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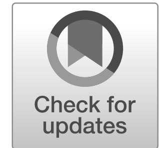
ORIGINAL ARTICLE



Sex-specific differences in rat soleus muscle signaling pathway responses to a bout of horizontal and downhill running

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Abstract
Males and females of many species, including humans, exhibit different muscle responses and adaptations to exercise stress; however, the molecular mechanisms that underlie these changes are poorly understood. Therefore, the present study assessed sex-related differences in intracellular signaling pathway responses to bouts of horizontal or downhill running in rat soleus muscles. Age-matched male and female Wistar rats (10 weeks old, $n = 18$/group) were either rested (control group) or subjected to an either a bout of horizontal (22 m/min, 20 min, 0° incline) or downhill (16 m/min, 10 min, − 16% incline) treadmill running. Soleus muscle samples were collected both prior to and immediately after exercise ($n = 6$/group). Intramuscular signaling responses to each type of exercise were determined via real-time (RT) PCR and western blot analyses. Although mTOR signaling (mTOR/S6K1/S6) responses to both horizontal and downhill exercise were found to be similar in both sexes, ERK phosphorylation levels were found to be significantly higher in male than in female rats after downhill exercise. Similarly, heat shock protein (Hsp) 72 and myostatin protein expression levels were both found to be significantly altered after downhill exercise: Hsp levels increased in male and decreased in female rats, whereas myostatin increased in female but decreased in male rats. Thus, the results of the present study suggest that downhill exercise may elicit sex-specific differential changes to Hsp72 expression, ERK phosphorylation, and myostatin-signaling activation in female compared with those in male rat soleus muscles. Further study is required to confirm these findings and to determine the way in which they impact sex-specific differences in exercise-induced muscle adaptations.

Keywords Sex differences · Intramuscular signaling · Treadmill exercise · Heat shock protein · Extracellular signal-regulated kinase · Myostatin

Introduction

Males and females in many species, including humans, have markedly different physiologies, including body composition [6], hormone concentration [3], muscle-fiber types [7], and substrate utilization [25]. Human studies have shown that these differences can affect muscle responses and adaptation to physical exercise. For example, men generally experience a greater hypertrophic response to resistance training in both young and older adults [10, 11]. However, the mechanisms underlying sex-related differences in exercise-induced muscle adaptation remain unclear.

Muscle mass is determined by the balance between muscle protein synthesis and breakdown. Muscle protein synthesis is primarily regulated by intracellular mechanistic target of rapamycin (mTOR) signaling. Notably, a previous human study demonstrated that resistance exercise activates muscle mTOR signaling equally in both sexes [4], and furthermore, resistance exercise-induced increases in muscle mTOR signaling have been shown to be unaffected by sex-dependent differences in post-exercise testosterone concentration in male and female subjects [28]. In contrast, previous studies have

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shown that female subjects exhibit greater increases in the expression of TGF-β-related genes, which are known to negatively regulate skeletal muscle mass in response to acute resistance exercise [16]. These data suggest that exercise-induced skeletal muscle signaling regulation may be gender-specific; however, evidence for this theory is notably limited.

Therefore, we herein evaluated sex-related differences in exercise-induced intramuscular signaling, by examining the activity levels of signaling pathways known to be involved in the maintenance of skeletal muscle mass in male and female rat soleus muscles after a bout of horizontal and downhill running. In general, compared with running horizontally, running downhill appears to incur both greater impact loads and a higher vertical velocity on landing, but downhill running incurs a lower metabolic cost; therefore, we hypothesized that the sex-specific differences may prominently appear after downhill running. A better understanding of the sex-specific changes in intracellular signaling that occur during exercise would offer novel insights into the mechanisms that underlie sex-dependent differences in muscle adaptations.

Materials and methods

Experimental animals

This study was approved by the Juntendo University Animal Care Committee (H28-15) and was conducted according to the guiding principles for the care and use of laboratory animals set forth by the Physiological Society of Japan.

Age-matched male and female Wistar rats (10 weeks old, n = 18/group) were used in this study. The rats were housed in a climate-controlled room (23 ± 1 °C, 55 ± 5% relative humidity, and 12:12-h light–dark photoperiod) and provided with standard rat chow and water ad libitum. After acclimation, both male (242.2 ± 1.9 g) and female (163.3 ± 1.3 g) rats were randomly assigned to either a non-exercised control (CT), horizontal running exercise (HR), or downhill running exercise (DR) group. Each group had 6 male and 6 female rats.

Treadmill exercise and sampling

Rats in each treatment group completed a bout of treadmill exercise as follows. In the HR group, untrained (i.e., sedentary) animals ran continuously on a motor-driven treadmill at a speed of 22 m/min for 20 min, on a 0° slope, and thus completed a bout of normal endurance exercise including both concentric and eccentric muscle contractions [22]. The DR group instead completed a bout (10 min) of downhill (grade, − 16%) treadmill running (16 m/min), in a manner that has been previously shown to be a physiologically relevant model of eccentric loading [1, 13, 20]. After exercise, blood and soleus muscle samples were collected, rapidly frozen in liquid nitrogen, and stored at − 80 °C until use. Similarly, the blood and soleus muscle samples of CT animals were collected. Blood samples were then centrifuged at 3000 rpm for 10 min to isolate serum samples, and blood parameters were measured by the Oriental Yeast Co., Ltd. (OYC, Tokyo, Japan).

