Exercise restores decreased physical activity levels and increases markers of autophagy and oxidative capacity in myostatin/activin-blocked mdx mice

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Hulmi JJ, Oliveira BM, Silvennoinen M, Hoogaars WM, Pasternack A, Kainulainen H, Ritvos O. Exercise restores decreased physical activity levels and increases markers of autophagy and oxidative capacity in myostatin/activin-blocked mdx mice. Am J Physiol Endocrinol Metab 305: E171–E182, 2013.—The importance of adequate levels of muscle size and function and physical activity is widely recognized. Myostatin/activin blocking increases skeletal muscle mass but may decrease muscle oxidative capacity and can thus be hypothesized to affect voluntary physical activity. Soluble activin receptor IIB (sActRIIB-Fc) was produced to block myostatin/activins. Modestly dystrophic mdx mice were injected with sActRIIB-Fc or PBS with or without voluntary wheel running exercise for 7 wk. Healthy mice served as controls. Running for 7 wk attenuated the sActRIIB-Fc-induced increase in body mass by decreasing fat mass. Running also enhanced/restored the markers of oxidative capacity and autophagy in mdx mice to or above the levels of healthy mice. Voluntary running activity was decreased by sActRIIB-Fc during the first 3–4 wk correlating with increased body mass. Home cage physical activity, quantified from the force plate signal, was decreased by sActRIIB-Fc the whole 7-wk treatment in sedentary mice. To understand what happens during the first weeks after sActRIIB-Fc administration, when mice are less active, healthy mice were injected with sActRIIB-Fc or PBS for 2 wk. During the sActRIIB-Fc-induced rapid 2-wk muscle growth period, oxidative capacity and autophagy were reduced, which may possibly explain the decreased running activity. These results show that increased muscle size and decreased markers of oxidative capacity and autophagy during the first weeks of myostatin/activin blocking are associated with decreased voluntary activity levels. Voluntary exercise in dystrophic mice enhances the markers of oxidative capacity and autophagy to or above the levels of healthy mice.

Myostatin; activin receptor IIB; exercise; skeletal muscle; hypertrophy

ADEQUATE SIZE AND FUNCTION of skeletal muscle are of paramount importance for health (3, 11, 49, 58, 60). Duchenne muscular dystrophy (DMD) is a disease characterized by progressive wasting of skeletal muscle (28). Restoration of dystrophin expression in all muscles of the body is difficult, and the effectiveness of such treatment likely depends on the muscle quality of DMD patients. Therefore, approaches aimed at stimulation of muscle growth and function are being developed that may complement dystrophin restoration approaches.

Type IIB activin receptors (ActRIIB) are expressed in skeletal muscle (21, 37) and mediate the signaling of myostatin and activins (37, 59). Myostatin and activin are efficiently blocked by systemic injection of a soluble ligand binding domain of ActRIIB fused to the Fc domain of IgG (sActRIIB-Fc) (38). sActRIIB-Fc rapidly increases muscle mass and function and alleviates the disease phenotype in a number of mouse models (1, 15, 22, 24, 46, 49, 60). Recent evidence showed that it also increases muscle mass without reported major side-effects in humans (5), supporting its possible therapeutic use in the future in some neuromuscular diseases or in muscle wasting.

Booth and Laye conclude in their review (7) that studying only sedentary animals and not allowing them to perform normal wild-type physical activity levels may lead to inaccurate conclusions. Adequate level of physical activity and exercise has well-established beneficial effects on skeletal muscle size and function as well as on overall health (32, 53). On the other hand, low aerobic capacity is a risk factor for cardiovascular and metabolic diseases (57). Although as mentioned above the lack or inhibition of myostatin seems to have various positive effects on muscle, there is also some evidence that it can decrease muscle oxidative capacity (2, 24, 41, 42, 50). Thus, treatments to positively complement myostatin-blocked or -deficient muscle are needed. Recent evidence indicates that muscle endurance seems to be positively affected by exercise when combined with myostatin blocking (35) and after myostatin deletion (41, 42). Because submaximal exercise seems to offer some benefits in mdx mice, a relatively mild disease model of DMD (9, 23, 34), exercise in combination with myostatin and activin blocking may offer further benefits in DMD treatment.

