGF-I injection, were assessed using a two-way ANOVA followed by a Bonferroni posttest. Fiber CSA distribution statistical analysis was performed using a χ^2 Pearson test. Statistical significance was set at $P < 0.05$.

RESULTS

Muscle Hypertrophy Caused by Mstn Inhibition Is Associated with Decreased Muscle IGF-I mRNA and Peptide Levels

IGF-IR mediated-signaling is required for FS to promote muscle hypertrophy (34). Since the IGF-IR is activated primarily by IGF-I, we investigated whether the muscle hypertrophy induced by Mstn inhibition is mediated by increased muscle IGF-I expression. First, muscle IGF-I mRNA levels were assessed in TA and GC muscles from different animal models of muscle hypertrophy induced by Mstn inhibition: mTr-FS, Mstn KO, and sActRIIB-Fc-treated mice. Surprisingly, we found that muscle IGF-I expression was systematically reduced in mTr-FS, Mstn KO, and sActRIIB-Fc-treated mice compared with their control littermates (Fig. 1B) in contrast to muscle mass (Fig. 1A). Interestingly, muscle IGF-I mRNA levels were already decreased (-44% , $P < 0.05$) in 4-wk-old mTr-FS mice, namely before any muscle hypertrophy. Then, the IGF-I peptide concentrations were determined in the TA muscle of mTr-FS. We confirmed that FS overexpression decreased not only IGF-I mRNA but also peptide in the skeletal muscle (mTr-FS, -41% ; 9 ± 1 vs. WT, 16 ± 2 ng/g, $n = 8/\text{group}$, $P < 0.05$; Fig. 1C). In the other animal models of Mstn inhibition, we are confident that changes in muscle IGF-I mRNA translated in parallel the changes in muscle IGF-I peptide. Indeed, in several animal models we observed parallel changes in IGF-I mRNA and peptide in skeletal muscle (data not shown). Circulating IGF-I concentrations were not affected by FS overexpression (Fig. 1D).

FS Induces Muscle Hypertrophy in HYPOX Animals Despite Very Low Concentrations of Circulating and Muscle IGF-I

To investigate the role of IGF-I in FS-induced skeletal muscle hypertrophy, we overexpressed FS by DNA electrotransfer in the TA muscle of HYPOX rats, characterized by very low concentrations of IGF-I. The IGF-I mRNA levels in the TA muscle were modestly decreased after hypophysectomy (-22% , $P < 0.01$). However, both muscle and circulating concentrations of the IGF-I peptide were severely reduced, respectively by 90% (pM1-transfected muscle: 2 ± 1 vs. 15 ± 1 ng/g, $n = 6/\text{group}$, $P < 0.001$) and by 95% (52 ± 3 vs. $1,097 \pm 57$ ng/ml, $n = 6/\text{group}$, $P < 0.001$) after hypophysectomy (Fig. 2, A and B). Interestingly, despite very low concentrations of muscle and circulating IGF-I, FS overexpression caused the same increase of muscle mass in HYPOX rats ($+23\%$, 217 ± 6 vs. 177 ± 5 mg, $P < 0.001$) and in their intact CTRL littermates ($+28\%$, 448 ± 27 vs. 351 ± 25 mg, $P < 0.001$, $n = 6/\text{group}$; Fig. 2C) 17 days after electroporation. Furthermore, FS overexpression induced an even larger rise of fiber CSA in HYPOX ($+102\%$, $1,766 \pm 166$ vs. 877 ± 39 μm^2 , $P < 0.001$) than in CTRL rats ($+57\%$, $2,420 \pm 175$ vs. $1,552 \pm 102$ μm^2 , $P < 0.001$, $n = 6/\text{group}$; Fig. 2D). These

