



Myostatin dysfunction is associated with reduction in overload induced hypertrophy of soleus muscle in mice

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3 **Myostatin dysfunction is associated with reduction in overload induced hypertrophy of**
4 **soleus muscle in mice**
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Abstract

The aim of the study was to investigate if myostatin dysfunction would promote the gain in muscle mass and peak isometric force (P_0) of soleus muscle (SOL) in response to functional overloading (FO) after ablation of the gastrocnemius muscle. Fifteen male Berlin high (BEH) mice homozygous for the compact mutation causing dysfunction of myostatin and seventeen mice with the corresponding wild type allele (BEH^{+/+}) were subjected to FO of SOL for 28 days at the age of 14 weeks. Compared to BEH^{+/+} mice, SOL of BEH was heavier (mean \pm SD, 13.5 ± 1.5 vs 21.4 ± 1.8 mg, respectively, $P < 0.001$). After FO, SOL mass increased relatively more in BEH^{+/+} than BEH strain (34.9 ± 11.5 vs 17.7 ± 11.9 %, respectively, $P < 0.01$). P_0 fell ($P < 0.01$) only in BEH strain which also showed an increase ($P < 0.01$) in optimal muscle length. Specific P_0 became even more depressed in BEH compared to BEH^{+/+} strain (8.4 ± 1.4 versus 10.8 ± 1.3 N/g, respectively, $P < 0.001$). Phosphorylation p70 S6 kinase did not differ between the strains. In summary, myostatin dysfunction impairs adaptation of SOL muscle to high functional demands.

Key words: Skeletal muscle, muscle hypertrophy, contractile properties, p70S6K, high resistance exercise

Introduction

Skeletal muscle mass is an important factor contributing to health and wellbeing (Wolfe, 2006), and there is a significant interest in ways to promote muscle mass and strength in humans of different ages and health status (Stewart *et al.*, 2013). Much of the debate has recently focused around myostatin (Smith and Lin, 2013).

Myostatin, a member of the TGF- β super family, is a potent inhibitor of muscle growth in mammals (McPherron *et al.*, 1997). Mouse models with impaired function of myostatin show an increase in skeletal muscle mass as a result of muscle fiber hypertrophy and hyperplasia (McPherron *et al.*, 1997). Myostatin dysfunction is associated with enhanced mammalian target of rapamycin (mTOR) signalling which induces muscle growth in response to functional overloading (Lipina *et al.*, 2010, Goodman *et al.*, 2011). Phosphorylation of p70 S6 kinase (p70S6K) which is associated with mTOR activation correlates with the increase in muscle mass after resistance training in mice (Baar and Esser, 1999). These observations suggested that inhibition of myostatin could preserve and restore contractile tissue in various muscle wasting conditions. Indeed, intraperitoneal injections of myostatin blocking antibodies induce an increase in muscle mass of dystrophic mouse model (Bogdanovich *et al.*, 2002). There is also evidence that acute resistance exercise decreases myostatin signalling through the activation of the TGF β inhibitor Notch (MacKenzie *et al.*, 2013). However, there are doubts about the physiological role of myostatin, as there was no difference in plasma myostatin levels between young men and older men showing significant loss of muscle mass and strength (Ratkevicius *et al.*, 2011). Effectiveness of myostatin inhibition as a treatment against loss of muscle function is unclear. Mice with dysfunctional myostatin show reduced specific force of the extensor digitorum longus (EDL) muscle (Mendias *et al.*, 2006, Amthor *et al.*, 2007). We have observed a reduction in specific force of *Xenopus* muscle fibers treated

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3 with SB431542 that acts to inhibit myostatin signaling through Smad transcriptional factors
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5 (Watt *et al.*, 2010).
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7 There are large knowledge gaps about the role of myostatin in adaptation of skeletal
8 muscle to exercise. Myostatin dysfunction leads to the shift towards faster contracting myosin
9 isoforms which is the likely mechanism underlying the reduced endurance capacity of
10 myostatin deficient mice (Matsakas *et al.*, 2010). Yet decreased or abolished myostatin
11 signalling might be advantageous for adaptations to resistance training since type II fibres
12 show greater enlargement in the cross sectional area compared to type I fibres (Verdijk *et al.*,
13 2009). However, to the best of our knowledge, this hypothesis has not been tested.
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23 Mouse soleus muscle (SOL) consists primarily of type I and type 2A fibers with some
24 (~10%) type 2X fibers and thus differs from the other appendicular muscles in rodents
25 dominated by type 2B, 2X and 2A fibers (Amthor *et al.*, 2007, Bloemberg and Quadriatero,
26 2012). This similarity to human muscles (Bloemberg and Quadriatero, 2012) makes mouse
27 SOL a prudent model for examining the role of myostatin in the adaptive response to
28 resistance training though higher metabolic rate in mice than humans complicates direct
29 comparisons (Darveau *et al.* 2002). Functional overloading (FO) after synergists ablation has
30 been widely used to study muscle hypertrophy in rodents (Lowe and Alway, 2002). The main
31 aim of our study was to test the hypothesis that myostatin dysfunction would increase the
32 gain in mass and isometric force of SOL after ablation of the gastrocnemius muscle. We
33 studied Berlin High mice which carried either a mutant myostatin alleles known as compact
34 (BEH) or wild type myostatin alleles, BEH^{+/+} (Amthor *et al.*, 2007, Lionikas *et al.*, 2013).
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53 **Materials and methods**