Maintaining autophagy at normal levels is important to rejuvenate organelles and to prevent accumulation of dysfunctional proteins (39, 40, 43). Recent evidence also suggests that autophagy may be impaired in muscle dystrophy (12) and that increased autophagy by exercise accounts for exercise benefits (18, 20). The effects of myostatin/activin blocking alone or in combination with exercise on autophagy are not known.

Myostatin/activin blocking rapidly increases skeletal muscle mass, but its effects on physical activity as well as the effects of combinatorial exercise are unclear. Here, we demonstrate the opposite and compensatory effects of exercise and myostatin blocking on the level of physical activity and skeletal muscle autophagy and oxidative capacity.

MATERIALS AND METHODS

Animals. In the experiments we used 6- to 7-wk-old male mice from the Jackson Laboratory (Bar Harbor, ME). The modestly dystrophic mdx mice were from a C57Bl/10ScSnJ background, and the wild-type mice were from the C57Bl/10ScSnJ strain. The mice were

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Myostatin/Activin Blocking and Exercise

Housed in standard conditions (temperature 22°C, light from 8:00 AM to 8:00 PM) and had free access to tap water and food pellets (R36, 4% fat, 55.7% carbohydrate, 18.5% protein, 3 kcal/g, Labtor, Stockholm Sweden).

Ethics statement. The treatment of the animals was in strict accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The protocol was approved by the National Animal Experiment Board (Permit No. ESLH-2009-08528/Ym-23).

Experimental design. In the long, 7-wk experiment, the mdx mice were randomly divided into four groups: 1) PBS control, 2) PBS running, 3) sActRIIB-Fc, and 4) sActRIIB-Fc running (n = 8 in each). Wild-type mice served as healthy controls (n = 5). All animals were housed in their individual cages. sActRIIB-Fc or PBS was injected intraperitoneally once a week with a 5 mg/kg dose of sActRIIB-Fc. The chosen exercise modality was voluntary wheel running. To allow the treatment to take effect, the running wheels were locked during the first injection day and the following day, preventing mice from exercising. On the last 2 days, the mice did not have access to running wheels, so that outcome effects would not reflect the acute effects of exercise.

In the short, 2-wk experiment, the C57Bl/10ScSnJ mice were randomly divided into three groups: 1) PBS, 2) sActRIIB-Fc once per week, and 3) sActRIIB-Fc twice per week (n = 6 in each). The 5 and 10 mg/kg subgroups in groups 2 and 3 were pooled for the further analysis as no differences were found between the 5 and 10 mg/kg doses (see Ref. 24).

In the third experiment, the C57Bl/10ScSnJ mice were divided into three groups (n = 6 in each group): 1) euthanized 1 or 2 days after a single injection of PBS, 2) euthanized 1 day after, and 3) euthanized 2 days after sActRIIB-Fc.

During the experiments, all the conditions were standardized. The mice were euthanized after the experiments by cervical dislocation, and tissue and blood samples were collected.

sActRIIB-Fc production. The recombinant fusion protein effectively blocking myostatin and activins (24) was produced and purified in house as described in detail earlier (4, 22, 24). In brief, the fusion protein contains the ectodomain (ecd) of human sActRIIB and a human IgG1 Fc domain. The protein was expressed in Chinese hamster ovary (CHO) cells grown in suspension culture.

Voluntary wheel running, feed intake, and muscle function. Voluntary wheel running was selected as the exercise modality in this study, as it may offer benefits in mdx mice (9, 34), whereas forced exercise, such as running, may sometimes even be detrimental to dystrophic mice or humans (16). Moreover, with voluntary monitored running we were able to determine whether the blocking of myostatin/activins affects running activity during the treatment period.

The mice were housed individually in cages where they had free access to custom-made running wheels (diameter 24 cm, width 8 cm) 24 h/day. Total running distance was recorded 24 h daily. Sedentary animals were housed individually in similar cages without the running wheel. Body mass and feed consumption were measured every week throughout the study.

Strength endurance and body coordination were tested by the hanging wire test according to published methods (56). Mice were suspended above a metal wire secured 30 cm above a padded cell and released. Depending on the functional ability of the mouse, use of all limbs and the tail were accepted during a 10-min hanging session. Three trials were given and the longest hanging time (up to the maximum of 10 min) was used for the further analysis.