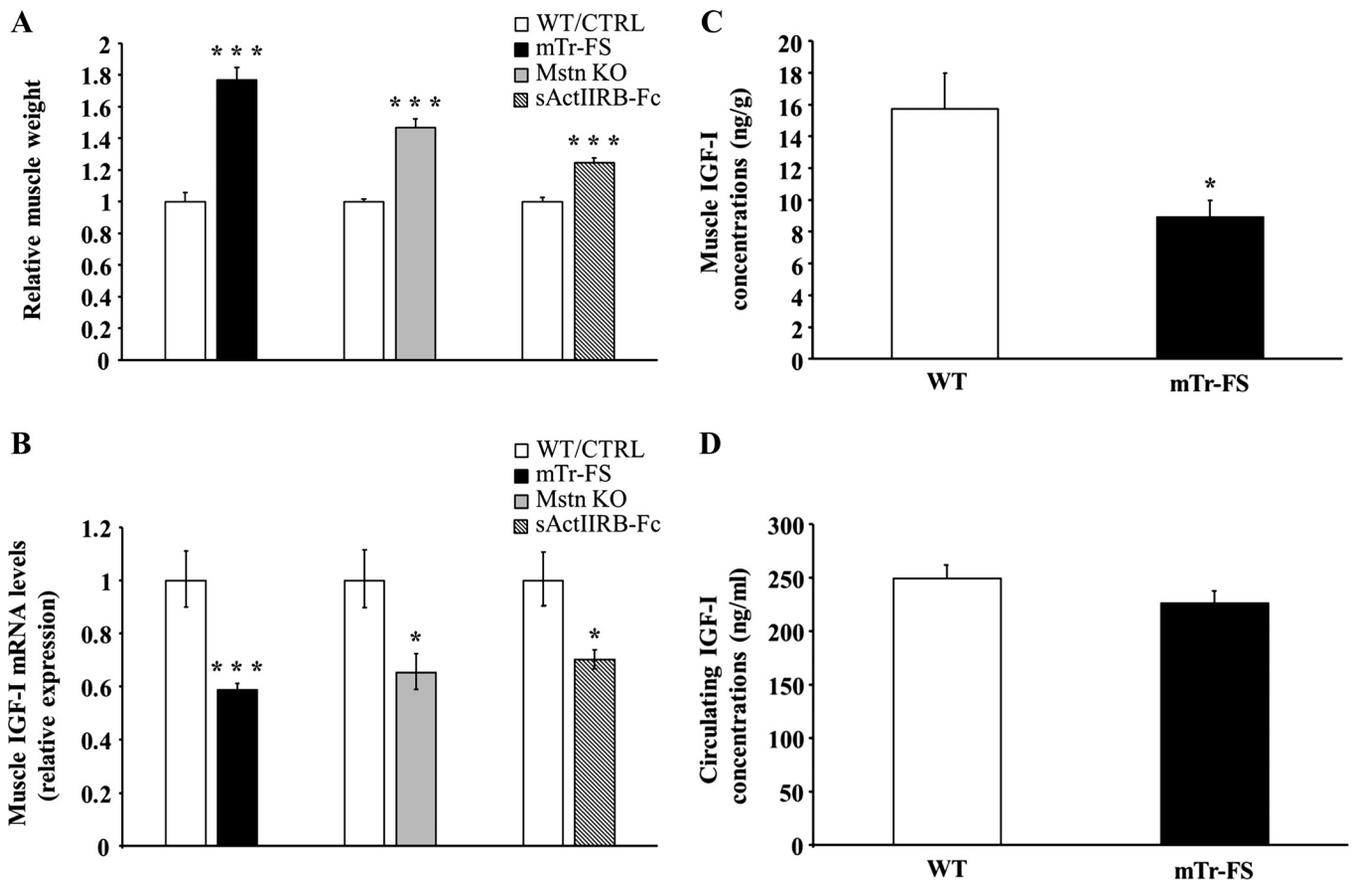


Fig. 1. Muscle hypertrophy caused by myostatin (Mstn) inhibition is associated with decreased muscle IGF-I mRNA and peptide levels. *A* and *B*: muscle weight and muscle IGF-I mRNA levels were assessed in tibialis anterior (TA) muscle of C57Bl/6 mTr-FS (follistatin) vs. WT mice ($n = 11$ /group) and FVB sActRIIB-Fc (soluble ligand binding domain of activin receptor type IIB fused to Fc domain IgG)-treated vs. saline-treated mice ($n = 6$ /group) and in gastrocnemius (GC) muscle of FVB Mstn KO vs. WT mice ($n = 7$ /group). *C* and *D*: IGF-I peptide concentrations were assessed in TA muscle (*C*) and in the circulation (*D*) of WT (open column) and mTr-FS (filled column) mice ($n = 8$ /group). sActRIIB-Fc, soluble ligand-binding domain of activin receptor IIB fused to the Fc domain IgG. Results are expressed as means \pm SE. Statistical analysis was performed using unpaired *t*-test ($P > 0.05$; * $P < 0.05$, *** $P < 0.001$).

results demonstrate that FS retains its full hypertrophic effect toward muscle despite very low concentrations of circulating and muscle IGF-I.

FS Overexpression Does Not Increase Skeletal Muscle IGF-I Sensitivity

The ability of FS to exert its full anabolic effect, even in the presence of very low IGF-I concentrations, suggests two hypotheses: either FS overexpression increases the muscle sensitivity to the anabolic effect of IGF-I, or, alternatively, IGF-I is not required for FS to promote muscle hypertrophy. The first hypothesis is suggested by the demonstration made previously that Mstn inhibition increases the insulin-stimulated activation of Akt and glucose uptake in skeletal muscle (29, 45, 66, 67). To investigate whether FS overexpression enhances IGF-I sensitivity in skeletal muscle, we assessed the activation of the protein kinase Akt, a downstream target of the IGF-IR crucial for the anabolic action of FS (34), after injection of graded doses of IGF-I into mTr-FS and WT mice (Fig. 3). Activation of IGF-I signaling normally increases the phosphorylation state of Akt, as seen in GC muscle from WT mice (4.4 ± 0.4 -fold, IGF-I 400 $\mu\text{g}/\text{kg}$ vs. 1 ± 0.4 -fold, Saline, $P < 0.001$, $n = 3$ –4/group). Unexpectedly, the increased phosphorylation of Akt in response to IGF-I was almost abolished in GC muscle

of mTr-FS mice (1.8 ± 0.6 -fold, IGF-I 400 $\mu\text{g}/\text{kg}$ vs. 1.1 ± 0.1 -fold, Saline, $P > 0.05$; Fig. 3, *A*, *B*, and *C*). In contrast, IGF-I injection (400 $\mu\text{g}/\text{kg}$) activated Akt to a similar extent in the liver from mTr-FS and WT mice (3.0 ± 0.6 -fold vs. 3.1 ± 0.5 -fold, NS, $n = 3$ –4/group; Fig. 3, *D*, *E*, and *F*). Levels of total Akt protein were increased in response to Mstn inhibition, as already reported (26, 45, 66). Taken together, our observations unravel a muscle-specific decrease of the IGF-I signaling in mTr-FS mice. To examine the mechanisms for this resistance, we assessed the expression of potential candidate proteins that might limit IGF-I responsiveness of the muscle in mTr-FS mice. The muscle hypertrophy caused by FS overexpression was associated with an increase in muscle IGF-binding protein (IGFBP)-5 mRNA levels (+64%, $P < 0.001$) together with a decrease in IGF-IR mRNA levels (–24%, $P < 0.01$), supporting the hypothesis of a decreased muscle sensitivity to IGF-I. Although muscle IGF-I sensitivity is decreased in adult mTr-FS mice, the possibility still exists that an increase in muscle IGF-I sensitivity might play a role at the early stages of hypertrophy. To test this hypothesis, we assessed the muscle IGF-I sensitivity caused by Mstn inhibition using mice treated with sActRIIB-Fc for a brief period as a model of incipient hypertrophy. In these conditions, the increase of Akt phosphorylation induced by IGF-I