54 **Animals and study design**

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3 All procedures involving mice were approved by the Lithuanian State Food and Veterinary
4 Service (Nr. 0223). Mice were housed in standard cages, one to three mice per cage at a
5 temperature of 22-24 °C and 40-60 % humidity as in our previous studies (Ratkevicius *et al.*,
6 2010, Kilikevicius *et al.*, 2012). Animals were fed standard chow diet and received tap water
7 ad libitum. BEH^{+/+} animals were generated by crossing animals from BEH and Berlin Low
8 (BEL) strains and then repeatedly backcrossing the offspring to BEH using marker assisted
9 selection for the wild type myostatin (Amthor *et al.*, 2007, Lionikas *et al.*, 2013, Bungler *et*
10 *al.*, 2004). We used males of the 17th or higher generation of backcrossing, homozygous for
11 the wild type allele of myostatin (BEH^{+/+}) and male BEH mice homozygous for the compact
12 allele.
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25 Fifteen BEH mice and eighteen BEH^{+/+} mice were subjected to **FO of SOL** for 28 days
26 starting at the age of 14 weeks. These animals were anesthetized with an intraperitoneal
27 injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and the gastrocnemius muscle
28 (GAS) was surgically removed from a randomly selected leg using similar methods as
29 described earlier (Hamilton *et al.*, 2010). Mice were given buprenorphine after surgery for
30 pain relief and were monitored on a daily basis. The contralateral SOL of these mice served
31 as internal control (**CL-CON**) in analysis of muscle mass, p70S6K phosphorylation and
32 cytochrome c levels. Twelve BEH^{+/+} and twelve BEH mice were not subjected to any
33 interventions and used as age matched independent controls (**CON**) in the analysis of **muscle**
34 **length and contractile properties, since this analysis took 40-50 min and could not be carried**
35 **out on FO and CL-CON muscles at the same time.**
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50 **Assessment of muscle properties**

51 Similar procedures as in our previous studies were used (Baltusnikas *et al.*, 2015). Mice were
52 euthanized at 18 weeks of age. Sutures were attached to the proximal and distal tendons of
53 Sol for measurements of contractile properties. The muscle was then excised and fixed
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3 between two platinum plate electrodes in 50 ml Radnotti tissue bath filled with the Tyrode
4 solution (121 mM NaCl, 5 mM KCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 0.4 mM NaH₂PO₄, 0.1
5 mM NaEDTA, 24 mM NaHCO₃, 5.5 mM glucose, pH adjusted to 7.4 when bubbled with 95
6 % O₂ and 5 % CO₂. The distal tendon of the muscle was attached to a hook and the proximal
7 end was tied directly to the lever of muscle test system (1200A-LR Muscle Test System,
8 Aurora Scientific Inc., Aurora, Canada). The muscle was then left to equilibrate in the
9 solution for 10 min. In the meantime the contralateral soleus muscle was dissected and placed
10 in the Tyroid solution bubbled with 95 % O₂ and 5 % CO₂. All experiments were carried out
11 at room temperature of ~23 °C. The length of the fixed muscle was increased in steps every
12 30 s just after delivery of electrical pulse to evoke a twitch contraction. This procedure was
13 continued until twitch force did not increase with the increase in muscle length. The muscle
14 was then photographed with the length scale in the background to assess muscle length with a
15 precision of 0.1 mm. Muscles were kept at this optimal length during the assessment of
16 contractile properties. Firstly, single twitches were generated and peak twitch force was
17 measured. The twitch contraction time was assessed as the time from the beginning of the
18 contraction to the peak of twitch force. Twitch half relaxation time was measured as the time
19 taken for force to decline from peak to 50 % of peak value. Afterwards, the muscle was
20 subjected to 900 ms trains of stimuli at 20, 50, 80, 100 and 150 Hz for assessment of peak
21 isometric force. Assessment of contractile properties was completed by eccentric exercise
22 consisting of 20 repeated eccentric contractions performed every 10 s. Each eccentric
23 contraction was induced by 1100 ms stimulation at a frequency needed to generate peak force
24 (80 or 100 Hz). During the last 200 ms of this stimulation a ramp stretch was imposed
25 followed by a 200 ms gradual return of the muscle to the initial length without any
26 stimulation. The amplitude of the stretch was 30 % of muscle fibre or 2.5 fiber lengths per
27 second assuming fibre length to muscle length ratio of 0.7 (Brooks and Faulkner, 1988). Peak
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3 isometric force was measured during the initial 900 ms of contraction. Then these muscles
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5 were incubated in 2 ml of Tyrode solution for 2 h at room temperature. 250 µl of Tyrode
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7 solution was sampled for assessment of CK activity using biochemical analyser (Spotchem™
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9 EZ SP-4430, Menarini Diagnostics, UK) with the reagent strips (Arkray Factory, Inc., Shiga,
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11 Japan). Following all measurements both control and experimental muscle were freed from
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13 tendons, blotted and weighed (Kern, ABS 80-4, Germany). Afterwards, muscles were dried
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15 for 48 h at a temperature of 40 °C and the weighed again for estimation of dry muscle weight.
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18 19 **Western blotting**