Home cage activity by force plate recording. Vertical ground reaction forces were measured using a custom-made force plate (linearity 99%, cross-talk <2%). Forces were measured by four uniaxial strain gauges which were rectangularly arranged under the corners of a glass plate on which the animal housing cage was located. A 14-bit A/D converter (DI-710; DATAQ Instruments, Akron, OH) was used for digitizing the force data at a sampling rate of 80 Hz. The measurement range of the strain gauge was ±250 g, while the digitization precision was ±0.02 g. The outcome variable distance was calculated similarly as previously (51). The force plate recording has been validated against video recording and found to be perfectly valid (51).

After the animal housing, the cage without the animal was positioned on the glass plate, and the force levels of all gauges were adjusted to zero. Twenty-four-hour measurements were conducted for the mice without running wheels. The three time points selected were 1–5 days before the first treatment injections, during the third week, and the end of the sixth week. The measurement was conducted in the animal’s own cage and in the same room with the other mice in this experiment.

Muscle sampling. Lower leg muscles soleus, gastrocnemius, muscle quadriceps femoris (MQF), extensor digitorum longus (EDL), and tibialis anterior were immediately removed, weighed, and frozen in liquid nitrogen. The muscle weights reported are always average weights of the left and right leg. The proximal part of gastrocnemius muscle was mounted in an O.C.T. embedding medium (Tissue Tek, Sakura Finetek Europe) with vertical orientation of muscle fibers and snap-frozen in isopentane cooled with liquid nitrogen. Hematoxylin and eosin (H&E) and succinate dehydrogenase (SDH) staining were conducted from 8- to 10-μm cross-sections cut with a cryocut. SDH analysis was conducted as earlier (52).

Tissue processing for protein analysis. The gastrocnemius muscle was pulverized and homogenized in liquid nitrogen and then homogenized in ice-cold buffer with proper inhibitors and further treated as previously reported (24, 25). The muscle homogenate was centrifuged at 10,000 g for 10 min at 4°C. Total protein content was determined using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL) with an automated KoneLab device (Thermo Scientific, Vantaa, Finland).

Western immunoblot analyses. Muscle homogenates were solubilized in Laemmli sample buffer and heated at 95°C to denature proteins. Samples containing 30 μg of protein were separated by SDS-PAGE and transferred to PVDF membrane. The membrane was blocked in TBS with 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk and incubated overnight at 4°C with primary antibodies. The membrane was then washed in TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Europe) for 1 h followed by washing in TBS-T. Proteins were visualized by ECL (SuperSignal west femto maximum sensitivity substrate; Pierce Biotechnology) and quantified (band intensity × volume) using a ChemiDoc XRS device in combination with Quantity One software (version 4.6.3, Bio-Rad Laboratories).

The uniformity of the protein loading was confirmed by staining the membrane with Ponceau S and by reprobing the membrane with an antibody against GAPDH (Abcam, Cambridge, MA). Our results were not markedly affected whether the results were normalized to GAPDH, to Ponceau S, or to total protein.

Autophagy. LC3 immunoblotting is an often-used marker for autophagy (20, 39). LC3 I is the cytosolic form of the protein, and LC3 II is conjugated to lipid phosphatidylethanolamine and is present in autophagosomes (26). We carefully analyzed the LC3 I and II isoforms by specific antibody and with gel run, separating protein bands at 16 and 14 kDa, respectively, as recommended (26, 44) (see below).

Antibodies. The antibodies recognized PGC-1α from Calbiochem (Merck, Darmstadt, Germany), estrogen-related receptor-α (ERRα) from Novus Biologicals (Littletton, CO), cytochrome c from Santa Cruz Biotechnology (Santa Cruz, CA), p-ULK1 (UNC-51-like kinase 1) at mTOR-stimulated site Ser757, FoxO1 at Thr24, and p-AS160 at Thr442 from Cell Signaling Technology and LC3B from Sigma-Aldrich. For ubiquitinated proteins, horseradish peroxidase-conjugated anti-ubiquitin antibody was used (Santa Cruz Biotechnology). Total FoxO1 was analyzed using specific antibody (Cell Signaling Technology) by reprobing the membrane after careful stripping of the...
membrane with Restore Western Blot Stripping Buffer (Pierce Biotechnology).

Serum creatine kinase, TNF-α, and blood hemoglobin and hematocrit. Blood samples were collected after the 7-wk experiment during euthanization of the animals. Blood hematocrit and hemoglobin were analyzed by microcuvette HemoCue (EKF-diagnostic, Barleben, Germany). Serum creatine kinase (CK) was analyzed by a kinetic photometric method with KoneLab (Thermo Scientific, Vantaa, Finland). Serum TNF-α was analyzed by an immunometric chemiluminescence method with an Immulite 1000 (DPC, Los Angeles, CA).