Muscle sample preparation

Frozen soleus muscles were powdered and portioned (~ 30 mg) for analysis. Subcellular fractions were prepared from the powdered muscles using a commercially available extraction kit (NE-PER, Thermo, Northumberland, UK). Briefly, ~ 30 mg of skeletal muscle was homogenized in CER-I buffer containing cOmplete EDTA-free (Roche, Penzberg, Germany) protease inhibitor cocktail and PhosSTOP (Roche, Penzberg, Germany), using a bead-cell disrupter (Microsmash, MS-100, Tomy Seiko Co., Ltd., Tokyo, Japan). Then homogenates were centrifuged at 500g for 5 min at 4 °C, and the resulting supernatants were collected. To obtain pure cytosolic fractions, the supernatants were centrifuged at 12,000g for 15 min at 4 °C, and the resulting supernatants were again collected. Protein concentrations were determined using a BCA Protein Assay Kit (Thermo, Rockford, USA).

Immunodetection

Proteins were loaded onto 4–15% TGX Stain-Free polyacrylamide precast gels (Bio-Rad, Copenhagen, Denmark) and electrophoresed at 150 V for 60 min. Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) and blocked with the PVDF Blocking Reagent for Can Get Signal (Toyobo, Tokyo, Japan). After washing thrice, the membranes were incubated with primary antibodies for β-actin, phosphorylated Ser2448-mTOR, mTOR, phosphorylated Thr389-p70s6k (S6K1), p70s6k, phosphorylated Ser235/236-S6, S6, phosphorylated Thr202/Tyr204 extracellular signal-regulated kinase (ERK)1/2, ERK (all 1:2000; Cell Signaling Technology (CST), Beverly, MA, USA), GDF-8/myostatin (1:2000; Abbiotec, San Diego, CA, USA), follistatin (1:2000; Aviva Systems Biology, San Diego, CA, USA), phosphorylated Ser465/467-Smad2/Ser423/425-Smad3, Smad2/Smad3 (both 1:2000; CST), LC3A/B, Beclin1, Atg7, Atg5 (all 1:2000; Cell Signaling), p62 (1:5000; Medical & Biological Laboratories Co., Ltd., Aichi, Japan), and α-Tubulin (1:5,000; Sigma-Aldrich, St. Louis, MO, USA). They were then incubated with anti-rabbit horseradish peroxidase (HRP)–conjugated secondary antibodies (1:5000; CST) in a dilution buffer (Can Get Signal, Toyobo) for 1 h at 25 °C. After several washes, protein bands were visualized using the ECL Prime reagent (GE Healthcare, Piscataway, NJ, USA) and photographed using the ChemiDoc™ Touch

Imaging System (Bio-Rad). To analyze Hsp72 expression levels, the membranes were further incubated firstly with an anti-Hsp72 alkaline phosphatase conjugate (1:2000; Stressgen, Victoria, BC, Canada) in T-TBS ,with 5% nonfat dry milk for 1 h at 25 °C, and secondly, with an alkaline phosphatase substrate (Immun-Star; Bio-Rad) at room temperature. Data were analyzed using Image Lab ver. 5.2.1 software (Bio-Rad). All phosphorylation ratios were normalized to that of the male CT group (n = 6). β-actin or α-Tubulin were used as a loading control, to confirm standardized protein loading and transfer.

Real-time PCR

Total RNA was isolated as described previously [30] and purified using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The concentration and purity (A260/280 and A260/230) of the RNA were then evaluated using the QIAxpert system (Qiagen), before total RNA samples (2 μg) were reverse transcribed using SuperScript VILO MasterMix (Invitrogen, Carlsbad, CA, USA). The expression of *heat shock protein* (*Hsp*) *72* (*HSPA1A*, Rn04224718_u1), *Myostatin* (*Mstn*, Rn00569683_m1), and *Myod1* (*MyoD*, Rn01457527_g1) mRNA was quantified via TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). The LightCycler 480 System II (Roche) was used to subject DNA to PCR cycling conditions comprising 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 1 min, before a final cycle of 37 °C for 30 s. mRNA concentrations were normalized to that of *18S* mRNA, and data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data were expressed as the mean ± standard error (SE). Statistical significance was determined by using a two-way (sex × exercise) analysis of variance (ANOVA) to separately consider two exercise conditions, followed by Sidak's multiple comparisons test to perform pairwise comparisons to detect significant interactions. P values < 0.05 were considered to indicate statistical significance. All analyses were conducted using Prism ver. 6.0 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Body and soleus muscle weight

Table 1 shows the body weights, and both absolute and relative (to body weight) soleus muscle weights exhibited by the analyzed male and female rats from the CT group. While both the body and absolute soleus muscle weights were significantly greater in male rats, the relative soleus muscle weights exhibited by male and female rats were similar (male, 0.37 ± 0.002 mg/g; female, 0.39 ± 0.002 mg/g).

Blood parameters

To further evaluate potential sex-specific differences in exercise responses, we analyzed blood parameters including creatinine (CRE), blood urea nitrogen (BUN), albumin (ALB), total protein (TP), and non-esterified fatty acid (NEFA) levels (Table 2). The results of these analyses showed that, although CRE, BUN, ALB, TP, and NEFA levels varied significantly (P < 0.0001) after horizontal exercise, they did not differ between the sexes. Similarly, the CRE, BUN, ALB, TP, and NEFA levels exhibited by the rats also varied significantly (P < 0.01) after downhill exercise, and while the CRE, ALB, and TP levels were found to be higher in female rats, the interaction between sex and exercise was not significant. In fact, no interaction between sex and exercise was observed in any of the analyzed parameters; thus, although some parameters were increased in the analyzed female rats, the overall responses of the male and female rats to acute exercise were very similar. Overall, no sex-specific changes to blood parameters were observed in response to acute horizontal or downhill exercise.

