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Excessive eccentric exercise-induced overtraining model leads to endoplasmic reticulum stress in mice skeletal muscles

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ABSTRACT

Aims: The present study verified the responses of selected endoplasmic reticulum (ER) stress proteins (i.e., BiP, ATF-6, pIRE1, pPERK, and peIF2alpha) in mice skeletal muscles after three different running overtraining (OT) protocols with same external load (i.e., intensity vs. volume), but performed in downhill, uphill and without inclination.
Materials and methods: The rodents were randomly divided into control (CT; sedentary mice), overtrained by downhill running (OTR/down), overtrained by uphill running (OTR/up) and overtrained by running without inclination (OTR) groups. The incremental load test and exhaustive test were used as performance parameters. Forty hours after the exhaustive test performed at the end of the OT protocols (i.e., at the end of week 8) and after a 2-week total recovery period (i.e., at the end of week 10), the extensor digitorum longus (EDL) and soleus muscles were removed and used for immunoblotting.
Key findings: For both skeletal muscle types, the OTR/down protocol increased the pIRE-1, pPERK and peIF2alpha, which were not normalized after the total recovery period. At the end of week 8, the other two OT protocols up-regulated the BiP, pPERK and peIF2alpha levels only for the soleus muscle. These ER stress proteins were not normalized after the total recovery period for the OTR/up group.
Significance: The above findings suggest that the OTR/down protocol-induced skeletal muscle ER stress may be linked to a pathological condition in EDL and soleus muscles.

1. Introduction

The endoplasmic reticulum (ER) is an intracellular organelle found in all eukaryotic cells and responsible for the biosynthesis, folding, assembly and modification of soluble and membrane proteins [17]. The ER also functions as a dynamic calcium storage responding to growth factors, hormones and stimuli that disrupt cellular energy homeostasis, nutrient availability or redox sate [36]. Physiological conditions increasing the protein folding demand or stimuli deregulating the reactions responsible by the protein folding lead to an imbalance between the protein folding load and the ER capacity, which causes the accumulation of unfolded or misfolded proteins inside the ER lumen [35,36].

This ER stress acts on the cells activating the unfolded protein response (UPR) to deal with stressful states and to solve protein folding defect [29,30]. The main UPR effectors are the activating transcription factor 6 (ATF6), the inositol requiring transmembrane kinase/endonuclease 1 (IRE1) and the double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK). These effectors are released from the abundant ER chaperone immunoglobulin-heavy-chain-binding protein (BiP), activating the transcription of UPR target genes by the inhibition of the PERK-mediated eukaryotic translation-initiation factor 2alpha (eIF2alpha) [11], the autophosphorylation of IRE1 [29,30] and the migration of a functional fragment of ATF6 to nucleus [12,34].

Considering the crosstalk between ER stress and inflammation, the IRE1 autophosphorylation alters its cytosolic domain allowing the bind of the adaptor protein tumor necrosis factor receptor-associated factor 2 (TRAF2), which activates the IκB kinase (IKK) and JUN N-terminal kinase (JNK), inducing the transcription of inflammatory genes [7,15,31]. The chronic increase of the interleukins 1-beta, 6 (IL-1beta and IL-6) and tumor necrosis factor alpha (TNF-alpha) also

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leads to ER stress, disrupting metabolic functions and causing more inflammation [36].

The regular moderate-intensity exercise is used to prevent and treat several inflammatory processes [10,32] and the ER stress induced by this type of exercise acts as a protective mechanism against current and future stressors [8,18,33]. In contrast, studying a downhill running-based overtraining (OT) model, Pereira et al. [26,27] observed high levels of IL-6, TNF-alpha, IKK and JNK in serum and skeletal muscles of mice. According to Meeusen and coworkers [22], OT is defined as a process of intensified training that may lead to functional overreaching (FOR), nonfunctional overreaching (NFOR), or OT syndrome (OTS). However, the responses of the ER stress in overtrained mice are unknown. Thus, we verified the effects of this OT protocol [26,27] on the BiP, ATF6, pIRE1 (Ser734), pPERK (Thr981) and peIF2alpha (Ser52) levels in mice skeletal muscles. Based on previous studies [7,15,31,36], we hypothesize that this OT protocol will up-regulate these proteins.