DNA isolation and mtDNA. Gastrocnemius muscle was pulverized and homogenized in liquid nitrogen and was weighed into TRIzol reagent (Invitrogen, Carlsbad, CA) and further mixed and homogenized utilizing a FastPrep FP120 apparatus (MP Biomedicals, Illkirch, France). Total DNA was extracted according to the manufacturer’s guidelines. The DNA concentrations were analyzed with Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham MA) in duplicate. mtDNA/gDNA levels were quantified with a real-time qPCR assay using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) in triplicates. The probes and primers used were predesigned transcripts validated by Applied Biosystems bioinformatics design pipelines. The PCR cycle parameters used were: 50°C for 2 min + 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. The mtDNA copy number was calculated based on the multiplex amplification of both the mitochondrial NADH dehydrogenase subunit 2 (ND2, mtDNA, ABI Assay ID Mm004225288_s1, VIC with limited primers) and the nuclear gDNA marker melanoma-associated antigen (mutated) 1-like 1 (Mum1l1, gDNA, ABI Assay ID Mm00615902_s1, FAM). PCR amplification was performed using 25 ng of DNA. In both assays, all primers and probes map within a single exon. The results are expressed as the ratio of mtDNA to gDNA in each specimen with the ΔC_T method. Quantification was performed in the exponential amplification phase.

Citrate synthase activity. The activity of citrate synthase (CS) in gastrocnemius muscle homogenate was measured using a kit (Sigma-Aldrich, CS0720) with an automated KoneLab device (Thermo Scientific).

Statistical methods. Differences between groups were evaluated by analysis of variance. Fisher’s least significant difference test was used for post hoc analysis. PASW statistics version 18.0 was used for the statistical analyses (SPSS, Chicago, IL). The level of significance was set at P ≤ 0.05. Data are expressed as means ± SE. Correlations were analyzed using Pearson’s product moment coefficient.

RESULTS

Exercise attenuates further increase in body mass due to myostatin and activin blocking by decreasing fat mass. The effects of sActRIIB-Fc administration with or without 7 wk of voluntary running were investigated in modestly dystrophic mdx muscle. Body mass increased rapidly during the first 2–3 wk after sActRIIB-Fc but thereafter tended to stabilize to the rate of growth in the PBS group (Fig. 1A). Voluntary running for 7 wk attenuated the sActRIIB-Fc-induced increase in body mass compared with PBS controls, mainly by decreasing fat mass (Fig. 1A and B). Running also decreased the sActRIIB-Fc-induced increase in absolute muscle mass, but significantly only in gastrocnemius muscle, not in other muscles (Fig. 1C). Relative to body mass, running in combination with sActRIIB-Fc actually increased soleus and EDL muscle mass compared with sActRIIB-Fc treatment alone (not shown). The mdx mice had bigger muscles and lower brown and white fat mass than the healthy mice (Fig. 1). ANOVA (2 × 2) also revealed a trend for running to decrease brown fat mass (P = 0.06). No effects of sActRIIB-Fc on absolute white or brown fat mass were observed (Fig. 1B). But since sActRIIB-Fc increased muscle and body mass, we found decreased epididymal body fat and a trend (P = 0.10) for a reduced retroperitoneal fat by sActRIIB-Fc when these variables were normalized to body weight (not shown). This was so even though the mice administered sActRIIB-Fc consumed more feed than their controls (Fig. 1A).

sActRIIB-Fc treatment for 7 wk did not increase liver size (not shown), in contrast to the previously published shorter 2-wk experiment (24). ANOVA (2 × 2) revealed a trend for sActRIIB-Fc treatment to increase heart mass (P = 0.06), but the effects of sActRIIB-Fc were not significant in the individual running or non-running groups (not shown).

sActRIIB-Fc and exercise: effect on the markers of muscle oxidative capacity. To estimate muscle oxidative capacity, both the number and capacity markers and regulators of mitochondria were examined in gastrocnemius muscle. Dystrophic mdx mice had lower mtDNA and PGC1-α protein content and tended to have decreased CS activity (P = 0.10) compared with control mice (Fig. 2). All these variables in addition to cytochrome c remained unchanged after 7 wk of sActRIIB-Fc treatment but were normalized in mdx mice to control levels or higher by running, showing the beneficial effects of exercise (Fig. 2). Increase in oxidative capacity by running compared with sedentary mice was also confirmed by more strongly stained mitochondrial enzyme SDH (Fig. 2D).

