Myostatin is a key mediator between energy metabolism and endurance capacity of skeletal muscle

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Mouisel E, Relizani K, Mille-Hamard L, Denis R, Hourdé C, Agbulut O, Patel K, Arandel L, Morales-Gonzalez S, Vignaud A, Garcia L, Ferry A, Luquet S, Billat V, Ventura-Clapier R, Schuelke M, Amthor H. Myostatin is a key mediator between energy metabolism and endurance capacity of skeletal muscle. Am J Physiol Regul Integr Comp Physiol 307: R444–R454, 2014. First published June 25, 2014; doi:10.1152/ajpregu.00377.2013.—Myostatin (Mstn) participates in the regulation of skeletal muscle size and has emerged as a regulator of muscle metabolism. Here, we hypothesized that lack of myostatin profoundly depresses oxidative phosphorylation-dependent muscle function. Toward this end, we explored Mstn−/− mice as a model for the constitutive absence of myostatin and AAV-mediated overexpression of myostatin propeptide as a model of myostatin blockade in adult wild-type mice. We show that mice from Mstn−/− mice, although larger and stronger, fatigue extremely rapidly. Myostatin deficiency shifts muscle from aerobic toward anaerobic energy metabolism, as evidenced by decreased mitochondrial respiration, reduced expression of PPAR transcriptional regulators, increased enolase activity, and exercise-induced lactic acidosis. As a consequence, constitutively reduced myostatin signaling diminishes exercise capacity, while the hypermuscular state of Mstn−/− mice increases oxygen consumption and the energy cost of running. We found an increased fatigability and reduced capacity for aerobic exercise capacity. We then compared this phenotype to the effect of myostatin blockade in adult muscle and likewise concluded that the impact of TGF-β signaling is not simply an indirect consequence of congenital fiber-type conversion following constitutive lack of myostatin or whether myostatin regulates muscle metabolism directly. Here, we hypothesized myostatin to regulate oxidative phosphorylation-dependent muscle function and that this would be independent of the muscle fiber-type composition. We first investigated in detail the muscle contractile, metabolic, and functional characteristics of constitutive Mstn−/− mice and found a profound deficit of aerobic exercise capacity. We then compared this phenotype to the effect of myostatin blockade in adult muscle and likewise found an increased fatigability and reduced capacity for aerobic exercise capacity.
bic exercise following overexpression of myostatin propeptide despite an unchanged fiber-type composition. These data and the role of myostatin in the regulation of peroxisome proliferator-activated receptor (PPAR) transcriptional activators comprehensively illustrate the importance of myostatin as a pivotal factor balancing size and strength of skeletal muscle against endurance through adaptation of its energy metabolism.

**METHODS**

**Animals**

$Mstrn^{+/+}, Mstrn^{+/−},$ and $Mstrn^{−/−}$ mice, on a C57BL/6 background (31), were bred using a heterozygous mating system in the animal facility of CERFE (Evry, France) and kept according to institutional guidelines. Investigations on mice (from 2 to 6 mo old) were carried out under the laboratory and animal facility licenses A75-13-11 and A91-228-107. Partly, C57BL/6 control mice ($Mstrn^{+/+}$) were purchased from Charles River (France). Body mass composition (lean tissue mass, fat mass, free water, and total water content) was analyzed using an Echo Medical Systems’ EchoMRI (whole body composition analyzers; EchoMRI, Houston, TX).

**Evaluation of Exercise Performance**

**Evaluation of the critical speed.** The critical speed (CSp) defines the proportional relationship between distance run and time to exhaustion at different velocities. Mice were exercised on a 10.6 × 30 cm double-lane treadmill (LE 8709; Bioseb, Chaville, France) as published previously (8). The protocol consisted of four runs at various speeds (between 20 and 80 cm/s, according to individual motor capacity, one run per day) leading to exhaustion between 3 and 45 min. CSp is based on the hyperbolic relationship between speed and time to fatigue during separate bouts of exhaustive runs performed at different speeds. Therefore, CSp was calculated from the slope ($a$) of the regression line, plotting the distance ($y$) vs. the time to exhaustion ($x$) from the four runs, according to the equation $y = ax + b$ ($b$ being the anaerobic distance capacity).