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21 Muscle homogenates were prepared as described **previously** (Ratkevicius *et al.*, 2010). Then
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23 samples containing 50 µg protein were loaded on 10% polyacrylamide gel, separated using
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25 SDS-PAGE electrophoresis **and** transferred to polyvinylidene fluoride (PVDF) membrane.
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27 Then membranes were washed with Tris buffered saline (TBS) containing 0.1 % (vol/vol)
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29 Tween-20 (TBS-T buffer) before two hour incubation in the blocking buffer (5 % (wt/vol)
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31 non-fat milk in TBS-T buffer). **Afterwards** the membranes were incubated for 18 h at 4 °C
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33 with a primary antibody at 1:1000 dilution (vol/vol) **in** TBS-T buffer supplemented with 5%
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35 bovine serum albumin. All antibodies were from Cell Signaling Technology (Danvers, MA,
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37 USA). The following primary antibodies were used; Phospho-p70 S6 Kinase (Thr389) or P-
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39 p70S6K (#9205), p70 S6 Kinase or p70S6K (#9202), cytochrome c or Cyt C (#4272) and β-
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41 Actin (#4967). After incubation with a primary antibody, membranes were washed in TBS-T
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43 buffer and exposed for 2 h to HRP-conjugated secondary antibody (#7071) at 1:2000 dilution
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45 in the blocking buffer. The imaging of blots was performed using ECL reagent (Amersham
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47 Biosciences, Buckinghamshire, UK) and Fluor-SMax Imager (Bio-rad, Hertfordshire, UK).
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49 The images were quantified using ImageJ (NIH, USA) software.
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52 53 54 **Statistical analysis**

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3 All data analysis was performed using IBM SPSS Statistics v21 and Prism 5.0 software. The
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5 two factor analysis of variance was used with strain (BEH or BEH+/+), treatment (FO or
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7 control) as main effects and strain by treatment interaction. A repeated measures design was
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9 followed in the analyses of muscle weight (two levels; overloaded and contralateral leg) and
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11 fatigue test (twenty levels). A Greenhouse-Geisser correction was applied in the fatigue test
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13 data analyses to compensate for the violation of sphericity. All the tests were two tailed with
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15 significance level was set at $P < 0.05$.
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20 21 **Results**