sActRIIB-Fc administration delays voluntary running activity in mdx mice. During the first 2–3 wk, when the mice were rapidly growing in the sActRIIB-Fc running group, their daily running distance was ~50% lower than that of the PBS running mice (Fig. 3A). However, during weeks 4–7, the difference in running distance disappeared. Therefore, the sActRIIB-Fc-treated mice did not reach their peak in daily voluntary running distance until ~week 6 compared with week 2 in the PBS group. More detailed analysis of the running data showed that during the first 4 wk the average velocity and running distance of each individual running bout were lower in the sActRIIB-Fc-treated mice than in the PBS mice (not shown, P < 0.05). In contrast, the number of running bouts did not differ between the groups (P = 0.32), and there was only a trend for longer time spent on the running wheels in the PBS running group compared with sActRIIB-Fc running group (P = 0.07). As expected, during the last 3 wk, no differences existed between the groups in the number, velocity, distance, or time of running bouts (P > 0.67).

Home cage activity. We also studied the level of habitual home cage physical activity in mdx mice without running wheels. Physical activity was measured by the distance of movement per day. The results obtained using the force plate system developed by our group (51) showed that also the sActRIIB-Fc-treated mdx mice living without access to running wheels were habitually less active than the PBS-injected control mice. Interestingly in the 3–wk time point the magnitude of the effect was similar to running distance in the running group, e.g., ~50% less (Fig. 3B). However, unlike the mice with access to running wheels seemingly getting accustomed with running, these sedentary mice with sActRIIB-Fc remained less active than PBS mice throughout the 7-wk period, although the difference between the groups was much smaller at the later time point (Fig. 3B).
Fig. 1. Changes in masses of body (A), fat (B), and muscle (C), and feed consumption (A) after 7 wk of administration of cActRIIB-Fc (soluble ligand binding domain of activin receptor IIB fused to the Fc domain of IgG) with or without physical exercise (Run). MQF, quadriceps; EDL, extensor digitorum longus. *Difference vs. PBS; #difference between sActRIIB-Fc and sActRIIB-Fc run.
**Strength endurance.** To roughly estimate functional strength endurance, a hanging wire test (56) was conducted during the last week of the study. As expected, at the age of ~13 wk, healthy control mice were able to hang for a significantly longer time than the mdx mice (Fig. 3D). No consistent differences existed between the treated or nontreated groups in hanging wire performance (Fig. 3D). Supporting these results, we recently reported that treatment for 5 wk of mdx mice with our sActRIIB-Fc increased muscle mass and myofiber size but had little effect on muscle function (22).

**sActRIIB-Fc administration with or without exercise: no effect on blood inflammation or damage markers, hemoglobin, or hematocrit.** The muscles of the mdx mice were more fibrotic, had centralized nuclei, and showed inflammatory cell invasion (Fig. 3E). No apparent visible differences, such as cytoplasmic inclusions, previously published for myostatin KO mice (2), or the amount of centralized nuclei were noticed in the H&E-stained muscle samples after 2 or 7 wk of treatments (Figs. 3E and Fig. 4D). Furthermore, serum CK was higher (Fig. 3F) and blood hematocrit/hemoglobin slightly lower in the mdx than in healthy control mice (not shown). Serum CK and TNF-α were not significantly affected by either sActRIIB-Fc or exercise (Fig. 3F). Running in combination with sActRIIB-Fc increased blood hemoglobin and hematocrit, but running or sActRIIB-Fc treatment alone did not have an effect (not shown).

**Decrease in voluntary running activity is accompanied by an increase in body mass and decrease in markers of muscle oxidative capacity.** To understand the possible reasons for the observed running activity results, a correlation analysis was

Fig. 2. A and B: markers and regulators (B) of oxidative capacity in muscle after 7-wk sActRIIB-Fc administration with or without physical exercise (Run). C: representative blot. D: oxidative properties of adult gastrocnemius muscle stained using SDH. Fibers stained dark contain high SDH activity. CytC, cytochrome c; PGC-1α, PPARγ coactivator 1α. *Significant difference vs. PBS. In mtDNA figure, #difference in sedentary mice vs. healthy controls.
first conducted. Running distance correlated negatively with the increase in body mass gained during the first 2–3 wk. Figure 3C shows the correlation \((r = -0.75, P = 0.001)\) between running activity at weeks 0–2 and the increase in body mass during that time.