Changes in Hsp72 mRNA and protein levels

Hsp72 mRNA and protein levels were measured after exercise (Fig. 1). *Hsp72* mRNA was resultantly shown to be significantly increased after horizontal and downhill exercise but to be similar between the sexes (Fig. 1d, e). In contrast, Hsp72 protein levels differed significantly (P = 0.0045) after downhill exercise and were higher in male than in female rats (Fig. 1c).

Changes in the expression of myostatin and related signal transduction proteins

We analyzed myostatin and follistatin protein levels and the mothers against decapentaplegic homolog (Smad) 2/3 phosphorylation ratio in both male and female rats after exercise (Fig. 2). The results of these analyses showed that follistatin protein levels were unchanged in both sexes after exercise (Fig. 2d, e). In contrast, while *myostatin* mRNA expression was not significantly altered after exercise (data not shown), myostatin protein levels were significantly increased (P = 0.0187; Fig. 2c) in female rats after downhill exercise. Similarly, significant sex-specific increases (HR, P = 0.0367; DR, P = 0.0267) to the Smad2 (Ser465/467)/Smad3 (Ser423/425) phosphorylation ratio were induced after both horizontal (P < 0.0001) and downhill (P < 0.0001) exercise (Fig. 2f, g). Moreover, horizontal and downhill exercise significantly decreased the expression of *MyoD* mRNA (Fig. 2h, i).

Table 1 Rat body and absolute and relative soleus muscle weights

	Absolute		Relative	
	Male	Female	Male	Female
Body weight (g)	260.9 ± 2.8	162.8 ± 1.0*		
Soleus weight (mg, mg/g)	96.4 ± 1.2	64.4 ± 0.7*	0.37 ± 0.002	0.39 ± 0.002

Values are presented as the mean ± SE. $n = 6$ per group. *$p < 0.05$ vs. male

Changes in mTOR and p44/42 mitogen-activated protein kinase ERK1/2 signaling phosphorylation levels

Figure 3 shows observed changes in mTOR (Ser2448), S6K1 (Thr389), S6 (Ser 235/236), and ERK (Thr202/Tyr204) expression and phosphorylation levels after exercise. Neither of the expression levels of mTOR, S6K1, S6, and ERK proteins was statistically changed in males or females of either experimental group (data not shown). Similarly, although S6K1 phosphorylation levels were higher in male compared with those in female rats (Fig. 3d, e), these differences were unaffected by exercise. In contrast, ERK phosphorylation levels were significantly increased in male compared with those in female rats ($P = 0.0354$; Fig. 3i) after downhill exercise.

Changes in the expression of autophagy-related proteins

We analyzed LC3 and autophagy-related protein levels in both male and female rats, after exercise (Fig. 4). The levels of autophagy-related proteins (Beclin1, Atg7, and Atg5) were unchanged in both sexes after exercise (Fig. 4f–k), whereas the ratio of LC3-II/I and LC3-II protein level significantly decreased in both male and female rats, regardless of the exercise routine ($P < 0.05$; Fig. 4b, c). Moreover, downhill exercise significantly altered the expression of p62 in female but not in male rats ($P < 0.05$; Fig. 4e).

Discussion

The results of the present study suggest that intracellular signaling response to downhill, but not horizontal running, is different between male and female rat soleus muscles. Thus, the study demonstrates, for the first time, that downhill exercise remarkably influences signaling responses that promote muscle mass maintenance, likely by exerting a higher impact on the soleus muscle than horizontal running. Importantly, our data also indicate that sex-related differences in exercise-induced skeletal muscle adaptation are likely caused by sex-specific intracellular signaling responses. Although the specific mechanisms that underlie these differences require further investigation, our findings implicate that they may include changes to Hsp72 expression, ERK phosphorylation, and/or myostatin-signaling activation in the rat soleus muscle.

To date, investigations into sex-specific differences in muscle signaling responses to treadmill exercise in humans have been impeded by the difficulty of applying an equal load to male and females muscles, when males and females with the same body mass index typically exhibit a different (approximately 2–5%) muscle-to-body mass ratio. In the present study, the absolute body and muscle weights were quite different between the male and female animals; however, the relative muscle weight was very similar; therefore, the physiological load that was applied to the soleus muscle during exercise was equivalent in the male and female rats. Combined with the fact that both horizontal and downhill exercise directly target the

Table 2 Blood parameters before and after exercise

	Male			Female			Two-way ANOVA (*P*)			
							HR		DR	
	CT	HR	DR	CT	HR	DR	Sex	Exercise	Sex	Exercise
CRE (mg/dL)	0.22 ± 0.01	0.28 ± 0.01	0.27 ± 0.01	0.24 ± 0.01	0.28 ± 0.01	0.30 ± 0.01	n.s.	< 0.0001	0.0376	< 0.0001
BUN (mg/dL)	17.1 ± 0.5	22.5 ± 1.0	19.8 ± 0.5	19.0 ± 1.2	22.5 ± 0.9	21.8 ± 1.3	n.s.	< 0.0001	n.s.	0.0092
ALB (g/dL)	3.70 ± 0.06	4.45 ± 0.05	4.43 ± 0.02	3.83 ± 0.10	4.47 ± 0.06	4.70 ± 0.07	n.s.	< 0.0001	0.0077	< 0.0001
TP (g/dL)	5.12 ± 0.09	6.13 ± 0.08	6.15 ± 0.03	5.15 ± 0.14	6.23 ± 0.10	6.53 ± 0.05	n.s.	< 0.0001	0.0268	< 0.0001
NEFA (μEq/L)	470.2 ± 40.4	860.2 ± 75.9	887.2 ± 98.6	487.2 ± 37.1	1058.0 ± 71.7	784.0 ± 152.1	n.s.	< 0.0001	n.s.	0.0012