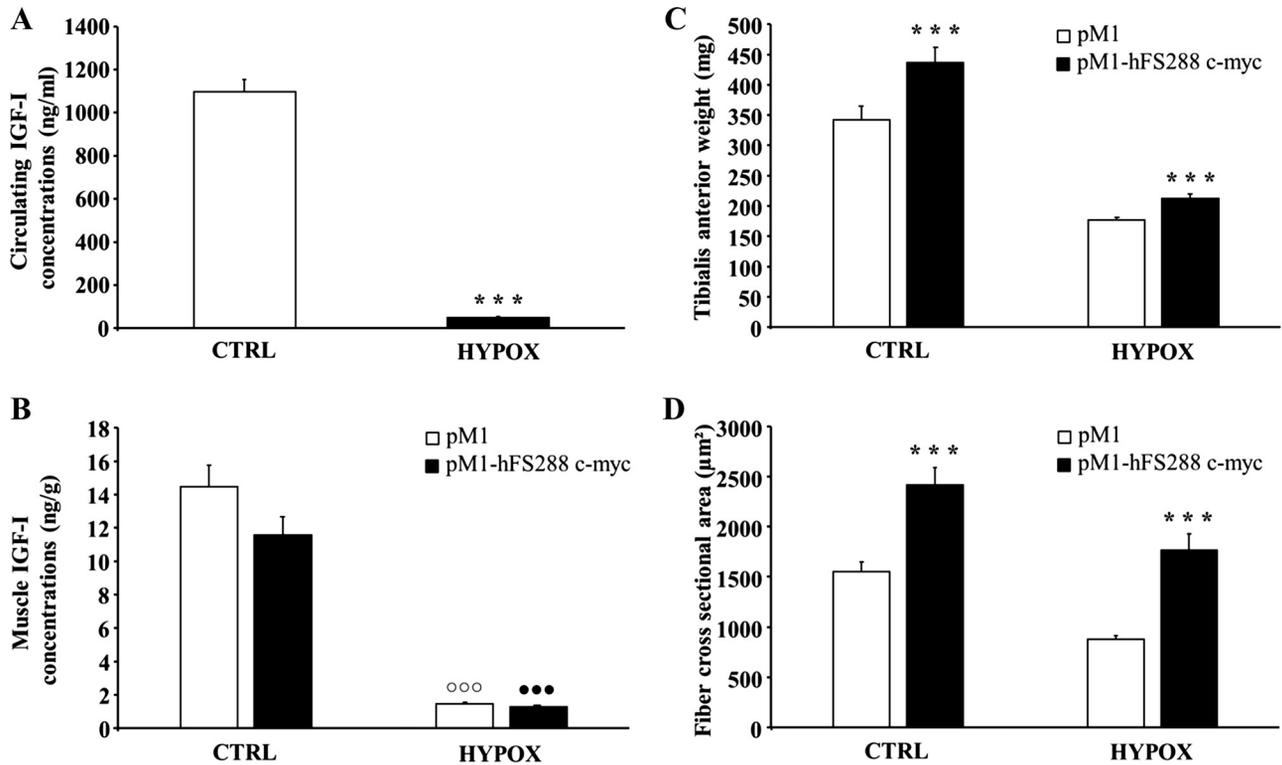


Fig. 2. FS induces muscle hypertrophy in hypophysectomized (HYPOX) animals despite very low concentrations of circulating and muscle IGF-I. A: circulating concentrations of IGF-I were assessed in CTRL (open column) and in HYPOX (filled column) rats ($n = 6/\text{group}$). Results are expressed as means \pm SE. Statistical analysis was performed using unpaired *t*-test ($***P < 0.001$). B–D: muscle concentrations of IGF-I peptide (B), TA muscle weight (C), and fiber cross-sectional area (CSA; D) were assessed 17 days after transfection of pM1 (open column) or pM1-hFS288 (filled column) in CTRL and HYPOX rats ($n = 6/\text{group}$). Results are expressed as means \pm SE. Statistical analysis was performed using 2-way ANOVA (muscle IGF-I concentrations: FS effect, NS, HYPOX effect, $P < 0.001$, interaction, NS; TA weight: FS effect, $P < 0.01$, HYPOX effect, $P < 0.001$, interaction, NS; fiber CSA: FS effect, $P < 0.001$, HYPOX effect, $P < 0.001$, interaction, NS) and Bonferroni posttest ($***P < 0.001$ vs. contralateral muscle; $○○○P < 0.001$ vs. CTRL pM1 muscle; $●●●P < 0.001$ vs. CTRL pM1-hFS288 c-myc muscle).

was not affected by the sActRIIB-Fc pretreatment (7.9 ± 10.7 -fold vs. 7.2 ± 1.4 -fold, NS, $n = 3$ – $4/\text{group}$; Fig. 4, A, B, and C). This last observation supports the view that the decreased muscle IGF-I sensitivity observed in mTr-FS is secondary to muscle hypertrophy. Taken together, our observations indicate that the muscle hypertrophy caused by Mstn inhibition does not result from increased IGF-I sensitivity.

FS-Induced Muscle Hypertrophy Is Attenuated in STZ-Diabetic Animals

Since the IGF-IR can be activated by either IGF-I or insulin, we investigated the role of insulin in skeletal muscle hypertrophy induced by FS overexpression. To investigate the role of insulin in FS-induced skeletal muscle hypertrophy, we overexpressed FS by DNA electrotransfer in the TA muscle of STZ-induced diabetic rats, characterized by low insulin concentrations. To attest the success of STZ injection, glycemia was determined 2 days after STZ injection and at the end of the experiment. As expected, the glycemia of STZ rats was markedly increased compared with CTRL (581 ± 11 vs. 116 ± 5 mg/dl, $n = 8/\text{group}$, $P < 0.001$) and restored to normal in STZ rats treated with insulin implants (115 ± 7 vs. 116 ± 5 mg/dl, $n = 8/\text{group}$, NS; Fig. 5A). Concentrations of IGF-I peptide were significantly decreased in STZ rats compared with CTRL rats both in the