Considering the singular characteristics of eccentric exercise [13] and knowing that other OT models were developed without the predominance of this type of contraction [14], we also compared the responses of the aforementioned ER stress proteins to the downhill running-based OT protocol [26,27] with other two OT protocols with same external load, but performed in uphill and without inclination [25]. Finally, to verify whether the effects of these OT protocols on the skeletal muscle ER stress are linked to pathological or non-pathological conditions [28], we measured the ER stress proteins after a 2-week total recovery period.

2. Methods

2.1. Experimental animals

Eight-week-old male C57BL/6 mice were maintained in individual cages with controlled temperature (22 ± 2 °C) on a 12:12-h light–dark inverted cycle with food (Purina chow) and water ad libitum. The present work was approved by the Ethics Committee of the University of Sao Paulo (ID 14.1.873.53.0) and adheres to the Brazilian law no. 11.794/2008 for the experimental use of animals. The rodents were randomly divided into control (CT; sedentary mice; n = 12), overtrained by downhill running (OTR/down; performed the OT protocol based on downhill running; n = 12), overtrained by uphill running (OTR/up; performed the OT protocol based on uphill running; n = 12) and overtrained by running without inclination (OTR; performed the OT protocol based on running without inclination; n = 12) groups. The mice were manipulated and/or overtrained in a dark room between 6 to 8 AM [24].

2.2. Incremental load test (ILT)

After adaptation to the treadmill running (INSIGHT®, Ribeirão Preto, São Paulo, Brazil) [24–27], the rodents performed the ILT. The initial intensity of this test was 6 $m \cdot min^{-1}$ at 0% with increments of 3 $m \cdot min^{-1}$every 3 min until exhaustion, which was defined when each mouse touched the end of the treadmill 5 times in 1 min. The rodents were encouraged using physical prodding and when they became exhausted without completing the stage, the exhaustion velocity (EV; $m \cdot min^{-1}$) was corrected according to Kuipers et al. [20]. The EV of each mouse was used to prescribe the intensity of the OT protocols [24–27]. On week 0, the experimental groups performed the ILT without inclination; however, at the end of weeks 4, 8, and 10, CT and OTR performed the ILT without inclination, OTR/down performed the ILT in downhill running, and OTR/up performed the ILT in uphill running [25].

2.3. Running OT protocols, 2-week total recovery period and performance evaluations

The 8-week running OT protocols performed in downhill, uphill and without inclination were applied as previously published [25], and each experimental week consisted of 5 days of training followed by 2 days of recovery. At the first four weeks of the OT protocols, the training intensity was maintained at 60% of the EV, the training volume was gradually increased from 15 min per day in the first week to 60 min per day in the fourth week, and rodents ran at a grade of 0%. At the fifth week of the OT protocols, while the training intensity and volume were maintained, the rodents ran at a grade of −14% (i.e., OTR/down group), 14% (i.e., OTR/up group) and 0% (i.e., OTR group). At the sixth week of the OT protocols, the training intensity increased to 70% of the EV. At the seventh week of the OT protocols, the training intensity and volume increased to 75% of the EV and 75 min, respectively. At the eighth week of the OT protocols, the number of the training daily sessions increased from one to two with a rest interval of 4 h.

The CT, OTR/down, OTR/up and OTR groups were re-evaluated at the end of week 10. During these two weeks (i.e., from the end of week 8 to the end of week 10), the rodents from OTR/down, OTR/up and OTR did not perform exercise sessions. The performance evaluations were applied on week 0 and 48 h after the last sessions of the OT protocols at the end of weeks 8 and 10, and consisted of the ILT [24–27] and exhaustive test [24–27]. The exhaustive test was performed 24 h after the ILT and each mouse ran at 36 $m \cdot min^{-1}$ with 8% treadmill grade until exhaustion, which was defined when the mice touched the end of treadmill 5 times in 1 min. The rodents were encouraged using physical prodding.

2.4. Body weight and food intake

The body weight and food intake of the experimental groups were registered daily. Indeed, the food intake was determined by the subtraction of the final food weight (i.e., the weight of the food put in the individual cage after 24 h) from the initial food weight (i.e., the weight of the food put in the individual cage on the previous 24 h) [25].

Table 1
Responses of the incremental load test ($m \cdot min^{-1}$) and exhaustive test (s) to CT, OTR/down, OTR/up and OTR groups at weeks 0, 8 and 10.