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23 Table 1 contains data on body mass of BEH+/+ and BEH mice in **the** experiments with FO of
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25 SOL muscle. BEH mice were heavier ($P < 0.001$) than BEH+/+ mice. Body mass of mice did
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27 not change **after** FO and did not differ from the age matched controls. Data on muscle mass,
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29 optimal length, peak isometric force and specific force are presented in Fig. 1. **SOL** was
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31 heavier in BEH mice compared to BEH+/+ mice ($P < 0.001$). FO induced a marked gain in
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33 SOL mass ($P < 0.001$). When adjusted for the weight of the contralateral SOL, the relative
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35 gain in BEH+/+ strain was greater than in BEH strain (mean \pm S.D., 34.9 ± 11.5 % vs $17.7 \pm$
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37 11.9 %, respectively, $P < 0.01$). These changes of muscle weight reflected greater relative
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39 gain in dry muscle mass for BEH+/+ compared to BEH strain (36.1 ± 11.8 % versus $16.0 \pm$
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41 10.5 %, respectively, $P < 0.01$). Muscle mass of the contralateral control SOL (**CON-CL**) did
42
43 not differ from **the** muscle mass of **the age-matched mice (CON)** that were not subjected to
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45 **FO**. There were no differences in optimal muscle length of SOL between BEH and BEH+/+
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47 **strains for CON mice**. FO induced an increase in **the optimal** muscle length ($P < 0.05$) **for**
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49 BEH mice, but not BEH+/+ mice. BEH **strain** generated greater peak force ($P < 0.01$)
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51 compared to BEH+/+ **strain** in CON mice, but the differences between the strains disappeared
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53 after **FO**; BEH mice showed a decrease in peak isometric force while this parameter did not
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3 change in BEH+/+ mice. Thus FO induced a decrease in specific force in both strains of
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5 mice, but specific force was lower in BEH compared to BEH+/+ mice at each time point ($P <$
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7 0.001).
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10 Twitch contractile properties are shown in Fig. 2. BEH mice generated twitches with
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12 shorter contraction time ($P < 0.001$) and half relaxation time ($P < 0.001$) compared to
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14 BEH+/+ mice. Twitch to tetanus force ratio was also lower in BEH mice compared to
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16 BEH+/+ mice. FO did not have any significant effect on these twitch properties.
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19 Data on changes in p70S6K phosphorylation and cytochrome c are presented in Fig. 3.
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21 There were no significant differences between BEH+/+ and BEH mice in these
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23 measurements. p70S6K phosphorylation did not change after FO. Cytochrome c to β -Actin
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25 ratio tended to be higher in BEH mice compared to BEH+/+, but the difference between the
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27 strains was not significant ($P = 0.085$). FO did not induce any change in this ratio.
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30 Data on the peak isometric force during 20 eccentric contractions and CK efflux after the
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32 exercise are presented in Fig. 4. Changes in the peak force differed depending on the strain
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34 and treatment (strain by treatment by time interaction, $P < 0.001$). The BEH muscles showed
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36 a greater drop in the peak force than BEH+/+ muscles ($P < 0.05 - 0.0001$) when studied in
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38 the control condition (CON). FO reduced the force loss in BEH muscles to the level of
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40 BEH+/+ strain which did not show any changes in the force loss after FO. There were no
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42 differences between BEH and BEH+/+ in muscle CK efflux after the exercise. Muscle CK
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44 efflux was not modulated by the adaptation to FO.
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49 Discussion

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52 The main aim of the study was to test the hypothesis that myostatin dysfunction promotes
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54 muscle hypertrophy and strength gain in response to FO. We studied the effects of ablation of
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56 the gastrocnemius muscle on muscle properties of SOL in BEH and BEH+/+ mice. The
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3 results reject our original hypothesis, since BEH mice showed smaller rather than greater gain
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5 in muscle mass compared to BEH+/+ and exhibited a decrease rather than an increase in peak
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7 isometric force after FO. Thus myostatin dysfunction might impair adaptations to increased
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9 functional demands in skeletal muscles with high oxidative capacity.
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11 12 13 14 *Contractile properties*

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16 SOL of BEH mice showed faster twitch speed and reduced specific force compared to
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18 BEH+/+ mice. This high twitch speed could be attributed to a shift in muscle fibre
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20 composition towards faster contracting myosin isoforms and fibre types in mice with
21
22 myostatin dysfunction (Amthor *et al.*, 2007, Matsakas *et al.*, 2010). However, the decrease in
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24 specific force is not expected from the changes in muscle fibre composition because type 2
25
26 fibres often show higher specific force than type 1 fibres (Bottinelli *et al.*, 1996). It appears
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28 that effects of myostatin inhibition are not limited to changes in the fibre type composition.
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30 SB431542 mediated inhibition of myostatin signalling lead to an increase in cross sectional
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32 area with no change in peak isometric force of muscle fibre in *Xenopus laevis* (Watt *et al.*,
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34 2010). One of the reasons for a decrease in specific force could be due to low levels of
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36 contractile proteins in muscle fibres of mice showing myostatin dysfunction (Qaisar *et al.*,
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38 2012). Enlarged myonuclear domains might limit protein synthesis and accumulation of
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40 contractile protein in muscle fibres showing excessive hypertrophy (Qaisar *et al.*, 2012).
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42 However, this is an oversimplified view since myostatin inhibits protein synthesis and
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44 removal of this inhibition is expected to have a positive effect on the overall protein synthesis
45
46 rates (Goodman *et al.*, 2013). Interestingly, specific muscle force increased in mice with
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48 myostatin knock out after a period of endurance training or food restriction which lead to a
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50 decrease in the cross sectional area of skeletal muscles (Matsakas *et al.*, 2012, Matsakas *et*
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52 *al.*, 2013). It has been suggested that accumulation of aberrant p62 proteins might interfere
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3 with force generation in muscle fibres of myostatin deficient mice fed ad libitum (Collins-
4 Hooper *et al.*, 2015). Interestingly, BEH and BEH+/+ mice did not differ in specific force of
5 SOL at the age of 31-35 days when muscle mass was less than half of the adult size
6 (Baltusnikas *et al.* in press). Adult C57BL/6 mice of similar muscle mass as young BEH mice
7 also show no effect of myostatin knock out on specific force of SOL muscle (Mendias *et al.*
8 2006). It might be that there is a critical muscle size beyond which muscle hypertrophy
9 interferes with force production causing a decrease in specific force. Biomechanical factors
10 such as an increase in muscle fibre pennation angles might limit force generating capacity of
11 hypertrophied muscles in addition to the factors acting at the single fibre level (Amthor *et al.*,
12 2007). Effects of myostatin dysfunction might also depend on the background strain of mice.
13 Thus it is important to study effects of myostatin dysfunction using various strains of mice.
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30 *Functional overloading (FO)*