**Blood Lactate Assessment During Exhaustive Exercise**

Lactate concentration was determined in blood samples (5 μl) collected from the tip of the tail using a Lactate pro LT device (Arkray, Kyoto, Japan) at the time points 0 and 5 min after treadmill running-induced exhaustion. Exhaustion was defined as the time point at which mice could not run anymore and stayed on the grid despite repeated electric stimulation. The running test started at the lowest speed of 5 cm/s to allow a warm-up and was increased by 1 cm/s every 30 s until exhaustion.

**Measurement of Voluntary Locomotion**

Total voluntary locomotor activities were determined in individual cages with bedding, food, and water (Labmaster; TSE Systems, Midland, MI). Animals were acclimated in individual cages for 48 h before experimental measurements. Each cage was embedded in a frame with an infrared light beam-based activity monitoring system, allowing measurement of total locomotor activity. Data were collected in intervals of 40 min during the whole experiment, and the activity level was recorded as the number of beam interruptions per 40 min. Mice had access to food and water ad libitum except for the 24-h fasting to stimulate locomotor activity of mice.

**Measurement of Contractile Properties**

The contractile properties of extensor digitorum longus (EDL) and soleus muscles were studied in vitro according to previously published protocols (2). Muscles were soaked in an oxygenated Tyrode solution (95% O₂ and 5% CO₂) containing 58.5 mM NaCl, 24 mM NaHCO₃, 5.4 mM KCl, 1.2 mM KH₂PO₄, 1.8 mM CaCl₂, 1 mM MgSO₄, and 10 mM glucose (pH 7.4) and maintained at a temperature of 22°C. One muscle tendon was attached to a lever arm of a servomotor system (300B, Dual-Mode Lever; Aurora Scientific, Aurora, ON, Canada). After equilibration (30 min), field electrical stimulation was delivered through electrodes running parallel to the muscle. Pulses of 1 ms were generated by a high-power stimulator (701B, Aurora). Absolute maximal isometric tetanic force ($P₀$) was measured during tetanic contractions (frequency of 50–100 Hz, train of stimulation of 1,500 ms for soleus and 750 ms for EDL). The muscle length was adjusted to an optimum (L₀) that produced $P₀$. Specific maximal isometric force ($sP₀$) was calculated by dividing the force by the estimated cross-sectional area (CSA) of the muscle. Assuming muscles have a cylindrical shape and a density of 1.06 mg/mm³, the CSA corresponds to the volume of the muscle divided by its fiber length (Lf). The Lf to L₀ ratio of 0.70 (soleus) or 0.45 (EDL) was used to calculate Lf. Maximal power ($P_{max}$) was determined from force-velocity data that were obtained by eliciting contractions (train of 1,000 ms, 150 Hz) at 3–5 different afterloads (10–40% $P₀$). Specific $P_{max}$ ($sP_{max}$) was calculated by dividing $P_{max}$ by muscle weight. Fatigue resistance was then determined after a 5-min rest period. The muscles were stimulated at 75 Hz during 500 ms, every 2 s, for 3 min. The time taken for initial force to fall by 50% (EDL) or 30% (soleus) was then measured. All data were recorded and analyzed on a microcomputer, using the PowerLab System (4SP; AD Instruments, Dunedin, New Zealand) and software (Chart 4; ADInstruments).