	Incremental load test ($m \cdot min^{-1}$)			Exhaustive test (s)		
	Week 0	Week 8	Week 10	Week 0	Week 8	Week 10
CT	24.5 ± 1.4	23.2 ± 1.3*	22.7 ± 1.0*	59.0 ± 6.1	56.2 ± 10.0	57.6 ± 10.8
OTR/down	23.2 ± 1.0$^{\Phi}$	16.2 ± 1.0$^{*,\lambda,\Phi}$	15.5 ± 1.6$^{*,\#,\lambda}$	69.9 ± 6.5	16.5 ± 3.4$^{*,\lambda}$	13.0 ± 2.1$^{*,\lambda,\Phi}$
OTR/up	25.7 ± 0.8	18.8 ± 0.9$^{*,\lambda}$	17.9 ± 1.0$^{*,\lambda}$	70.0 ± 6.8	17.4 ± 2.5$^{*,\lambda}$	20.2 ± 3.0$^{*,\lambda}$
OTR	24.4 ± 0.6	17.9 ± 1.8$^{*,\lambda}$	16.1 ± 0.6$^{*,\lambda}$	71.8 ± 6.1	17.1 ± 2.2$^{*,\lambda}$	18.6 ± 3.8$^{*,\lambda}$

OTR/down: overtrained by downhill running; OTR/up: overtrained by uphill running; OTR: overtrained by running without inclination.
* $P < 0.05$ vs. week 0 for the same experimental group.
$^{\#}$ $P < 0.05$ vs. week 8 for the same experimental group.
$^{\lambda}$ $P < 0.05$ vs. the CT group for the same experimental week.
$^{\Phi}$ $P < 0.05$ vs. the OTR/up group for the same experimental week.

Table 2
Responses of body weight (g) and food intake (g) to CT, OTR/down, OTR/up and OTR groups at weeks 0, 8 and 10.

	Body weight (g)			Food intake (g)		
	Week 0	Week 8	Week 10	Week 0	Week 8	Week 10
CT	22.7 ± 0.4	26.4 ± 0.4*	26.8 ± 0.4*	25.0 ± 1.2	26.3 ± 0.9*	27.0 ± 0.8
OTR/down	21.0 ± 0.3$^{\lambda}$	23.1 ± 0.5$^{*,\lambda,\tau}$	24.6 ± 0.3$^{*,\lambda,\Phi}$	22.7 ± 0.8	22.1 ± 0.7$^{\lambda,\Phi,\tau}$	21.9 ± 0.9$^{\lambda,\Phi}$
OTR/up	20.4 ± 0.4$^{\lambda}$	24.1 ± 0.5$^{*,\lambda}$	26.0 ± 0.4$^{*,\#}$	23.5 ± 0.8	28.8 ± 1.3*	28.2 ± 1.4$^{*,\tau}$
OTR	21.3 ± 0.4$^{\lambda}$	24.3 ± 0.2$^{*,\lambda}$	25.6 ± 0.6*	24.3 ± 0.5	24.6 ± 0.8$^{\Phi}$	21.7 ± 0.9$^{\#,\lambda}$

OTR/down: overtrained by downhill running; OTR/up: overtrained by uphill running; OTR: overtrained by running without inclination.
* $P < 0.05$ vs. week 0 for the same experimental group.
$^{\#}$ $P < 0.05$ vs. week 8 for the same experimental group.
$^{\lambda}$ $P < 0.05$ vs. the CT group for the same experimental week.
$^{\Phi}$ $P < 0.05$ vs. the OTR/up group for the same experimental week.
$^{\tau}$ $P < 0.05$ vs. the OTR group for the same experimental week.

2.5. Skeletal muscle extractions and immunoblotting analysis

The mice were anesthetized 40 h after the exhaustive test performed at the end of the OT protocols (i.e., at the end of week 8) and after 2-week total recovery period (i.e., at the end of week 10). After a fast period of 6 h, the mice were anesthetized with an intraperitoneal (i.p.) injection of 2-2-2 tribromoethanol 2.5% (10–20 $\mu L \cdot g^{-1}$). As soon as anesthesia was confirmed by the loss of the pedal reflexes, due to their different fiber type composition [2], the extensor digitorum longus (EDL) and soleus muscles of both hindlimbs were removed and homogenized in extraction buffer (1% Triton X-100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF and 0.1 $mg \cdot mL^{-1}$ aprotinin) at 4 °C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35), operated at maximum speed for 30 s.