In order to further understand what happened during the first weeks, mice were euthanized after a 2-wk myostatin and activin blocking period. Similarly to capillary density, which was reported earlier (24), the concentrations of proteins important for muscle oxidative capacity, PGC1-\(\alpha\) and cytochrome \(c\), decreased after 2 wk of sActRIIB-Fc treatment, with a similar trend in ERR-\(\alpha\) (Fig. 4). No changes were yet seen acutely 1 or 2 days after a single injection. AS160 is a critical factor for skeletal muscle glucose uptake (31). No changes were observed in the phosphorylation of p-AS160 (not shown). Therefore, a lower capillary density (see Ref. 24), and trends for...
lower markers of aerobic capacity were observed after 2 wk of sActRIIB-Fc injections, suggesting that some proteins or organelles such as mitochondria or tissue structures such as capillaries do not seem to be in balance with the rapid muscle growth.

A further correlation analysis supported the hypothesis that the rapid increase in muscle size during the first weeks of myostatin and activin blocking was associated with decreased oxidative capacity. It was found that, after 2 wk of myostatin blocking, muscle fiber size correlated negatively with capillary density ($r = -0.87$, $P < 0.001$) and CS activity ($r = -0.61$, $P = 0.02$).

**Autophagy.** LC3 immunoblotting was examined as marker for autophagy (20, 39). Both cytosolic (LC3 I) and autophagosomal (LC3 II) forms of LC3 decreased after the 2-wk sActRIIB-Fc administration (Fig. 5). In particular, the content of LC3 II, not, e.g., the LC3 II/LC3 I ratio, has been suggested to be the best indicator of autophagy (44). Supporting the 2-wk results in healthy mice, also 7 wk of sActRIIB-Fc in mdx mice decreased LC3 II content (2 ANOVA sActRIIB-Fc treatment effect $P < 0.05$), even though in post hoc analysis the effect of sActRIIB-Fc alone only tended to be ($P = 0.1$) significant (Fig. 5A). In contrast, running for 7 wk increased LC3 II (2 ANOVA running effect $P < 0.05$; Fig. 5A). Post hoc analysis revealed that LC3 II also increased significantly compared with the non-running group in the exercise-only mice (PBS running). Our preliminary microarray data did not, however, reveal major changes in most of the autophagy genes (e.g., Atg5, Atg7, LC3A, LC3B, p62, Beclin 1, Bnip1–5) due to 7 wk of exercise or myostatin blocking. However, various anti- or proautophagy BCL2 and BCL2-like genes either in-

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**Fig. 4.** In gastrocnemius muscle, altered markers (A) and regulators (B) of oxidative capacity after short-term sActRIIB-Fc administration. C: representative blot. D: representative hematoxylin and eosin-stained gastrocnemius cryosections from PBS and sActRIIB-Fc after 2 wk of treatment. Western blot results are shown as relative to control (PBS) = 1. ERRα, estrogen-related receptor-α. *Difference vs. PBS ($P < 0.05$); in A.
creased or decreased due to the treatments (not shown). BCL2-regulated autophagy has been recently shown to be required for some of the positive responses of exercise (20).

Increased mTORC1 signaling by sActRIIB-Fc reported earlier by us (24) is in theory capable of directly inactivating autophagy-initiating kinase Ulk1 by phosphorylating (p) it at Ser757 (27). sActRIIB-Fc did not, however, affect p-ULK1 at either 1 or 2 days, 2 wk, or 7 wk. In partial support of the LC3II result, p-ULK increased in the combined sActRIIB-Fc and running group. It was also noticed that the mdx mice had elevated p-ULK1 levels. No clear and consistent effects of the treatments were found, but protein ubiquitination tended to be decreased or increased due to the treatments (not shown).