31 We used 4 week exposure to FO. It is a popular model to study muscle hypertrophy in
32 mammals (Lowe and Alway, 2002). This type of muscle overloading leads to an increase in
33 muscle mass with less pronounced increase in force generating capacity in mouse **plantaris**
34 muscle (Bodine and Baar, 2012). A similar force deficit is observed in skeletal muscles of
35 rats (Kandarian and White, 1989). We focused on SOL which can be excised intact for
36 assessment of contractile properties and contains a mixture of muscle fibre types resembling
37 human muscles (Bloemberg and Quadrilatero, 2012). It is expected that the effects of FO and
38 myostatin inhibition would vary between skeletal muscles that differ in muscle fibre type
39 composition and other properties. SOL showed a relatively small increase in muscle mass and
40 no increase in peak isometric force which is in contrast to the previous findings on plantaris
41 after FO (Bodine and Baar, 2012). The majority of studies on the role of myostatin in rodents
42 focused on the muscles dominated by type 2B fibres (Amthor *et al.*, 2007, Matsakas *et al.*,
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3 2010, Matsakas *et al.*, 2012). The observed effects of myostatin on the tibialis anterior and
4
5 extensor digitorum muscles are typically large and stimulated the interest in it as a possible
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7 pharmacological target (Mendias *et al.*, 2006). However, human muscles are rather different
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9 as no 2B fibres are detected (Smerdu and Erzen, 2001). The present study on the role of
10
11 myostatin indicated that, contrary to expectation, the potential for gain in muscle mass and
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13 function might be limited in the muscles comprised of type 1 and type 2A fibres.
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16 A particular feature of adaptation to FO in BEH strain was an increase in optimal muscle
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18 length which did not change in BEH+/+ strain. Severe muscle exercise is associated with
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20 muscle damage, increased heterogeneity of sarcomere length and increased muscle optimal
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22 length (Proske and Morgan, 2001). Functional overloading does induce muscle damage
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24 especially during the first week of its application (Lowe and Alway, 2002). It appears that
25
26 myostatin dysfunction increases muscle susceptibility to damage after exercise (Mendias *et*
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28 *al.*, 2006, Baltusnikas *et al.*, in press). Indeed, SOL of BEH mice showed a faster drop in
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30 peak force in repeated eccentric contractions compared to BEH+/+ strain eventhough muscle
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32 CK efflux was similiar. Muscle damage and the associated inflammatory response can inhibit
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34 accumulation of contractile proteins and lead to a decrease in specific force (Pizza *et al.*,
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36 2002). Inhibition of signalling by transforming growth factor- β (TGF- β) superfamily, which
37
38 myostatin is a member of, impairs muscle regeneration and leads to a long term deficit in
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40 force production after eccentric exercise (McPherron *et al.*, 1997, Gumucio *et al.*, 2013). It
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42 appears that myostatin dysfunction interferes with the adaptation of SOL to the overloading.
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44 There were no changes in mitochondrial cytochrome c levels, and reduction in force loss
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46 during eccentric exercise was not due to improved oxidative capacity of muscles in BEH
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48 mice after FO. It is likely that a decrease in specific force might have played an important
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50 role in this phenomenon.
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3 SOL of BEH and BEH^{+/+} mice showed similar cytochrome c levels, but twitch
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5 contraction time was faster in BEH mice. Myostatin dysfunction leads to an increase in
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7 content of faster contracting type IIA, IIX and IIB fibres and reduction in type I fibres in SOL
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9 (Amthor et al. 2007, Matsakas et al. 2013). Thus faster twitch contraction time of SOL in
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11 BEH compared to BEH^{+/+} is in agreement with such a shift in fibre type composition. On the
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13 other hand, cytochrome c reflects mitochondrial content which is sensitive to physical
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15 activity (Holloszy et al. 1976). Our results suggest that there is no significant difference in
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17 mitochondrial content between SOL of BEH and BEH^{+/+} mice. There was also no difference
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19 in percentage of oxidative fibres in SOL of mice C57BL/6 with and without myostatin KO
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21 (Collins-Hooper et al. 2015). Mouse SOL shows particularly high involvement in postural
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23 and locomotor activity compared to other limb muscles (Roy et al. 1991). It is likely that
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25 these high levels of physical activity help to maintain high oxidative capacity of SOL in BEH
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27 mice with dysfunctional myostatin. Indeed, exercise training of C57BL/6 mice with
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29 myostatin KO induces a marked increase in content of oxidative fibres even in the fast
30
31 contracting extensor digitorum longus (EDL) with only minor changes in fibre type
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33 composition (Matsakas et al. 2013).
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40 *Anabolic signalling*