The isometric contractile properties of gastrocnemius (+soleus) were studied in situ, as previously described (22). Mice were anesthetized (pentobarbital sodium, 50 mg/kg ip). Supplemental doses were given as required to maintain deep anesthesia throughout the experiments. The foot was fixed with a clamp, and the knee was immobilized using stainless-steel pins. The distal tendon of the plantaris muscle was cut. The Achilles tendon was attached to an isometric force transducer (Harvard). Great care was taken to ensure that the blood and nerve supply remained intact during surgery. The sciatic nerves were severed proximally and stimulated distally by a bipolar
silver electrode using supramaximal square wave pulses of 0.1-ms duration. All isometric measurements were made at an initial muscle length of L0. Force productions in response to tetanic stimulations (P0) were successively recorded (pulse frequency from 50 to 150 Hz, train duration of 500 ms), and at least 1 min was allowed between each contractions. The muscle mass (m) was measured to calculate sP0. Fatigue resistance was then determined after a 5-min rest period. The muscle was stimulated during 500 ms at 100 Hz, every 2 s, for 3 min. The duration corresponding to a force decreased by 50% was noted. After contractile measurements, the animals were killed with an overdose of pentobarbital. Muscles were then weighed, frozen in liquid nitrogen or in isopentane precooled in liquid nitrogen, and stored at −80°C until histology or biochemical analyses.

Mitochondrial Respiration and Cytosolic Enzyme Measurements

Measurement of OXPHOS activity. The mitochondrial respiration was studied in vitro in saponin-skinned fibers. Briefly, fibers were separated under a binocular microscope in solution S at 4°C (see below) and permeabilized in solution S with 50 μg/ml of saponin for 30 min. After being placed 10 min in solution R (see below) to wash out adenine nucleotides and creatine phosphate, skinned separated fibers were transferred into a 3-nl water-jacketed oxygraphic cell (Strathkelvin Instruments, Glasgow, UK), equipped with a Clark electrode, as previously described (5), under continuous stirring. Solutions R and S contained the following: 2.77 mM CaK2EGTA, 7.23 mM K2EGTA (100 mM free Ca2+), 1 mM free Mg2+, 20 mM taurine, 0.5 mM DTT, 50 mM potassium-methane sulfonate (160 mM ionic strength), and 20 mM imidazole (pH 7.1). Solution S also contained 5.7 mM Na2ATP, 15 mM creatine-phosphate, while solution R contained 5 mM glutamate, 2 mM malate, 3 mM phosphate, and 2 mg/ml FA free bovine serum. After the experiments, fibers were harvested and dried, and respiration rates were expressed as micromoles of O2 per min per gram dry weight. Solution R was similar to solution R without substrates and was used to determine maximal VO2 rates for different substrates.

Measurement of the maximal muscular oxidative capacities. After the determination of the basal respiration rate VO2, the maximal fiber respiration rate was measured at 22°C in the presence of a saturating (2 mM) ADP concentration as phosphate acceptor and glutamate-malate as mitochondrial substrates (VGmax). The acceptor control ratio was VGmax/VO2 and represented the degree of coupling between oxidation and phosphorylation.

Measurement of the respiratory chain complexes. When VGmax was recorded, electron flow goes through complexes I, II, III, and IV. Then 4 min after this VGmax measurement, the complex I was blocked with amyntal (2 mM), and then complex II was stimulated with succinate (VS). After that, cytochrome-c oxidase (complex IV) was studied by 10.220.33.3 on June 15, 2017 http://ajpregu.physiology.org/ Downloaded from