Figure 5. Changes in LC3 II and LC3 I protein (A) and phosphorylated ULK1 at Ser757 levels (B) after short- and long-term sActRIIB-Fc (ActRIIB) administration with or without physical exercise (Run) in gastrocnemius muscle. In addition to the short-term effect, 7 wk of sActRIIB-Fc in mdx mice decreased LC3 II content (2 × 2 ANOVA sActRIIB-Fc treatment effect P < 0.05), whereas running increased LC3 II (2 × 2 ANOVA running effect P < 0.05). Representative blots of LC3 and p-ULK1 (C) and ubiquitinated proteins and p-FoxO1 and total FoxO1 (D). Results are depicted as relative to PBS = 1. *Difference vs. PBS (P < 0.05). P = 0.06 depicts a trend of a difference in LC3 II at A2D vs. PBS.

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elevated in the muscles of the mdx mice. In support of that result, FoxO1 (8) was dephosphorylated in the mdx mice compared with the control group (see representative blots in Fig. 5).

DISCUSSION

The importance of an adequate size and function of skeletal muscle for health and well-being have been recognized during the past few years (3, 49, 58, 60). In the present study, we provide data that contribute to a better understanding of the processes that occur during muscle growth when myostatin/activin signaling is blocked in inactive, but also in active, exercised muscles. This is important, as studying animals in a restricted environment that does not allow their normal wild-type physical activity levels, may sometimes lead to inaccurate conclusions (7).

In DMD, correction of the cause of the disease, i.e., restoration of dystrophin expression, may not be enough if muscle wasting is in a progressed state. Therefore, novel treatment modalities complementing the efficacy of gene defect correction approaches are being studied. Previously, exercise has improved muscle function in myostatin-deficient (41, 42) and in myostatin-blocked healthy mice (35). In the present study, exercise decreased the myostatin/activin blocking-induced increase in body mass by reducing fat mass. In early DMD and in mdx mice, in addition to possible hypertrophy of muscle fibers, so-called increase in nonfunctional muscle size or “pseudohypertrophy” and thus increase in body mass is common (30). Thus, conceivably, the prevention of an excessive increase in body mass is beneficial when treating muscle dystrophies to optimize muscle mass to body mass ratio.

Some, but not all, markers of oxidative capacity were decreased in the muscle of mdx mice. This is supported by earlier evidence of a decrease in genes important for oxidative energy production in DMD (55) and decreased mitochondrial function in mdx mice (33) in skeletal muscles. Importantly, exercise enhanced or restored the oxidative phenotype of mostly dystrophic muscle to or above the levels of healthy wild-type mice. This improvement in oxidative phenotype occurred in dystrophic muscles without any major effect of 7 wk of myostatin and activin blocking. This is in contrast to the effects of the short-term myostatin and activin blocking in healthy mice (see below).

The protein content of PGC1-α, one of the main regulators of muscle oxidative capacity (10), was decreased in mdx mice. However, voluntary running restored the decreased PGC1-α content in mdx mice to or above the levels of healthy mice. In the long run, this may be important, because in addition to the positive influence on oxidative capacity (10), overexpression of PGC1-α in muscle has been also reported to ameliorate DMD pathology (19). We could not, however, notice any improvements for mdx muscle dystrophic phenotype and in a muscle endurance test (hanging wire) after running. This is even though mdx mice could increase their daily voluntary running from the first days of the experiment. Increased amount of voluntary running suggests improved ability in addition to motivation for running (14). Previously, voluntary running has been reported to be beneficial for mdx mice (9, 23, 34), but also some negative effects have been reported (23, 54).

Further studies are needed to investigate the effects of different modes, intensities, and doses of exercise in muscle dystrophy.

Low aerobic fitness and low level of physical activity are associated with various health risks (32, 57). In this study, we show that rapid muscle growth induced by sActRIIB-Fc decreased voluntary running during the first few weeks. Moreover, it delayed the time at which peak voluntary running activity was reached. Our finding suggests that at least mdx mice require extra time to adjust to the effects of the blocking of myostatin and activins in order to tolerate a typical, fairly large amount of voluntary running. We also showed that, in normal cage conditions without access to running wheels, the sActRIIB-Fc treated mdx mice were habitually less active than the nontreated mice throughout the 7-wk intervention but especially at first weeks. Thus, we postulate that 5–6 wk of exercise may be required for the previously sedentary dystrophic mice to tolerate increased levels of physical activity after myostatin and activin blocking. Previously, postdevelopmental myostatin depletion and thus increased muscle mass decreased voluntary running in healthy mice, and this difference tended to last throughout the 12-wk period (48). In contrast to this and our results, myostatin blocking by neutralizing antibody has been reported to increase habitual physical activity and performance in ob/ob mice (6). The difference between the studies may be the experimental model used. Obese mice seem to benefit functionally from the increased muscle mass and decreased fat mass caused by myostatin blocking (6, 29).