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42 There was no difference between SOL of BEH^{+/+} and BEH mice in P70S6K
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44 phosphorylation which has been linked to muscle growth in rats (Baar and Esser, 1999). In
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46 contrast to the other limb muscles, rat SOL showed only a small increase in P70S6K
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48 phosphorylation immediately after electrical stimulation mimicking high resistance exercise
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50 and this change became insignificant within 3 hours of recovery. It appears that mouse SOL
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52 also shows a reduced signalling through mTOR - P70S6K axis compared to the
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54 gastrocnemius muscle which showed increased activation in C57BL/6 mice with myostatin
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3 knockout (Lipina *et al.*, 2010). Density of activin type IIB receptors (ActRIIB) which mediate
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5 myostatin signalling is higher in the faster contracting muscles such as EDL compared to the
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7 slower contracting SOL (Medias *et al.* 2006). Thus myostatin signalling is likely to be weaker
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9 in SOL compared to EDL and the gastrocnemius muscle. Indeed, SOL shows smaller
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11 hypertrophy compared to both gastrocnemius and EDL in mice with myostatin dysfunction
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13 (Amthor *et al.* 2007). Importance of mTOR signalling for muscle growth varies with time of
14
15 exposure to the hypertrophic stimuli. P70S6K phosphorylation returns to baseline levels
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17 within 21 days of FO in fast twitch plantaris (Hamilton *et al.*, 2014). In general, our results
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19 agree with the contention that mTOR signaling is not a predictor of muscle hypertrophy in
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21 response to the long term functional overloading.
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25 26 27 *Perspectives*

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29 Myostatin inhibition has attracted a considerable interest as a strategy for improvement of
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31 muscle function. A significant effort has been spent in studying effects of myostatin
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33 inhibition on muscle cells and skeletal muscles in various mouse models. Much less attention
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35 has been devoted to effects of myostatin dysfunction on the muscle adaptations to various
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37 types of functional overloading and muscle exercise. Our results show that BEH mice had
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39 experienced a smaller rather than greater gain in SOL mass and, in contrast to BEH^{+/+} mice,
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41 exhibited a decrease in muscle force generating capacity after functional overloading. These
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43 results suggest that myostatin dysfunction has a negative effect on adaptation to increased
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45 functional demands in skeletal muscles with high levels of motor activity. In our
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47 experimental model muscle adaptations to myostatin dysfunction occurred prior to functional
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49 overload (FO). Studies employing a model of the conditional myostatin knockout limited to
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51 the period of FO are needed to confirm these findings.
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56 **Competing interests**

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3 There are no competing interests
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Table 1. Body mass of experimental BEH+/+ (n=12) and BEH (n=9) mice that were subjected to functional overloading (FO) of soleus (SOL) muscle and body mass of aged matched BEH+/+ (n=12) and BEH (n=12) mice that were not subjected to any interventions.

	Experimental mice		Control mice
	Before FO 14 weeks	After FO 18 weeks	18 weeks
BEH+/+ (g)	54.0 ± 3.3	54.1 ± 3.3	52.0 ± 3.6
BEH (g)	61.0 ± 4.0***	60.4 ± 4.4***	60.2 ± 3.1***

Values are means ± SD *** $P < 0.001$ between BEH+/+ and BEH mice.

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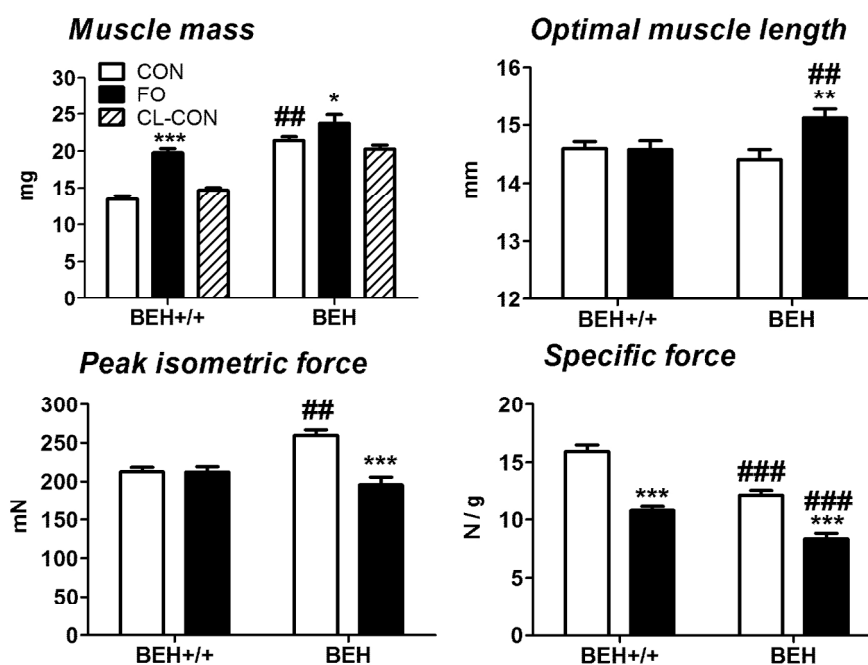