RT-quantitative PCR

Total RNA was extracted from frozen muscle after pulverization in liquid nitrogen and from cultured C2C12 myoblast cells with the TRIzol (Invitrogen, Carlsbad, CA) extraction protocol. 2.25 μg of total RNA were reverse-transcribed using the ThermoScript RT-PCR System (Invitrogen) with random hexamers in a 60-μl reaction volume, of which we used 4 μl for each subsequent quantitative PCR (qPCR)-reaction and 2 μl of a 1:10 dilution for the 18S reference gene. We used the following oligonucleotide primers for qPCR: 18S rRNA (reference gene): [forward (F)] 5′-CAT TCG AAC GTC TGG CCT ATC-3′; [reverse (R)] 5′-CTC CCT CTC CGG AAT CGA AC-3′; Ppara: [F] 5′-GGG CAA GAG AAT CCA CGA AG-3′; [R] 5′-CGT CTT CTC GGC CAT ACA CA-3′; Pparbld: [F] 5′-AGC CAC AAC GCA CCC TTT-3′; [R] 5′-CGG TAG AAC ACG TCA CTG ACA-3′; Ppargc1a: [F] 5′-CGA TGG TGT GGG GAT AAA GC-3′; [R] 5′-GGA TCC GGC AGT TAA GAT CA-3′; Ppargc1a: [F] 5′-GAA AGC GCC AAA CAG AGA GA-3′, [R] 5′-GTA AAT CAC ACG GCC
CCTT-3’. The qPCR for each sample was run with the SYBR Green protocol (Applied Biosystems, Foster City, CA) in triplicate on an ABI PRISM 7700 sequence detection system (Applied Biosystems) with a hotstart Taq polymerase. A 10-min denaturation step at 94°C was followed by 45 cycles of denaturation at 94°C for 10 s and annealing/extension at 60°C for 30 s. Before sample analysis we had determined for each gene the PCR efficiencies with a standard dilution series (10^6–10^0 copies/μl), which subsequently enabled us to calculate the copy numbers from the Ct values, using the −ΔΔCt method.

Statistical Analysis

Data were analyzed using either one-way ANOVA, followed by a Tukey post hoc test, or paired/unpaired Student’s t-tests. Values are presented as means ± SE. The significance level was set at P < 0.05.

RESULTS

Decreased Endurance Exercise Capacity and Voluntary Locomotor Activity in Myostatin Deficiency

An index for endurance exercise capacity is the maximal oxygen uptake per body weight (VO2max [ml O2·min⁻¹·kg⁻¹]), which was determined by running on a treadmill at incremental speeds in a metabolic cage. Initially, the oxygen uptake is proportional to the running speed but levels off at a plateau, the so-called VO2max, beyond which no further increase is possible. Nevertheless, running velocity can still increase beyond the speed at VO2max to reach the maximal velocity [vPeak (m/min)] before exhaustion of the animals. VO2max of Mstn⁻/⁻ mice was reduced by 10% (P = 0.004; Fig. 1A), and a similar tendency was shown for vPeak compared with Mstn⁺/+ mice (P = 0.13; Fig. 1B), while heterozygous Mstn⁺/+ mice had an intermediate phenotype. Hence, such decreased oxygen consumption in vivo parallels the decreased oxidative phosphorylation rates in vitro (Fig. 2G). However, absolute VO2max (ml/min) of Mstn⁻/⁻ mice was increased by 14% (P = 0.004; Fig. 1C), owing to a respective 20% and 24% increase of total and lean body mass (P < 0.01; data not shown). In consequence, the energy cost of running (running economy at 13 m/min) was increased by 15% in Mstn⁻/⁻ compared with Mstn⁺/+ mice (P = 0.01; Fig. 1D).

“Critical speed” accurately reflects the capacity for aerobic exercise and is based on the proportional relationship between distance run and time to exhaustion at different velocities (8). Mstn⁻/⁻ mice became exhausted more rapidly, resulting in a 30% lower critical speed compared with Mstn⁺/+ mice (15.9 ± 1.2 vs. 22.9 ± 1.2 m/min, P < 0.001; Fig. 1E). These findings provide further evidence that the double muscle phenotype that we observed in Mstn⁻/⁻ mice (16.4 ± 0.3 vs. 8.4 ± 0.2 mg for soleus muscle, compared with Mstn⁺/+ mice, P < 0.001; similar observations were made for other hind limb muscles) cannot compensate for inefficient energy metabolism to maintain endurance capacity. The respiratory exchange ratio (RER), CO2eliminate/O2consumed, indicates the type of fuel being metabolized to supply the body with energy. Resting and maximal RER were slightly increased in Mstn⁻/⁻ mice and even further increased in Mstn⁻/⁻ mice compared with wild-type animals (Fig. 1F). This implicates a preference for glycolysis over fatty acid oxidation, which is considered to be disadvantageous for endurance exercise (46).