circulation (-38% , 920 ± 73 vs. $1,476 \pm 69$ ng/ml, $n = 8/\text{group}$, $P < 0.001$) and in the muscle (-51% , pM1-transfected muscle: 14 ± 1 vs. 30 ± 3 ng/g, $n = 8/\text{group}$, $P < 0.001$; Fig. 5, C and D). They were restored to normal levels in STZ rats treated with insulin implants both in the circulation ($1,518 \pm 54$ vs. $1,476 \pm 69$ ng/ml, $n = 8/\text{group}$, NS) and in the muscle (pM1-transfected muscle: 28 ± 3 vs. 30 ± 3 ng/g, $n = 8/\text{group}$, NS; Fig. 5, C and D). In contrast to hypophysectomy, insulinopenia decreased the FS-induced hypertrophy. Indeed, FS overexpression for 17 days induced a lower rise of fiber CSA in STZ rats ($+33\%$, $2,440 \pm 166$ vs. $1,835 \pm 80$ μm^2 , $P < 0.05$) than in CTRL rats ($+57\%$, $3,191 \pm 150$ vs. $2,037 \pm 56$ μm^2 , $P < 0.001$; Fig. 5E). To examine some potential mechanisms for this attenuation, we assessed the expression of MAFbx/atrogen-1 and REDD1, two factors that inhibit muscle protein synthesis (14, 19) and might thus limit the responsiveness to FS. The expression of MAFbx/atrogen-1 and REDD1 was significantly increased in the muscle of STZ animals (respectively in pM1-transfected muscle: 2.7-fold, $P < 0.001$ and 1.9-fold, $P < 0.001$), supporting the idea of decreased protein synthesis in response to FS in the case of insulinopenia. Moreover, insulin implants in STZ rats restored the full hypertrophic effect of FS as assessed by the fiber CSA ($+60\%$, $3,501 \pm 199$ vs. $2,181 \pm 97$ μm^2 , $P < 0.001$) as well as the baseline expression levels of MAFbx/atrogen-1 and REDD1.

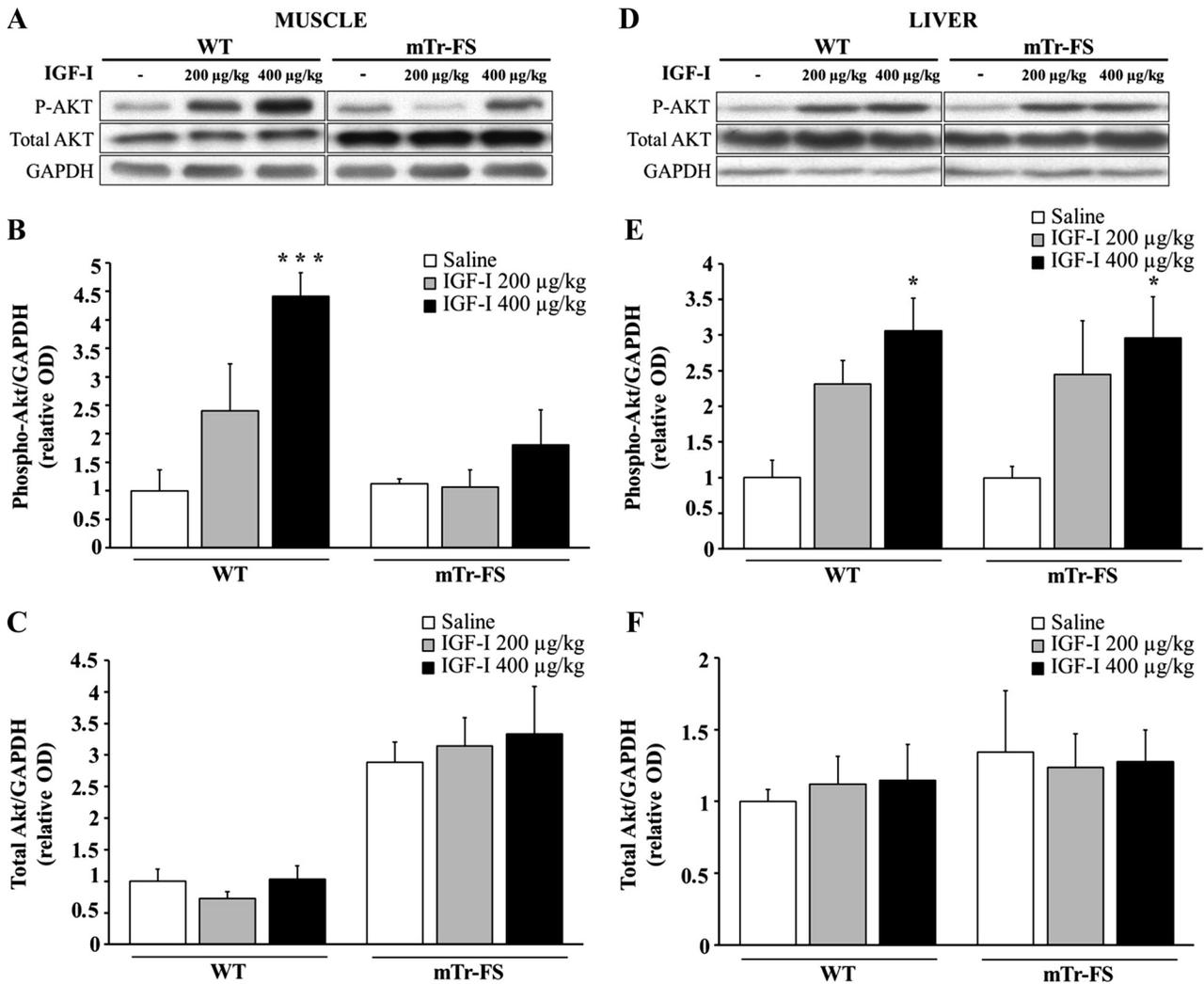


Fig. 3. FS does not increase muscle IGF-I sensitivity. A–C: increase of muscle pAkt (Ser⁴⁷³) induced by acute IGF-I injection (200 µg/kg, gray column, or 400 µg/kg, filled column vs. saline, open column) is severely blunted in mTr-FS mice ($n = 3-4$ /group). Muscle activation of Akt was assessed by Western blot analysis (A) and densitometric analysis of pAkt/GAPDH (B) and total-Akt/GAPDH (C). Results are expressed as means \pm SE. Statistical analysis was performed using 2-way ANOVA (pAkt/GAPDH: IGF-I effect, $P < 0.01$, mTr-FS effect, $P < 0.01$, interaction, $P < 0.05$; total-Akt/GAPDH: IGF-I effect, NS, mTr-FS effect, $P < 0.001$, interaction, NS) and Bonferroni posttest ($***P < 0.001$ vs. saline). D–F: increase in liver pAkt (Ser⁴⁷³) induced by acute IGF-I injection (200 µg/kg, gray column, or 400 µg/kg, filled column vs. saline, open column) is similar in WT and mTr-FS mice ($n = 3-4$ /group). Liver activation of Akt was assessed by Western blot analysis (D) and densitometric analysis of pAkt/GAPDH (E) and total-Akt/GAPDH (F). Results are expressed as means \pm SE. Statistical analysis was performed using 2-way ANOVA (pAkt/GAPDH: IGF-I effect, $P < 0.01$, mTr-FS effect, NS, interaction, NS; total-Akt/GAPDH: IGF-I effect, NS; mTr-FS effect, NS, interaction, NS) and Bonferroni posttest ($*P < 0.05$ vs. saline).