Fig. 1. Muscle mass, optimal length, peak isometric force and specific force of soleus muscles (SOL) in the control mice (CON) and after 28 days of functional overloading (FO) with the respective contralateral controls (CL-CON) where appropriate for BEH+/+ (n=12) and BEH (n=9) mice. Values are means \pm SEM; * P < 0.05, *** P < 0.001 between CON and FO; ## P < 0.01, ### P < 0.001 between BEH+/+ and BEH muscles.

169x121mm (300 x 300 DPI)

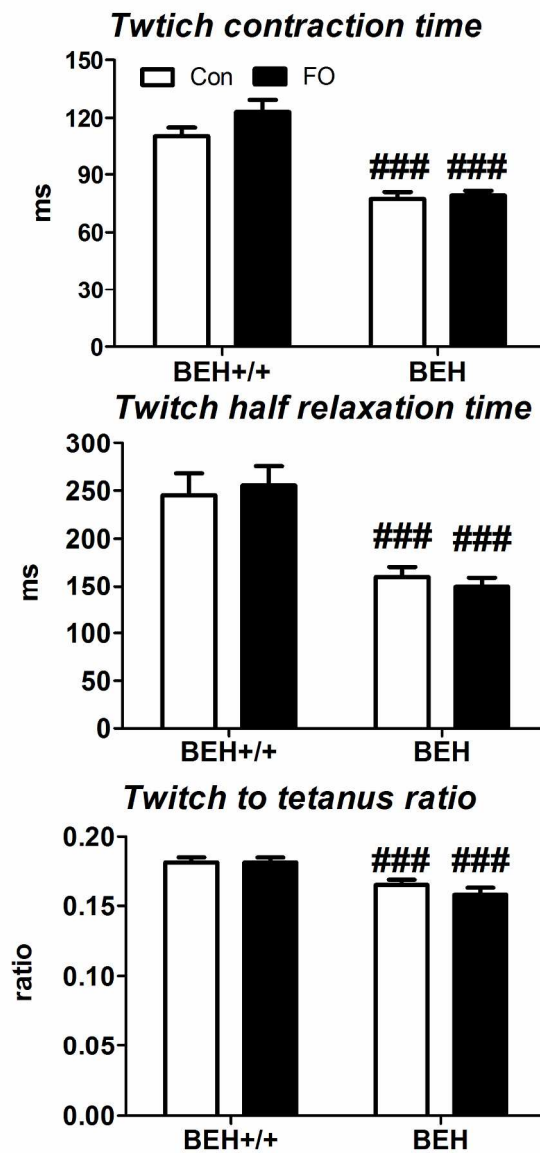


Fig 2. Twitch contractile properties of soleus muscle (SOL) in the control (CON) BEH+/+ (n=12) and BEH (n=12) mice and after 28 days of functional overloading (FO) for BEH+/+ (n=12) and BEH (n=9) mice. Values are means \pm SEM; ### P < 0.001 between the respective BEH+/+ and BEH mice.
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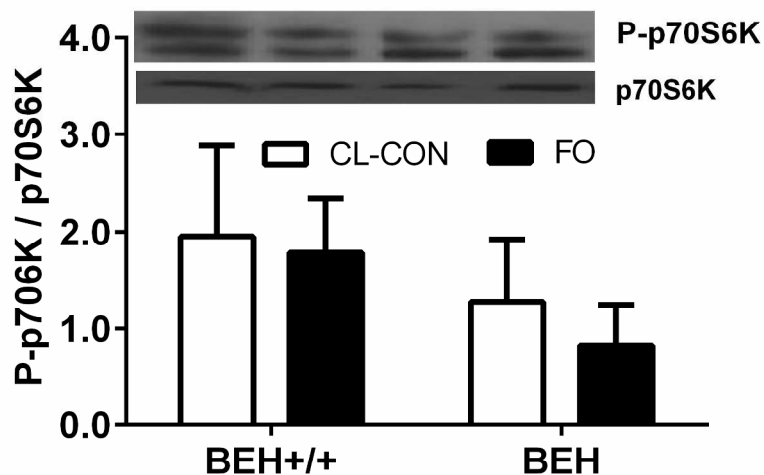
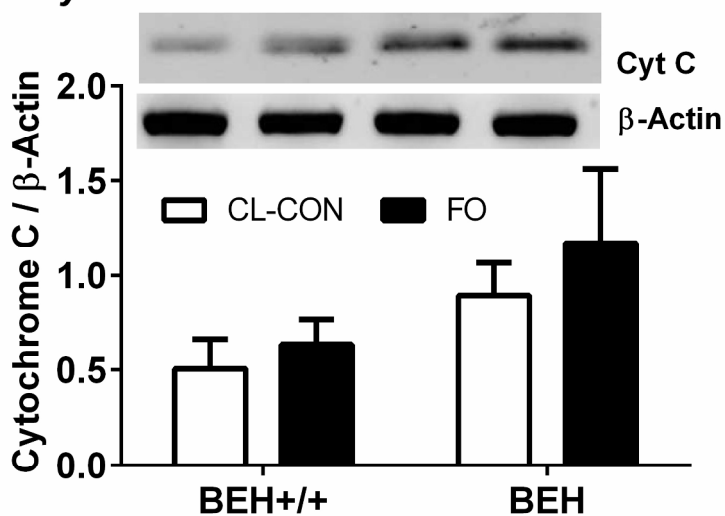
p70S6K phosphorylation***Cytochrome C***