To evaluate the impact of decreased endurance capacity on voluntary locomotion, total night-time activity was measured and revealed no significant difference between Mstn⁻/⁻ and Mstn⁺/+ mice, although we observed a trend toward lower total locomotor activity in myostatin deficiency (1,792 ± 279 counts/12 h and 2,333 ± 255 counts/12 h respectively, P = 0.16). However, upon a metabolic challenge consisting of food deprivation, Mstn⁻/⁻ mice failed to increase their locomotor activity compared with the marked increase seen in Mstn⁺/+ mice (Fig. 1G), which further demonstrates that myostatin deficiency impairs locomotion.

Profound Fatigability of Myostatin-Deficient Skeletal Muscle

To assess the contribution of skeletal muscle fatigability to the decrease of endurance capacity in Mstn⁻/⁻ mice, we next determined how muscle force was maintained upon repetitive stimulation. Soleus muscle from Mstn⁻/⁻ mice fatigued far more rapidly following repetitive stimulation [t(30% P0) = 72 s] compared with Mstn⁺/+ soleus [t(30% P0) = 100 s; P < 0.001], while heterozygous Mstn⁺/+ muscle had an intermediate phenotype (Fig. 2A). Remarkably, myostatin-deficient muscle, despite being about twice as strong at the beginning of the experiment (389 ± 11 vs. 233 ± 5 mN for soleus absolute maximal tetanic force P0, compared with Mstn⁺/+ mice, P < 0.001), fatigued so rapidly that absolute maximal force dropped to Mstn⁺/+ levels after 3 min of repetitive tetanic stimulation (Fig. 2B). This rapid force decline caused the specific force of Mstn⁻/⁻ muscles to decrease from 91% at the start to 59% at the end of the fatigue protocol compared with Mstn⁺/+ muscles (P = 0.04 and P < 0.001, respectively; Fig. 2C). Interestingly, similar results were found for the fast glycolytic extensor digitorum longus muscle (EDL, −21% concerning the fatigue index in Mstn⁻/⁻ vs. Mstn⁺/+), as well as for the entire posterior lower leg compartment (fatigue index was decreased by 48% in Mstn⁻/⁻ vs. Mstn⁺/+), for which measurements were performed in situ to maintain blood perfusion during the stimulation protocol (data not shown).

Increased Glycolysis and Decreased Mitochondrial Respiration Rates in Myostatin Deficiency

To investigate whether increased muscle fatigability in the absence of myostatin resulted from increased anaerobic glucose metabolism-induced muscle acidosis, we determined serum lactate levels after exhaustive treadmill exercise. In Mstn⁻/⁻ mice, serum lactate was already elevated at resting state and increased disproportionately to 12.1 ± 1.1 mmol/l at 5 min postexercise compared with 5.1 ± 0.4 mmol/l in controls (P < 0.001; Fig. 2D). The elevated serum lactate in myostatin-deficient mice concurred with an increased enzymatic activity of enolase (Fig. 2E), a key component of glycolysis. To determine whether lactate accumulation resulted from defective oxidative phosphorylation (OXPHOS), we investigated mitochondrial respiration rates in situ for OXPHOS complexes I, II, and IV. Mstn⁻/⁻ muscles revealed higher respiration rates for the predominantly oxidative soleus muscle compared with the predominantly glycolytic EDL muscle (Fig. 2G). Remarkably, the absence of myostatin decreased OXPHOS rates of the soleus muscle to the level of Mstn⁺/+ EDL muscles, and Mstn⁻/⁻ EDL further lost OXPHOS activity of up to 42% (Fig. 2G). It is unlikely that such OXPHOS reduction was merely due to mitochondrial depletion, because complex I (CxI) activity remained unaltered. The CxII/CxI and CxIV/CxI ratios decreased in myostatin-deficient muscle, which might be an indicator for qualitative changes in the assembly of the cyto-
chrome-c oxidase (complex IV) and of the entirely nuclear encoded succinate dehydrogenase (complex II), (Fig. 2F). In fact, the biochemical profile of mitochondria from Mstn−/− soleus muscle resembled that of Mstn+/− EDL mitochondria and suggested a shift of mitochondrial qualities from the “slow oxidative” to the “fast glycolytic” type. This shift in metabolic activity was accompanied by a profound conversion of the contractile phenotype of Mstn−/− soleus muscle away from slow/oxidative MHC-1 toward fast/glycolytic MHC-2x/MHC-2b (Fig. 3, A–C). In line with these observations are the findings that the Km(ADP) in resting soleus muscle was much higher than that for EDL muscle (Fig. 2H). Km(ADP) was decreased by the addition of creatine, in both EDL and soleus muscle, demonstrating the coupling between mitochondrial creatine kinase and the adenine nucleotide translocase. The absence of myostatin lowered the Km(ADP) of the soleus muscle toward the level of the fast glycolytic EDL muscle, and the Km(ADP) of Mstn−/− EDL decreased even further (Fig. 2H).