Values are presented as the mean ± SE. $n = 6$ per group. No significant interactions between sex and exercise responses were observed. *CRE*, creatinine; *BUN*, blood urea nitrogen; *ALB*, albumin; *TP*, total protein; *NEFA*, non-esterified fatty acids; *CT*, control; *HR*, horizontal running; *DR*, downhill running; *n.s.*, not-significant

soleus muscle, the design of the present study facilitated an effective analysis of sex-specific soleus muscle signaling responses to two type of treadmill exercise, which applied equivalent mechanical loads in male and female animals.

The data generated by the present study firstly indicated that downhill, but not horizontal running, induces sex-specific impacts on Hsp72 induction. The bout of downhill exercise used in this study is of short duration, but comprises a high impact, mainly eccentric contractions in a manner that has been previously shown to cause damage to the soleus muscle [1, 13, 20]. Thus, the observed higher level of Hsp72 induction in the male rats may be associated with an increased capacity for musculoskeletal protection and/or recovery after high impact mechanical stimulation [26]. Female Hsp72 induction has been previously suggested to be reduced by higher levels of estrogen production. For example, a previous study showed that after exercise (treadmill running at 30 m/min for 60 min), *Hsp70* mRNA and protein induction in gastrocnemius (both red and white) skeletal muscle was greater in male than in female, and in placebo- than in estrogen-treated ovariectomized female rats [23]. These data suggest that estrogen levels may modulate the post-exercise induction of Hsp72; however, they do not explain why downhill, but not horizontal running, induced sex-specific Hsp72 responses observed in the present study. In fact, this observation suggests that sex-specific Hsp72 responses may be dependent upon on the type of exercise stress being experienced. It may be that Hsp72 functions to protect, aids the recovery of, and facilitates the adaptation of muscles following high impact mechanical stimulation (eccentric exercise) [26]. The fact that *HSPA1A* (*Hsp72*) mRNA levels in the present study were upregulated by acute exercise in both sexes implies that, while both sexes were susceptible to this form of muscular stress, the male rats were more severely affected, and thus exhibited an increased response to downhill exercise, likely via the induction of translational and/or post-translational mechanisms. Further studies will be required to confirm this hypothesis.

The present study also showed that mTOR signaling (mTOR/S6K1/S6) was similarly activated by both downhill and horizontal treadmill exercise in the male and female rats (although, notably, S6K1 phosphorylation was found to be higher in male than in female control animals). These findings support that the achieved mechanical load on the target soleus muscle was similar in both sexes as intended and suggest that during eccentric exercise, mTOR signaling induction is likely not a key factor that regulates sex-specific muscle signaling