IGF-I Treatment Restores the Full Hypertrophic Effect of FS in STZ-Diabetic Animals

To investigate whether restoration of the full anabolic effect of FS was due to insulin infusion or to normalization of IGF-I or glycemia, we administrated IGF-I to STZ rats electroporated with FS. IGF-I infusion restored the body growth of STZ rats without normalizing glycemia (Fig. 6, A and B). Furthermore, we showed that IGF-I treatment also restored to normal the FS-induced muscle hypertrophy as assessed by the measurement of the fiber CSA (STZ+IGF-I: +59%, $P < 0.001$ vs. STZ: +28%, $P < 0.05$; Fig. 6C). Accordingly, we demonstrated that FS does not exert its full anabolic effect in the absence of insulin associated with a decrease of IGF-I. Interestingly, in the case of insulinopenia, IGF-I infusion could restore the FS hypertrophy and therefore compensate for the lack of insulin despite the persistence of hyperglycemia.

DISCUSSION

Our study investigated the role of IGF-I and insulin in muscle hypertrophy caused by FS overexpression. Indeed, we showed in a previous experiment that the hypertrophic action of FS on skeletal muscle requires the activation of the IGF-IR/Akt/mTOR pathway (34). Since the IGF-IR can be activated either by IGF-I or by insulin and because insulin signaling could also contribute to the regulation of skeletal muscle mass (48), we specifically investigated the role of these ligands in FS-induced muscle hypertrophy. Our results show that FS causes a decrease in muscle IGF-I expression, which contrasts with the marked muscle hypertrophy. Moreover, FS retains its full anabolic effect toward muscle even in the presence of very low concentrations of IGF-I. Finally, FS does not increase muscle sensitivity to IGF-I in stimulating phosphorylation of Akt but, surprisingly, decreases it once hypertrophy is present.

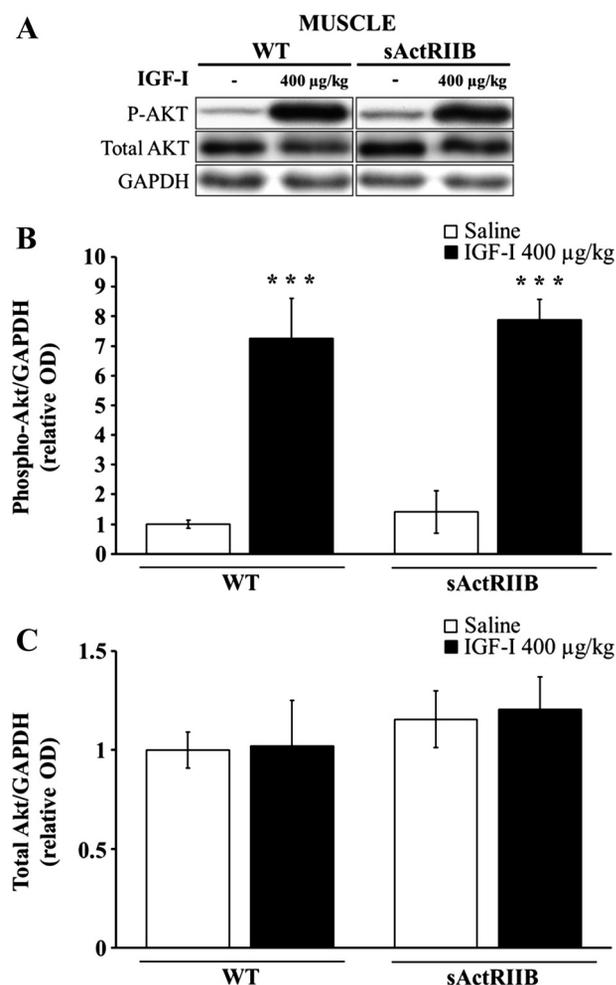


Fig. 4. sActRIIB-Fc does not increase muscle IGF-I sensitivity. A–C: increase of muscle pAkt (Ser⁴⁷³) induced by acute IGF-I injection (400 g/kg, filled column, vs. saline, open column) is not affected in sActRIIB-Fc-treated mice ($n = 3\text{--}4/\text{group}$). Muscle activation of Akt was assessed by Western blot analysis (A) and densitometric analysis of pAkt/GAPDH (B) and total-Akt/GAPDH (C). Results are expressed as means \pm SE. Statistical analysis was performed using 2-way ANOVA (pAkt/GAPDH: IGF-I effect, $P < 0.001$, sActRIIB effect, NS, interaction, NS; total-Akt/GAPDH: IGF-I effect, NS, sActRIIB effect, NS, interaction, NS) and Bonferroni posttest ($***P < 0.001$ vs. saline).

Taken together, our observations indicate that increased IGF-I production or sensitivity does not contribute to the muscle hypertrophy caused by FS. In contrast, insulinopenia induced by STZ injection attenuates the hypertrophic action of FS on skeletal muscle. Moreover, the full anabolic response to FS can be restored in this condition by insulin but also by IGF-I infusion. Therefore, our results indicate that insulin is required for the anabolic action of FS but can be substituted by IGF-I in the case of insulinopenia.