**Decreased Expression of PPAR Transcription Factors in Myostatin-Deficient Muscle**

We next aimed to gain insight into the molecular mechanism of the metabolic dysregulation observed in myostatin deficiency. We hypothesized that myostatin might act in a signaling cascade upstream of PPAR transcriptional regulators because inactivation of myostatin, as well as of Pparβ/δ (43),
both resulted in a similar loss of the oxidative phenotype. In wild-type mice, as expected, Pparβ/d, Ppara, and Pparg mRNA expression levels were 2–3 times higher in the predominantly oxidative soleus muscle than in the predominantly glycolytic EDL muscle. As predicted, PPAR mRNA levels in the soleus muscle of Mstn/H11002/H11002 mice (Fig. 3D) fell to about the level seen in Mstn/H11001/H11001 EDL muscle (Fig. 3E), while in the EDL muscle of Mstn/H11002/H11002 mice fell below the already low values found in Mstn/H11001/H11001 EDL, although this was statistically significant only for Ppara (Fig. 3E). Together, these results suggest that myostatin may promote high oxidative metabolism in skeletal muscle via PPAR signaling.

Myostatin Blockade by AAV-Propeptide in Adult Mice Increases Fatigability

To determine the role of myostatin on energy-dependent muscle function during adulthood, we overexpressed myostatin propeptide using AAV as expression vectors. Injection of AAV2/8-propeptide into the femoral artery led to robust transgene expression (Fig. 4A) and a slight increase of the lower leg muscle weight (Fig. 4B). Importantly, soleus muscle fatigued more rapidly after propeptide treatment (Fig. 4D), despite only minimal changes of absolute maximal and specific force at the start of the fatigue protocol (Fig. 4D) and an unaltered fiber-type composition (Fig. 4C). Interestingly, mRNA levels of Pparβ/d and Ppargc1a transcripts were reduced (Fig. 4E), suggesting changes in the regulation of oxidative metabolism independent of muscle fiber-type composition. Moreover, myostatin propeptide treatment diminished exercise capacity 6 mo after systemic intravenous treatment with myostatin AAV2/8-propeptide (Fig. 4, F and G), despite similar body weights (24.1 ± 0.7 g vs. 24.4 ± 0.6 g for mice treated with PBS vs. AAV2/8-propeptide, respectively; P = 0.79). Hence, we were able to show that myostatin blockade in adult wild-
type mice caused a similar deficit in aerobic muscle properties, as shown for Mstn−/− mice and that these effects were independent of muscle fiber-type composition.