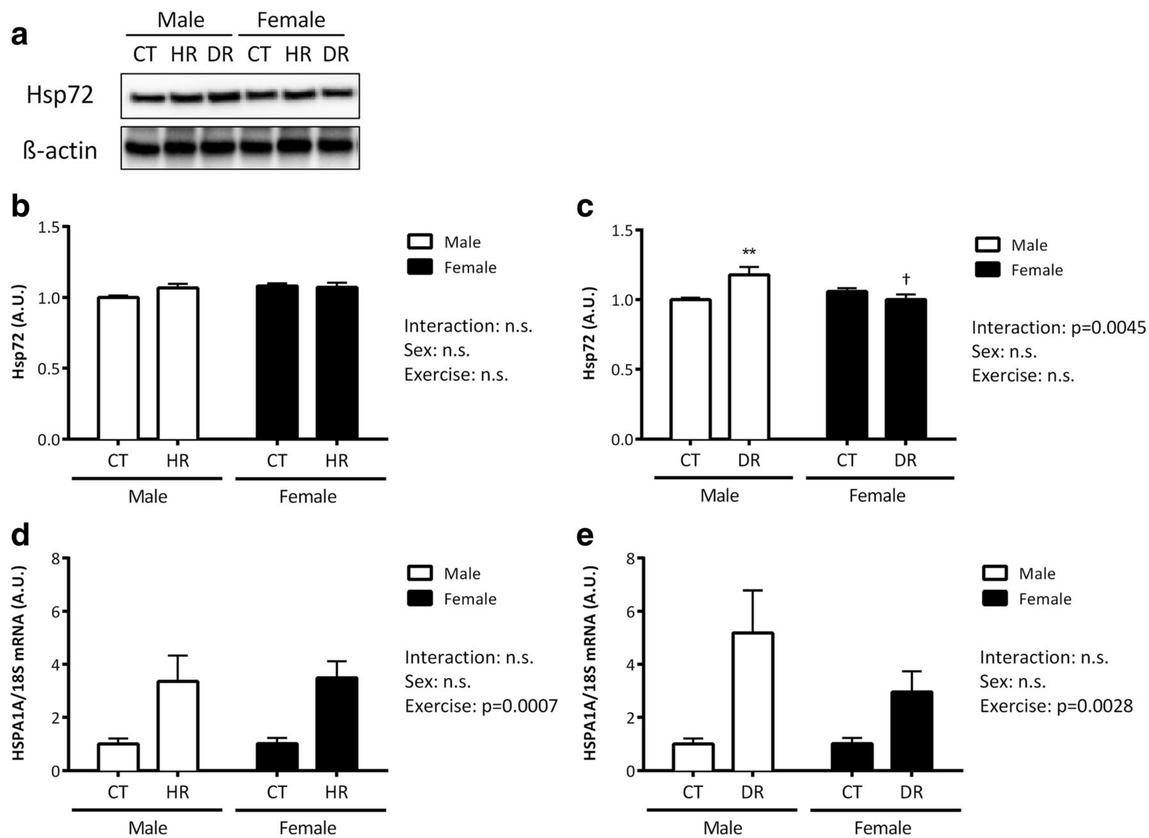


Fig. 1 Heat shock protein (Hsp)72 protein (**a**–**c**) and (*HSPA1A*) mRNA (**d**, **e**) expression were analyzed after a bout of horizontal (HR) or downhill (DR) exercise in male and female rats. Data were analyzed via a two-way ANOVA and presented as the mean ± standard error (SE). $n = 6$ per group. β-actin was used as a loading control. $^{**}P < 0.01$ vs. CT, $^{\dagger}p < 0.05$ vs. male DR. CT, control group; n.s., not-significant; A.U., arbitrary unit

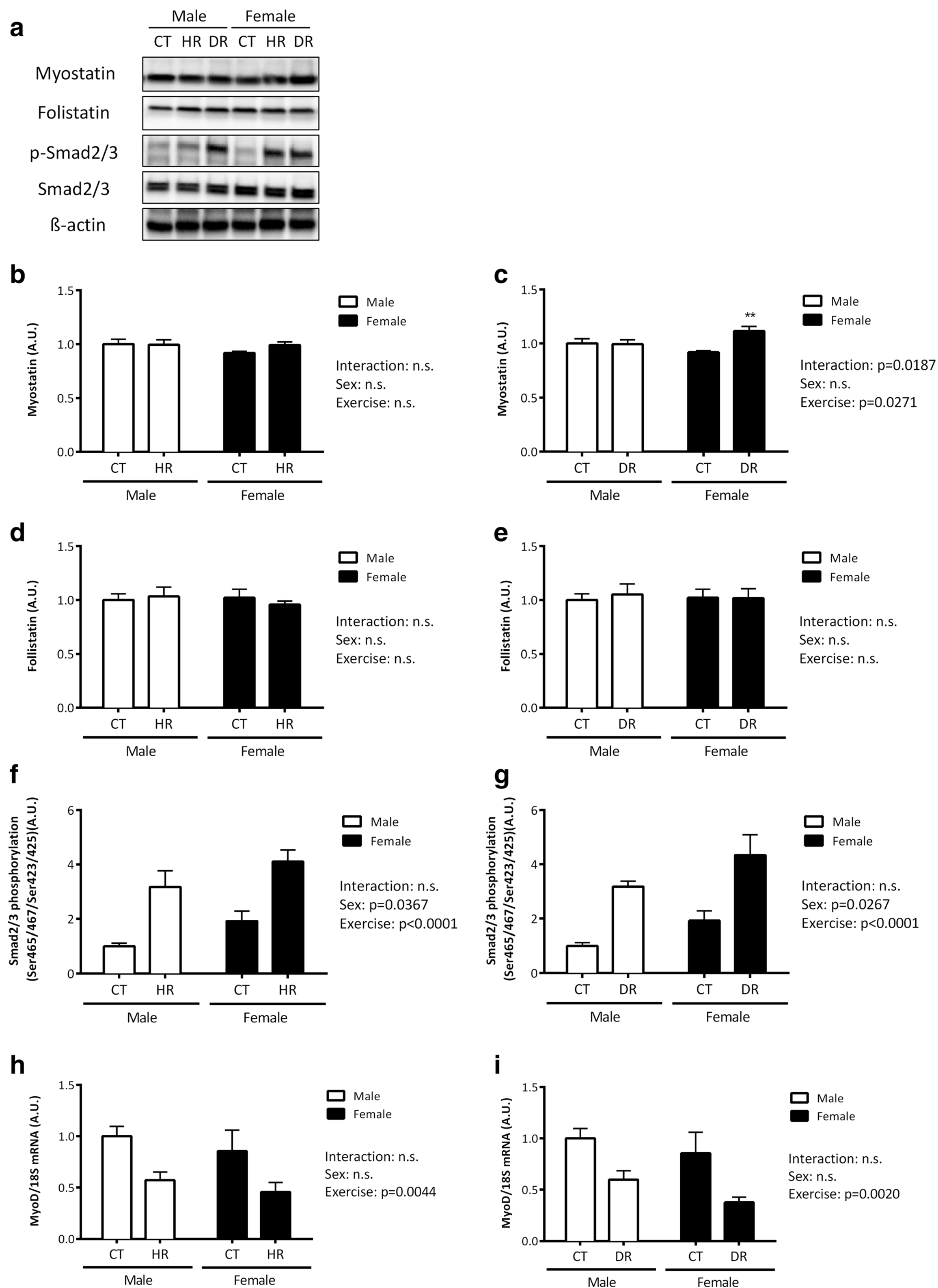


Fig. 2 Representative blots (**a**) demonstrating the expression of myostatin and related signal transduction proteins in the analyzed rats. Myostatin (**b**, **c**) and follistatin (**d**, **e**) expression, the Smad2 (Ser465/467)/Smad3 (Ser423/425) phosphorylation ratio (**f**, **g**), and *MyoD* mRNA expression (**h, i**) were all analyzed after bouts of horizontal (HR) or downhill (DR) exercise in male and female rats. Data were analyzed via a two-way ANOVA and presented as the mean ± standard error (SE). n = 6 per group. β-actin was used as a loading control. $**p < 0.01$ vs. CT. CT, control group; n.s., not-significant; A.U., arbitrary unit