The regulation of IGF-I during the muscle hypertrophy caused by Mstn inhibition remains debated. Such regulation has been suggested by the observation of increased muscle mRNA expression and circulating concentrations of IGF-I in response to Mstn inhibition (35, 62, 64). However, some authors have also reported a decrease in muscle IGF-I mRNA expression in the same conditions (12, 23, 62). The reasons for this discrepancy are unclear. They might result from the choice

of primers for qRT-PCR, which may assess different IGF-I mRNA transcripts (7). In our work, we used specific primers located on exons 3 and 4 to measure the expression levels of all IGF-I mRNA transcripts, and not only the mechanogrowth factor (MGF) (65). Moreover, the approaches to inhibit Mstn differ from one study to another. It is known that FS and sActRIIB-Fc inhibit both Mstn and activin A (ActA) (24, 38, 57) in contrast to a Mstn KO model in which only Mstn is inactivated. Finally, the mechanism of muscle hypertrophy caused by Mstn inhibition may result from fiber hypertrophy and/or hyperplasia according to the stage of development at which the inhibition occurs (28, 44). In this study, we assessed IGF-I mRNA expression in different Mstn inhibition models: prenatal deletion of the Mstn gene (Mstn KO), resulting in fiber hyperplasia (23); postnatal inhibition of Mstn and ActA by sActRIIB-Fc resulting in fiber hypertrophy (11); and prenatal inhibition of Mstn and ActA by FS overexpression, resulting in fiber hyperplasia and hypertrophy (37). In all conditions, we showed that muscle IGF-I expression is systematically reduced regardless of the approach used to inhibit Mstn. Moreover, we demonstrated that this decrease of muscle IGF-I mRNA levels is correlated with low muscle IGF-I peptide concentrations. The possibility that this decrease in muscle IGF-I peptide results from a decrease in extracellular matrix as observed in response to Mstn inhibition (9, 10, 60) is unlikely. Indeed, immunohistochemical studies showed that IGF-I protein is localized mainly in muscle fiber and not in extracellular matrix (40).

The role of IGF-I during the muscle hypertrophy caused by Mstn inhibition has never been investigated. Not only does IGF-I by itself increase skeletal muscle mass (2, 13, 47), but its increased muscle expression in response to several anabolic stimuli suggests that it may contribute to muscle hypertrophy in many conditions (overloading, testosterone, overexpression of PGC-1 α , etc.) (1, 20, 39, 40, 52). Evidence suggests that IGF-I might contribute to FS-induced muscle hypertrophy. Indeed, FS induces muscle hypertrophy by increasing protein synthesis and satellite cell activation, as IGF-I does (5, 6, 24, 58). In addition, Mstn inhibition stimulates the Akt/mTOR/S6K pathway, the pathway mainly responsible for the muscle hypertrophy caused by IGF-I (41, 45, 51). Finally, MKR mice, which are characterized by downregulation of muscle IGF-IR, are resistant to the anabolic effect of FS (34). To investigate the role of IGF-I in FS-induced hypertrophy, we used a model of HYPOX rats. Indeed, this model had already been successfully used to assess the role of IGF-I in testosterone- and load-induced skeletal muscle hypertrophy (25, 56). Furthermore, this animal model is probably the only nonlethal model characterized by a profound and global IGF-I deficiency (4, 42, 49). This model allowed us to demonstrate that the GH-IGF-I axis is not required for FS to exert its anabolic action. Since HYPOX animals still present very low concentrations of IGF-I, we cannot exclude the possibility that these low IGF-I concentrations were sufficient to allow the FS-induced muscle hypertrophy. However, these concentrations were clearly not sufficient to support body and muscle growth. Except if FS increases the muscle sensitivity to the anabolic action of IGF-I, the role of IGF-I in the FS-induced muscle hypertrophy is not supported by our observations.

The possibility for FS to increase muscle sensitivity to IGF-I is suggested by some reports showing that Mstn inhibition