Possible explanations for the decreased physical activity after sActRIIB-Fc treatment are intriguing. We found that sActRIIB-Fc decreased running activity the more body mass increased. Muscle growth and increased protein synthesis, which were observed after blocking myostatin and activins (24), is an energy-consuming process. We previously reported a rapid sActRIIB-Fc-induced increase in the phosphorylation of AMPK, a marker of energy stress (24). Large muscles and high body mass are also not necessarily beneficial for efficient running. For instance, selective breeding of mice for high levels of voluntary running has favored a phenotype with markedly reduced muscle mass (13). Other changes during the rapid muscle growth were also observed. Previously, we observed decreased capillary density per muscle area after 2 wk of sActRIIB-Fc treatment (24). Expanding on this finding, we show here a decrease in the markers of oxidative energy production capacity per muscle area after 2 wk of myostatin blocking. The result is supported by a previous microarray study with sActRIIB-Fc (50) and studies using myostatin-null mice (2, 41, 42), all of which showed a reduction in various markers of aerobic capacity or metabolism. Thus, in addition to increased body and muscle mass per se and energy stress, decreased oxidative energy production capacity per muscle mass may also explain, in part, the reduced amount of voluntary running during the first few weeks after the blocking of myostatin and activins.

Autophagy is the major cellular route for the degradation of long-lived proteins and cytoplasmic organelles (20, 45, 61). After the first 2 wk of myostatin and activin blocking, concurrently with the decreased running after sActRIIB-Fc administration, we also observed a decreased level of LC3 II protein, a marker of autophagy (44). Previously, autophagy has been shown to be increased by treating cells with myostatin in vitro (36). Maintaining autophagy at normal levels is important to
rejuvenate organelles and to prevent accumulation of dysfunctional proteins (39, 40, 43). Recent evidence suggests that autophagy is needed to maintain muscle mass in some cachectic states (39) and that induction of autophagy can rescue mdx and some other forms of muscle dystrophies (12, 17, 47). Moreover, inhibition of autophagy can induce muscle dystrophy (40). Therefore, the decrease of autophagy by myostatin-blocked mice can be speculated to also contribute to the decrease the voluntary activity of mice. Our results suggest that there are various factors such as rapidly increased body mass and energy stress and decreased autophagy and oxidative capacity, all of which may explain why the muscles of dystrophic mice are not fully functionally adapted for running and why they are habitually less active during the first weeks after myostatin and activin blocking.

Until recently, the effects of exercise on autophagy in muscles were unknown. Autophagy seems to be required for some of the responses possibly explaining the beneficial nature of exercise for health (20). Recent studies reported that key autophagy-related genes were upregulated after exercise, especially in myostatin-deficient mice (41), and in another study only in wild-type mice but not in collagen VI-deficient muscles (18). The present study is the first to show that autophagy can be induced by exercise in mdx mice, a model of DMD, and that myostatin/activin blocking and exercise have slight but opposite effects on autophagy. Thus, physical exercise by increasing autophagy may be a promising treatment modality in muscle dystrophies (40). In the future, studies with elegant research models and technology (20, 45) in particular are needed to obtain a more complete understanding of the importance of muscle autophagy in pharmacological and exercise treatments of dystrophic muscle diseases.

In conclusion, blocking of myostatin and activins rapidly increases muscle mass but decreases voluntary activity in dystrophic mice. Decreased voluntary activity is associated with increased body mass and reduced markers of muscle aerobic capacity and autophagy. These changes may negatively contribute to the decreased level of physical activity and, thus, be potentially associated with various health risks if the inactivity were to last a longer period (32, 57). However, with long-lasting myostatin/activin blocking treatment in combination with exercise, muscles adapt to tolerate running. Adaptation to exercise is important, as exercise at least partially restored the reductions in the markers of muscle oxidative capacity in dystrophic mice and increased autophagy that was especially acutely decreased by myostatin blocking. Exercise can also, together with blocking myostatin and activins, potentially optimize body composition in dystrophic mice. Thus, myostatin/activin blocking and exercise are complementary and may therefore provide a suitable combination for treating various diseases and other conditions in which muscle loss is detrimental (3, 25, 49, 58, 60).

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REFERENCES


DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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