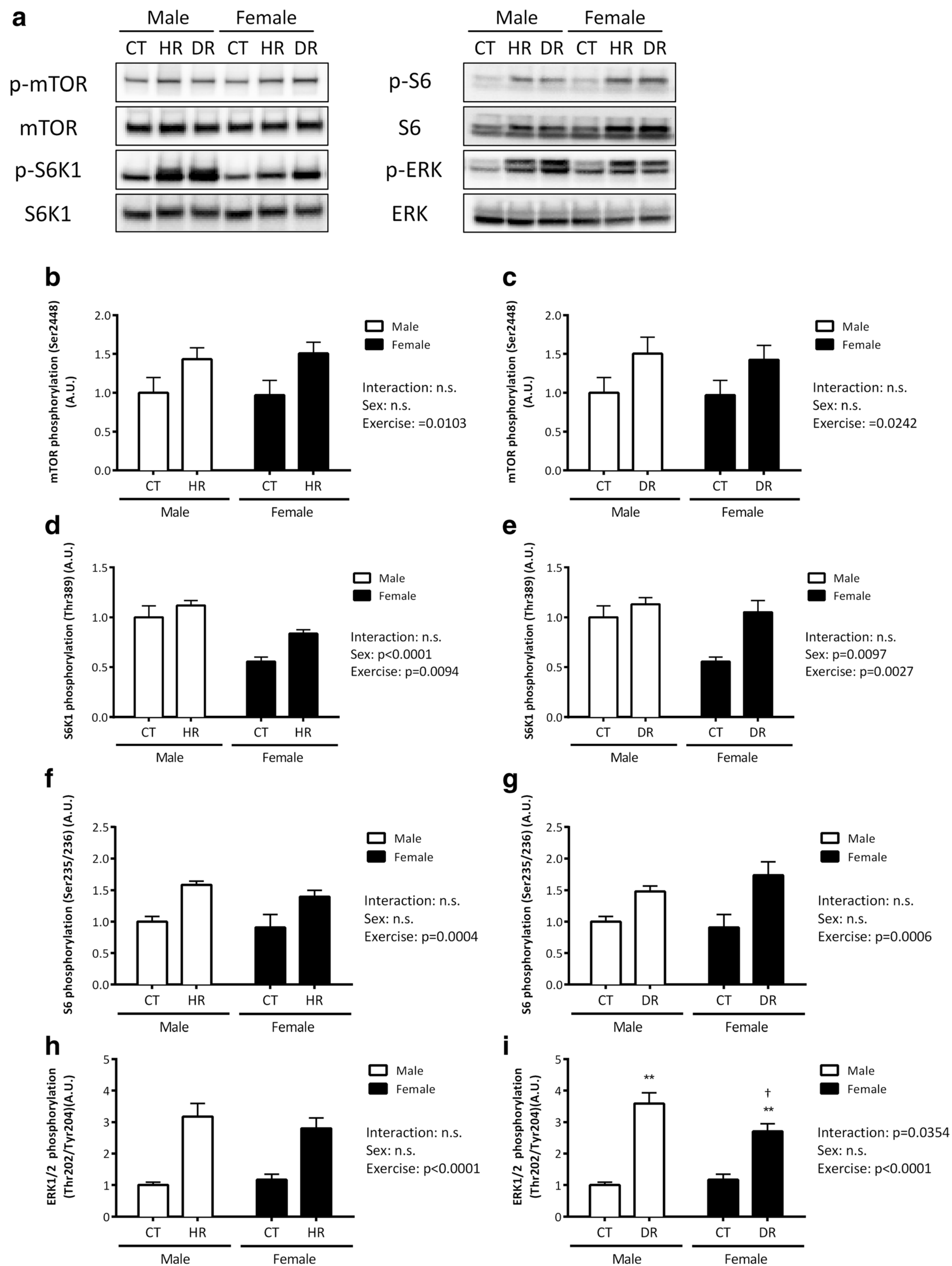
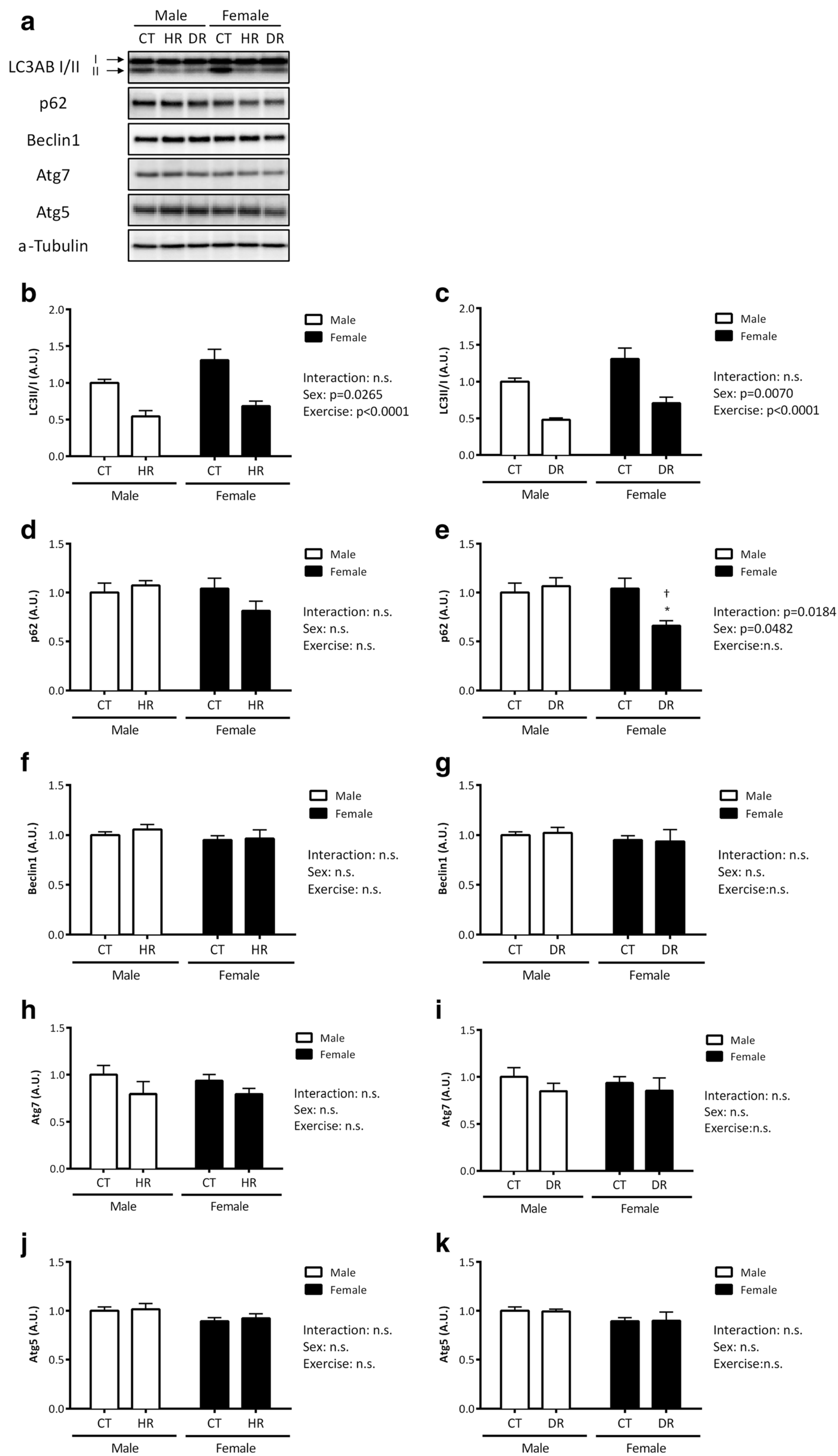