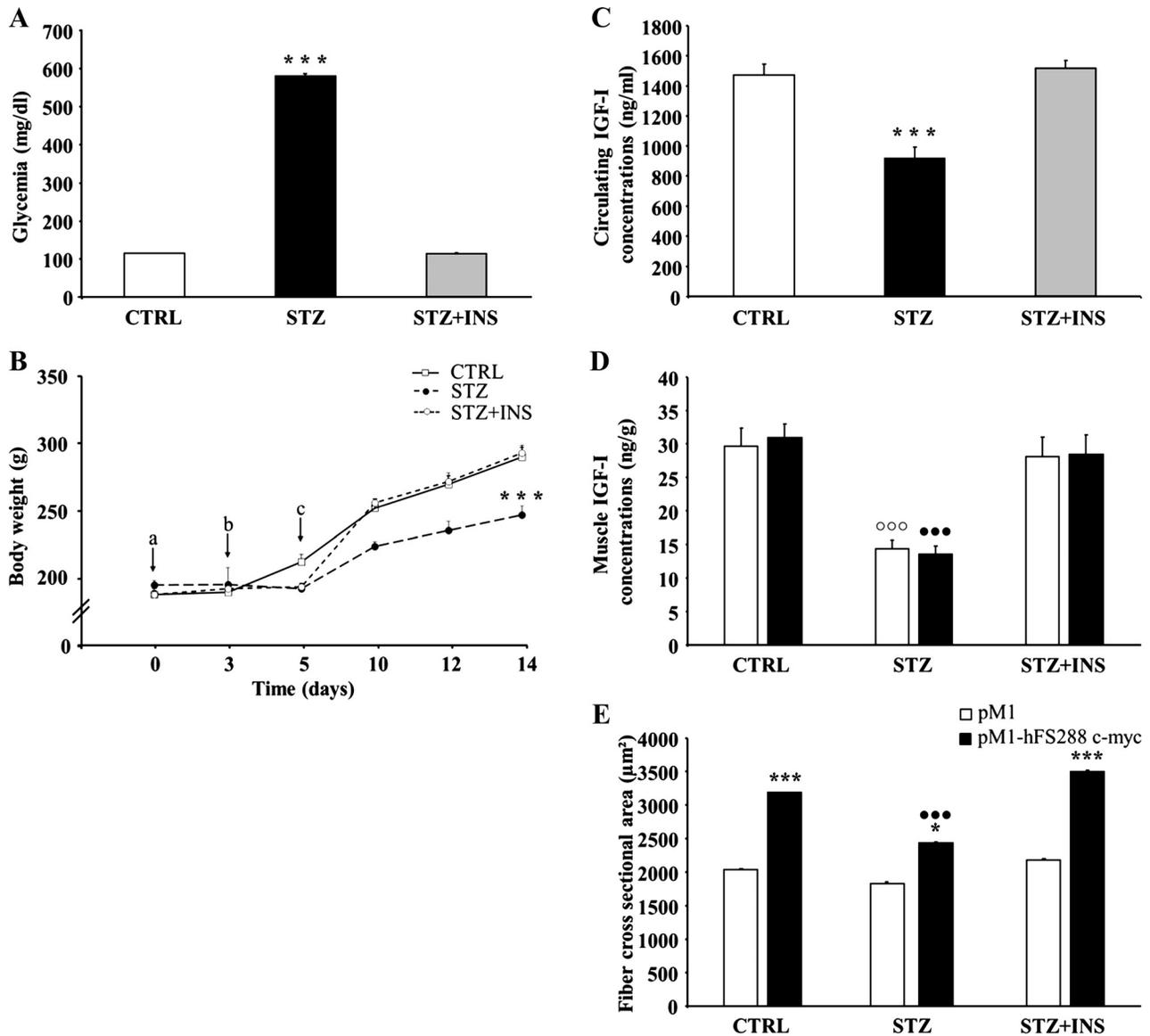


Fig. 5. FS-induced muscle hypertrophy is attenuated in STZ-diabetic animals. **A:** glycemia is significantly higher in STZ-treated rats (filled column) vs. CTRL rats (open column) and is restored to normal levels in STZ rats treated with insulin implants (gray column, $***P < 0.001$ vs. CTRL, $n = 8-10$ /group). **B:** evolution of body weight of CTRL (—), STZ-treated rats (—), and STZ rats treated with insulin (---). Three days after DNA injection (a), rats were treated with STZ (60 mg/kg) (b); 2 days later rats were treated with insulin (4U/day, $***P < 0.001$ vs. CTRL) (c). **C and D:** circulating (C) and muscle (D) IGF-I concentrations are significantly decreased in STZ rats vs. CTRL rats and are restored to normal levels in STZ rats treated with insulin implants. Fiber CSA (E) were assessed 14 days after transfection of pM1 (open column) or pM1-hFS288 (filled column) in CTRL, STZ, and STZ+INS rats ($n = 8-10$ /group). Results are expressed as means \pm SE. Statistical analysis was performed using 1-way ANOVA and Bonferroni posttest ($*P < 0.05$, $***P < 0.001$ vs. contralateral muscle; $oooP < 0.001$ vs. CTRL pM1 muscle; $***P < 0.001$ vs. CTRL pM1-hFS288).

increases the insulin-stimulated activation of Akt and glucose uptake in skeletal muscle (29, 45, 66, 67). Although IGF-I and insulin signaling pathways share multiple intracellular mediators, no study has investigated the muscle IGF-I sensitivity in case of Mstn inhibition. It has been demonstrated that IGF-I and FS promote muscle hypertrophy via the Akt/mTOR signaling pathway leading to the stimulation of protein synthesis (34, 51). Therefore, since Akt is an important mediator in the hypertrophy process, we assessed its activation after injection of IGF-I to investigate the IGF-I sensitivity of the muscle from mTr-FS mice. For the first time, our results demonstrated a decreased IGF-I sensitivity of muscle overexpressing FS. This absence of Akt activation in response to IGF-I contrasts with

the increased pAkt in response to insulin observed in Mstn KO mice (29). The mechanisms operational in this reduction of muscle sensitivity to IGF-I are unknown. The possibility has been suggested for Mstn to interact with the IGF signaling by modulating the local expression of IGF-BPs (16, 62). Interestingly, we observed an increase of muscle IGF-BP-5 expression in response to Mstn inhibition. Since IGF-BP-5 has been shown to inhibit the IGF-I action towards muscle cells (33, 46, 59), its induction by FS could reduce the local bioavailability of IGF-I and attenuate the IGF-I action specifically in the skeletal muscle and not in the liver. Furthermore, as previously described (34), we also showed a decrease in the muscle IGF-IR mRNA expression in response to Mstn inhibition. Therefore,