DISCUSSION

Myostatin exerts a dual function on skeletal muscle as it limits its size, while promoting oxidative properties. Here, we show that myostatin acts to economize muscle energy expenditure, because smaller muscle requires less oxygen during exercise. The higher OXPHOS activity and lower respiratory exchange ratio point toward increased fatty acid consumption as a preferred fuel in the presence of myostatin and suggests higher energy efficiency compared with the energetically less efficient glycolysis in states of myostatin deficiency. The emerging property of myostatin to save fuel combined with a simultaneous increase in running endurance and maximal running velocity might explain the high conservation of myostatin during evolution and the rare occurrence of myostatin mutations. The comparison of muscle physiology between hypermuscular myostatin knockout and wild-type mice sheds light on the fact that myostatin-deficient muscle confers little functional advantage over wild-type muscle due to its rapid fatigability. We have demonstrated that the fatigability and diminished capacity for forced and voluntary locomotor activities seen in Mstn−/− described by us and others (18, 29, 39, 42) goes in parallel with a reduction of muscle OXPHOS activities. Interestingly, recent in vivo investigations using 31P-magnetic resonance spectroscopy supports our findings as the relative contribution of oxidative ATP production to total ATP turnover was reduced following repeated isometric contractions of Mstn−/− muscles, whereas the ATP cost of contraction was increased (15). Thus, muscle strength due to myostatin deficiency comes at the cost of exercise intolerance, which is often seen in patients with mitochondrial disorders, such as MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) or MERRF (myoclonic epilepsy with ragged red fibers) syndrome (24). Interestingly, muscle cramps are frequently observed in whippet dogs with Mstn mutation (37). Moreover, “double muscle cattle”, several breeds of which have been identified to carry Mstn mutations (17, 30), are prone to exercise-induced lactic acidosis and severe rhabdomyolysis (20, 21).

Fig. 3. Effect of myostatin deficiency on myofiber-type composition (A–C) and expression of PPAR transcription factors (D–E). A: images of fiber-type composition of soleus muscle from Mstn+/− mice and Mstn−/− mice. Immunohistochemistry was performed to depict MHC-1 fibers (green), MHC-2a fibers (purple), MHC-1/2a hybrid fibers (orange), nonstained MHC2x or MHC2b fibers (black), and laminin (blue). B: relative fiber-type distribution from entire transverse sections of the soleus muscle following immunostaining described above. C: SDS-PAGE electrophoresis of MHC isoforms shows an additional band of MHC-2b expression in Mstn−/− mice. D and E: relative Ppara, Ppargc, and Pparg mRNA copy numbers in the soleus muscle (D) and EDL muscle (E) from Mstn+/− mice and Mstn−/− mice, as expressed per 106 18S rRNA copies. Values are expressed as means ± SE. Number (n) of muscles analyzed: n ≥ 5 for each genotype.
However, a number of questions result from our work. We ask whether the observed decrease in oxidative metabolism and energy-dependent muscle function might be an indirect effect and a consequence of the profound congenital fiber-type change that is typically found in the constitutive absence of myostatin. Thus, we blocked myostatin in adult wild-type mice using myostatin propeptide, which did not affect fiber-type composition. This is in agreement with previous studies fol-

Fig. 4. Muscular and systemic effects of AAV-propeptide-mediated adult myostatin blockade. A–E: functional, morphometric, and metabolic analysis of the hind limb muscles 1 mo after injection of AAV2/8-myostatin-propeptide or PBS into femoral arteries of 2-mo-old C57BL/6J mice. A: RT-PCR depicting exogenous myostatin-propeptide expression only after AAV2/8 transfection (lane 1: molecular weight marker; lanes 2–6: individual muscles). B: wet weights for soleus, extensor digitorum longus (EDL), plantaris, and tibialis anterior (TA) muscles. C: soleus relative myofiber-type distribution. D: fatigue index (left) and specific tetanic force (right) of the soleus muscle at the beginning and at the end of the fatigue protocol. E: relative *Pparβd* and *Pgc1α* mRNA copy numbers in the TA muscle. F and G: exercise capacity of C57BL/6J mice treated systemically (intravenously) with AAV2/8-myostatin-propeptide or PBS. F: effect of AAV2/8-myostatin-propeptide on critical speed before and 6 mo after systemic application. G: plot depicts the proportional relationship between distance run (y-axis) and time to exhaustion (x-axis) at different velocities. The slope of the regression line indicates the critical speed. Values are shown as means ± SE. Number (n) of muscles or mice examined: n = 4–6.
ollowing blockade of myostatin or its activin IIB receptor (ActRIIB) signaling (3, 11, 12, 36). Importantly, treatment with soluble ActRIIB-Fc to block myostatin and homolog signaling factors caused an mRNA expression profile away from oxidative metabolism (41). Supporting the hypothesis that myostatin regulates oxidative metabolism independently of muscle fiber composition, we found here that treatment with myostatin propeptide caused muscle fatigability and decreased aerobic exercise capacity.