Fig. 3 Representative blots (**a**) and graphs showing the results of the conducted western blot analysis of mTOR (Ser2448) (**b**, **c**), S6K1 (Thr389) (**d**, **e**), S6 (Ser235/236) (**f**, **g**), and ERK (Thr202 and Tyr204) (**h**, **i**) expression and phosphorylation after a bout of horizontal (HR) or downhill (DR) exercise in male and female rats. Data were analyzed via a two-way ANOVA and presented as the mean ± standard error (SE). $n = 6$ per group. $^{**}p < 0.01$ vs. CT, $^{\dagger}p < 0.05$ vs. male DR. CT, control group; n.s., not-significant; A.U., arbitrary unit

responses or adaptations [10, 11]. In contrast, ERK phosphorylation was found to be increased in male compared with that in female rats after downhill, but not horizontal, exercise. ERK is known to regulate the activity of several nuclear transcription


a
Male
Female
CT HR DR CT HR DR
LC3AB I/II
I
II
p62
Beclin1
Atg7
Atg5
a-Tubulin
b
LC3II/I (A.U.)
Male
Female
Interaction: n.s.
Sex: p=0.0265
Exercise: p<0.0001
c
LC3II/I (A.U.)
Interaction: n.s.
Sex: p=0.0070
Exercise: p<0.0001
d
p62 (A.U.)
Interaction: n.s.
Sex: n.s.
Exercise: n.s.
e
p62 (A.U.)
†
*
Interaction: p=0.0184
Sex: p=0.0482
Exercise:n.s.
f
Beclin1 (A.U.)
Interaction: n.s.
Sex: n.s.
Exercise: n.s.
g
Beclin1 (A.U.)
Interaction: n.s.
Sex: n.s.
Exercise:n.s.
h
Atg7 (A.U.)
Interaction: n.s.
Sex: n.s.
Exercise: n.s.
i
Atg7 (A.U.)
Interaction: n.s.
Sex: n.s.
Exercise:n.s.
j
Atg5 (A.U.)
Interaction: n.s.
Sex: n.s.
Exercise: n.s.
k
Atg5 (A.U.)
Interaction: n.s.
Sex: n.s.
Exercise:n.s.