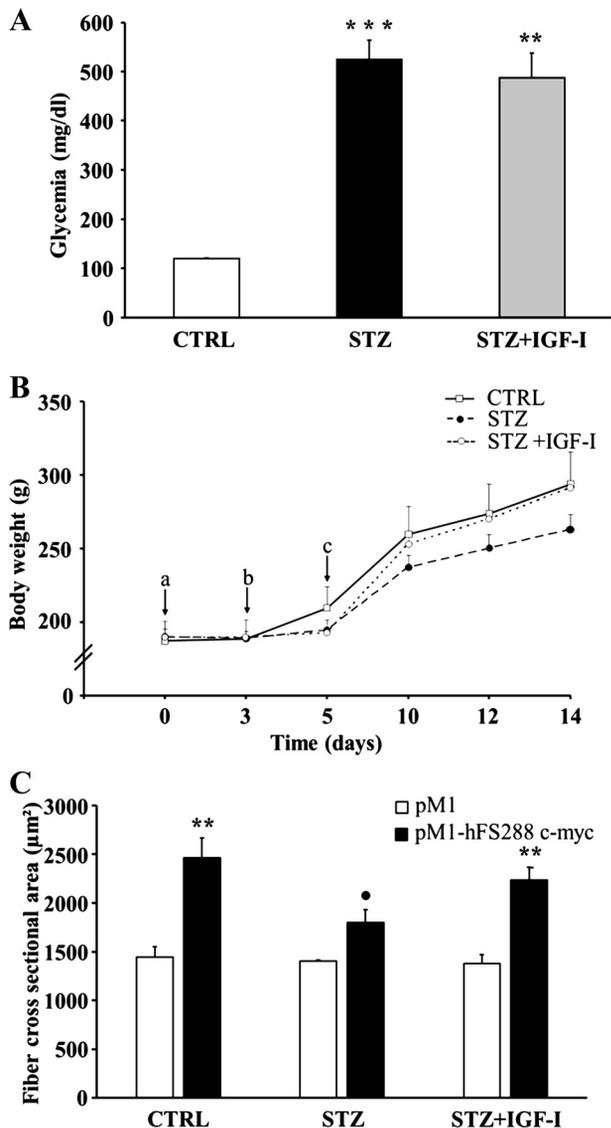


Fig. 6. IGF-I treatment restores FS-induced muscle fiber hypertrophy in STZ-diabetic animals. *A*: glycemia is significantly higher in STZ-treated rats and STZ rats treated with IGF-I vs. CTRL rats (** $P < 0.01$, *** $P < 0.001$ vs. CTRL; $n = 3$ /group). *B*: evolution of body weight of CTRL (—), STZ-treated (---), and STZ rats treated with IGF-I (· · ·). Three days after DNA injection (*a*), rats were treated with STZ (60 mg/kg) (*b*); 2 days later, rats were treated with IGF-I (3 mg·kg⁻¹·day⁻¹) (*c*). *C*: fiber CSA were determined 14 days after transfection of pM1 (open column) or pM1-hFS288 (filled column) in CTRL, STZ, and STZ+IGF-I rats ($n = 3$ /group). Results are expressed as means \pm SE. Statistical analysis was performed using 1-way ANOVA and Bonferroni posttest (** $P < 0.01$ vs. contralateral muscle; * $P < 0.05$ vs. CTRL pM1-hFS288).

we cannot exclude that the decrease of muscle IGF-I sensitivity observed in mTr-FS mice might result from a down regulation of the muscle IGF-IR. Finally, alterations in muscle vascularization in mTr-FS might also impair the access of IGF-I to the membrane IGF-IR. Indeed, Mstn inhibition has been shown to decrease muscle capillary density together with VEGF expression (31, 50). However, this hypothesis is unlikely, as this decrease in muscle capillary density does not seem to impair the stimulation of muscle Akt and glucose uptake by insulin (29). Collectively, these results lead thinking that in response to FS, muscle cells put in place mechanisms inhibiting the

IGF-I actions as a negative feedback loop to prevent greater hypertrophy. This view is supported by the normal muscle IGF-I sensitivity observed at early stages of hypertrophy induced by the sActRIIB-Fc, suggesting that the decreased muscle IGF-I sensitivity observed in mTr-FS mice is secondary to muscle hypertrophy. Therefore, neither increased IGF-I production nor increased IGF-I sensitivity contribute to the muscle hypertrophy caused by FS.

Since the IGF-IR can be activated by IGF-I but also insulin, we investigated the role of these ligands in the skeletal muscle hypertrophy induced by FS. Our results show that low insulin, unlike low IGF-I, severely blunts the FS-induced muscle hypertrophy. This conclusion is also suggested by the results of Wang et al. (61), reporting a reduced hypertrophic effect of sActRIIB-Fc treatment in mice treated with STZ. Since muscle hypertrophy induced by FS results mainly from a stimulation of protein synthesis via the Akt/mTOR pathway (34, 58, 64), insulinopenia could impair the effect of FS through altered protein synthesis. This hypothesis is supported by several observations. First, STZ decreases the activation of the Akt/mTOR pathway (32) required for the anabolic effect of FS (34). Second, insulinopenia stimulates the muscle expression of REDD1, a well-recognized translational repressor inhibiting Akt/mTOR signaling (21, 32), and MAFbx/atrogen-1 (17), a muscle-specific ubiquitin ligase that targets for degradation the protein translation initiation factor eIF3f (14, 15, 36). As we showed an increase in REDD1 and MAFbx/atrogen-1 mRNA expression, it seems that the anabolic effect of FS toward muscle requires the action of insulin, probably to allow stimulation of protein synthesis.

Restoration of the full anabolic effect of FS by IGF-I in STZ animals, despite the persistence of hyperglycemia, indicates that IGF-I may substitute for the anabolic properties but not for the hypoglycemic effect of insulin. A similar observation was already made for bone growth. Indeed, IGF-I treatment restored the longitudinal bone growth of STZ-treated rats without normalization of glycemia (55). The fact that FS retains its hypertrophic action in IGF-I-treated STZ animals despite the presence of hyperglycemia suggests that the failure of FS to stimulate muscle hypertrophy in STZ animals is not due to glucose toxicity. However, the observation that IGF-I restores growth in STZ-diabetic animals without restoring normoglycemia cannot be interpreted as the result of the IGF-I binding to the IGF-IR. Indeed, our work did not investigate which, IGF-IR or insulin receptor (INS-R), is required for FS to exert its anabolic action. Although the ligands insulin and IGF-I can substitute one for the other, this phenomenon does not seem to apply to their receptors. Indeed, deletion of the INS-R (48), like the IGF-IR (43), is associated with decreased muscle growth, suggesting that both receptors are required for proper physiological muscle growth. Moreover, expression of the dominant-negative form of IGF-IR, or MKR, which inhibits the FS-induced muscle hypertrophy (34), impairs both insulin and IGF-I signaling in muscle due to hybrid receptor formation (22). Therefore, since the two receptors are mandatory for proper physiological muscle growth, we cannot specify which of them, INS-R or IGF-IR, is involved in the FS-induced muscle hypertrophy. Ultimately, our study establishes the need of insulin or IGF-I for the full anabolic effect of FS toward skeletal muscle. When insulin or IGF-I alone is missing, FS retains its full anabolic effect. But, when both are deficient, as

in STZ animals, FS fails to simulate muscle growth. In physiological conditions, both hormones probably allow FS to promote muscle hypertrophy. Thus, IGF-I is not absolutely required for FS to induce skeletal muscle hypertrophy except in case of insulinopenia.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.B., S.K., A.L., and J.-P.T. conception and design of research; C.B., S.K., A.L., P.L., and B.F. performed experiments; C.B., S.K., A.L., and P.L. analyzed data; C.B., S.K., A.L., and J.-P.T. interpreted results of experiments; C.B. and S.K. prepared figures; C.B., S.K., and J.-P.T. drafted manuscript; C.B., S.K., A.L., O.R., and J.-P.T. edited and revised manuscript; C.B., S.K., A.L., O.R., P.L., B.F., and J.-P.T. approved final version of manuscript.

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