Fig. 3. p70S6K phosphorylation (P-p70S6K / p70S6K ratio) and cytochrome c (Cyt C) in the soleus muscle (SOL). Western blotting was performed on the muscles after 28 days of functional overloading (FO) for BEH+/+ (n=6) and BEH (n=6) mice and the respective contralateral controls (CL-CON). The representative Western blots are shown for each of the four studied muscle groups in the same order as bars in the figure.

Values are means \pm SEM.
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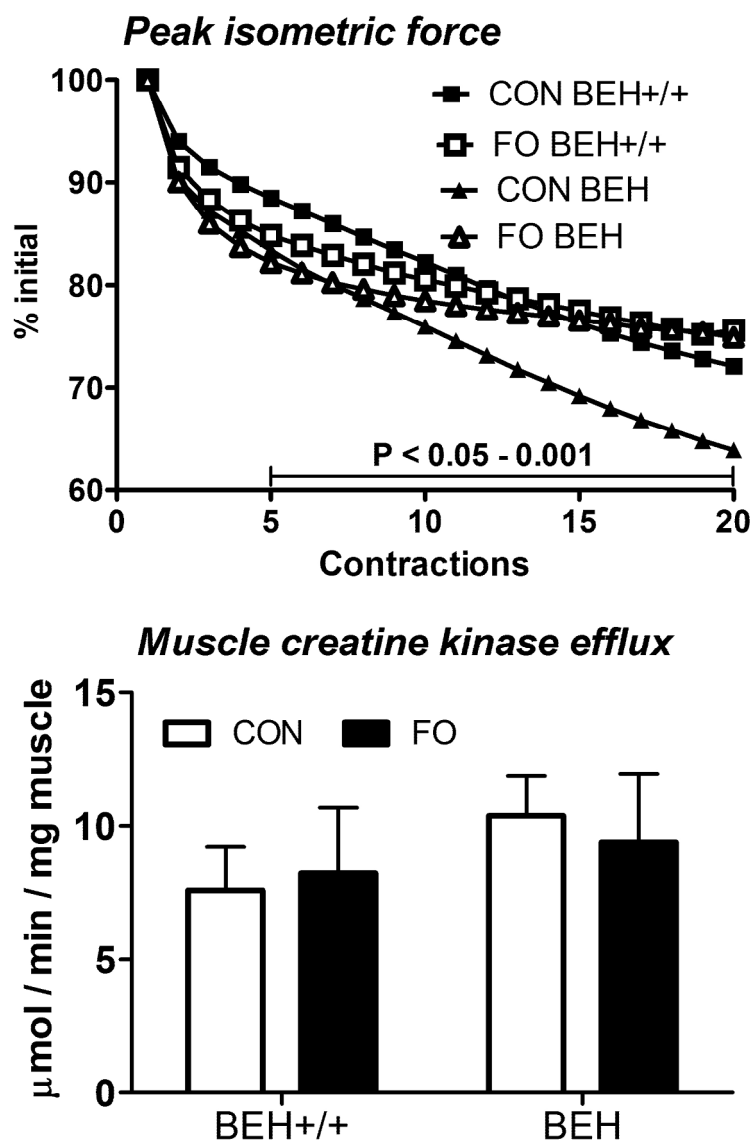


Fig 4. Peak isometric force in eccentric exercise and the exercise induced creatine kinase efflux for soleus muscle (SOL) in the control (CON) BEH+/+ (n=12) and BEH (n=12) mice as well as after 28 days of functional overloading (FO) for BEH+/+ (n=12) and BE (n=9) mice, respectively. Values are means \pm S.E.M. As indicated for the control condition, the difference between BEH+/+ and BEH ranged from $P < 0.05$ after 5 contractions to $P < 0.0001$ after 20 contractions.
193x257mm (300 x 300 DPI)