◀ **Fig. 4** Representative blots (**a**) and graphs showing LC3-II/I (**b**, **c**), p62 (**d**, **e**), Beclin1 (**f**, **g**), Atg7 (**h**, **i**), and Atg5 (**j**, **k**) expression after horizontal (HR) or downhill (DR) exercise in male and female rats. Data were analyzed via a two-way ANOVA and are presented as the mean ± standard error (SE). $n = 6$ per group. α-Tubulin was used as a loading control. $^{*}p < 0.05$ vs. CT, $^{\dagger}p < 0.05$ vs. male DR. CT, control group; n.s., not-significant; A.U., arbitrary unit

factors in response to diverse systemic stimuli, including insulin and growth factors, and local stressors, such as muscle contractions [29]. Furthermore, ERK has been shown to regulate muscle cell proliferation and differentiation; therefore, the increased ERK induction in male rats in the present study may be indicative of a greater myogenic response to downhill exercise. Notably, sex-related differences in myostatin levels were also observed after downhill (eccentric) exercise. Specifically, the female rats exhibited slightly increased levels of known negative regulators of myogenesis [5, 19] (although expressions of the myostatin antagonist, follistatin, were similar in both sexes). Myostatin is known to bind to the activin type IIB receptor (ActRIIB) and to thereby mediate Smad2/3 phosphorylation, activation, and complex formation with Smad4 [24]. Nuclear translocation of the Smad2/3/4 complex subsequently blocks the transcription of myogenesis-associated genes [5]. Notably, while the present study showed that the response of Smad2/3 phosphorylation levels was similar in male and female rats after exercise, Smad2/3 phosphorylation status after downhill exercise was found to be greater in the female than in the male rats. Given that a recent report showed that female subjects exhibit greater increases in TGF-β signaling than males in response to acute resistance exercise [16], the increased myostatin signaling responses observed here may represent a mechanism by which muscle mass maintenance is negatively regulated in females following eccentric (resistance) exercise training. Moreover, ERK can also directly inhibit the nuclear translocation of the Smad2/3/4 complex [2]; therefore, it may be that these molecules act together to regulate myogenesis and muscle regeneration after high-impact exercise. It is possible that patterns of decreased ERK activation and increased myostatin expression similar to those that were observed in the female rats in the present study after downhill exercise may underlie the differential adaptation of female (compared with male) human skeletal muscle to resistance exercise [10, 11].

Importantly, the modulation of Hsp72, ERK1/2 phosphorylation, and/or myostatin levels is involved in the regulation of autophagy. Autophagy (macroautophagy) is a bulk degradation system occurring within lysosomes, and recent evidence revealed that in addition to its role in cellular homeostasis, it acts as an essential modulator of adaptation to exercise [15, 18, 27]. In the current study, LC3-II level and the ratio of LC3-II/LC3-I, which is the most widely used biomarker of autophagic flux, were similarly decreased by both downhill and horizontal treadmill exercise in male and female rats, suggesting that the changes in autophagic regulation via LC3 activation is similar in both sexes regardless of the type of exercise. This may be related to a similar mTOR activation, which regulates autophagy in the skeletal muscle. On the other hand, we found that p62 expression, a notorious maker of selective autophagy, was exclusively decreased in females after downhill exercise, but not significantly altered after horizontal exercise. Accumulation of p62 has been used as a marker for inhibition of autophagy [14]; thus, the decline in p62 level indicates the enhancement of autophagy in female muscle. Although we could not elucidate the reason for this discrepancy, the significant decline in p62 levels observed in the female muscles could be related to higher baseline levels of autophagy in female than in male rats. While p62 leads to the degradation of ubiquitinated proteins, not only through autophagy but also via the proteasome [17], the contribution of p62 to autophagy induction following downhill exercise remains unclear. Moreover, given that LC3-II expression suddenly decreased after exercise and rose during the recovery periods (0.5–3 h) in cardiac muscle [21] and acute endurance exercise activates autophagy at the gene and protein levels in rodent skeletal muscle [12], additional time-course studies are warranted to clarify the role of sex-specific regulation of autophagy in the muscle adaptation to different types of exercise.

The present study was limited by the fact that the effects of the estrous cycle on muscle adaptation in female rats was not examined, despite the fact that previous studies have shown that estrogen can affect stress and anabolic responses to acute exercise [8, 23]. Moreover, while an endpoint immediately after the acute exercise was selected to evaluate acute signaling responses to exercise (since the activation of most intramuscular signaling pathways in response to acute exercise is transient in rat skeletal muscle [9]), the study did not consider long-term muscle adaptation to exercise. Further studies are required to clarify the roles of the identified sex-specifically expressed signaling molecules and pathways in muscle adaptation to different types of exercise. Nonetheless, the present study provides novel and invaluable insights into sex-specific differences in signaling responses to different types of treadmill exercise in rat soleus muscle.

Conclusions

The data presented here indicate that intracellular signaling response to downhill, but not horizontal running, is different in male and female rat soleus muscles. Furthermore, our findings suggest that, in the rat soleus muscle, these may include changes to Hsp72 expression, ERK phosphorylation, and/or myostatin-signaling activation. These data indicate that sex-related differences in exercise-induced skeletal muscle adaptation may be mediated by the differential induction of intracellular signaling pathways; further studies are required to clarify the significance of these sex-related differential intramuscular signaling responses in skeletal muscle adaptation.

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Author contributions T.Y., R.K., and H.N. conceived and designed the research; T.Y., S.C., T.T., and T.N. performed the experiments; T.Y. S.C., and T.N. analyzed the data; T.Y. and R.K. interpreted the results of the experiments; T.Y. prepared the figures; T.Y. drafted the manuscript; T.Y., S.C., T.T., T.N., R.K., T.S., and H.N. edited and revised the manuscript; T.Y., S.C., T.T., T.N., R.K., T.S., and H.N. approved the final version of the manuscript.

Compliance with ethical standards

This study was approved by the Juntendo University Animal Care Committee (H28-15) and was conducted according to the guiding principles for the care and use of laboratory animals set forth by the Physiological Society of Japan.

Conflict of interest The authors declare that they have no conflict of interest.

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