We show that myostatin deficiency has an influence on the expression of Ppar-α/β/γ transcription factors, which control metabolic properties but not muscle mass (43, 45). This indicates that myostatin may control the muscle oxidative phenotype notably via PPAR activity. Indeed, downstream targets of Ppar-β, such as Pgc1-α and Cox4, were downregulated in Mstn−/− mice (26). Furthermore, knockout of Pparb, similar to the findings in Mstn−/− mice, reduced oxidative properties of skeletal muscle (43). Importantly, we show here that myostatin blockade in adult mice following overexpression of myostatin propeptide also reduced expression of Pparb and Pgc1a, supporting the hypothesis that myostatin directly regulates oxidative metabolism. However, the exact molecular mechanism remains to be elucidated, and, as yet, little is known about direct molecular targets of myostatin signaling.

Interestingly, distinct but complementary effects on the metabolic profile of obese insulin-resistant mice occur when Ppar-β is activated and myostatin inhibited (7). Moreover, work on myostatin-mediated effects through AMPK (23, 44, 48) raise a number of questions concerning mediators and signaling pathways implicated on muscular metabolic effects of myostatin. It would be of interest to substantiate these findings by an analysis of the muscle microRNA network, as this was recently shown to control metabolism via nuclear receptors, such as PPARs (14).

A further question concerns the problem of whether muscle hypertrophy following lack of myostatin changes the oxidative muscle metabolism. In fact, we previously have shown that long-term exercise improved contractile and metabolic features of Mstn−/− mice, however, these improvements were associated with a loss of muscle hypertrophy (28). These results suggest that regulation of muscle size and metabolic phenotype by myostatin are linked. It remains to be determined, however, whether the regulation of both processes can be dissociated from each other.

Can we generalize the conclusion of our work that lack or blockade of myostatin always negatively affects aerobic muscle function? It is important to note that previous work demonstrated a beneficial effect of myostatin blockade during aging (38). It is quite likely that myostatin blockade in conditions of muscle atrophy through pathological upregulation of myostatin (e.g., in sarcopenia, cardiac cachexia, and tumor cachexia) might outweigh the negative effect on muscle metabolism by far (19, 27, 49). Similarly, myostatin blockade improved running performance in obese and insulin-resistant (ob/ob) mice (7). Again, benefits to insulin signaling and glucose metabolism may largely outweigh potential negative effects on muscle oxidative metabolism, especially if combined with a Ppar-β agonist. It should be noted that treatment of adult mice under a high-fat diet with soluble ActRIIB did not alter fat mass and glucose metabolism (33), whereas treatment of obese and insulin-resistant mice (ob/ob) with antymostatin antibodies improved glucose homeostasis and glucose tolerance (7). In fact, the metabolic changes following myostatin blockade could be beneficial for patients with insulin resistance, and recently, it was shown that AAV-propeptide overexpression mediated higher glucose uptake in skeletal muscle, which is likely mediated by an upregulation of membrane glucose transporters (12). Thus, further work is required to define under which circumstances myostatin blockade could exert beneficial effects to combat insulin resistance and overweight.

In conclusion, our results suggest that myostatin increases oxidative metabolism of skeletal muscle, thereby improving exercise endurance. These fundamental functions of myostatin should be taken into account when developing therapies based on myostatin blockade. Further investigations are required to answer the question of whether emerging therapies based on PPAR agonists might prevent adverse effects of myostatin blockade on oxidative metabolism and exercise tolerance.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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