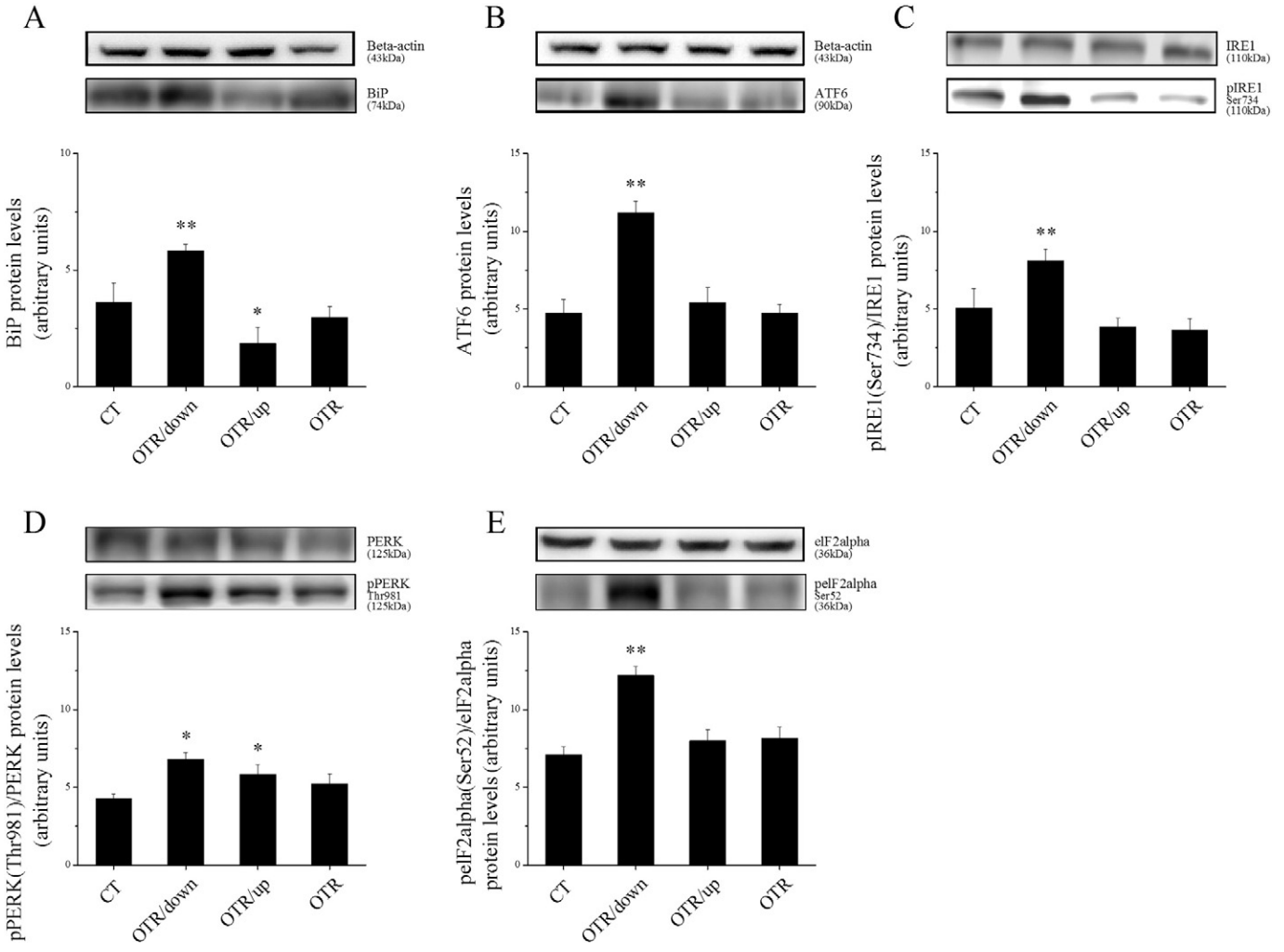

Fig. 1. Responses (arbitrary units) of BiP and its respective beta-actin (A), ATF-6 and its respective beta-actin (B), pIRE1 (Ser734)/IRE1 (C), pPERK (Thr981)/PERK (D) and peIF2alpha (Ser52)/eIF2alpha (E) measured at the end of week 8 in EDL for the experimental groups. Data correspond to means ± SE of n = 6 mice. CT: sedentary mice; OTR/down: overtrained by downhill running; OTR/up: overtrained by uphill running; OTR: overtrained by running without inclination. *P < 0.05 vs. the CT group. **P < 0.05 vs. all experimental groups.

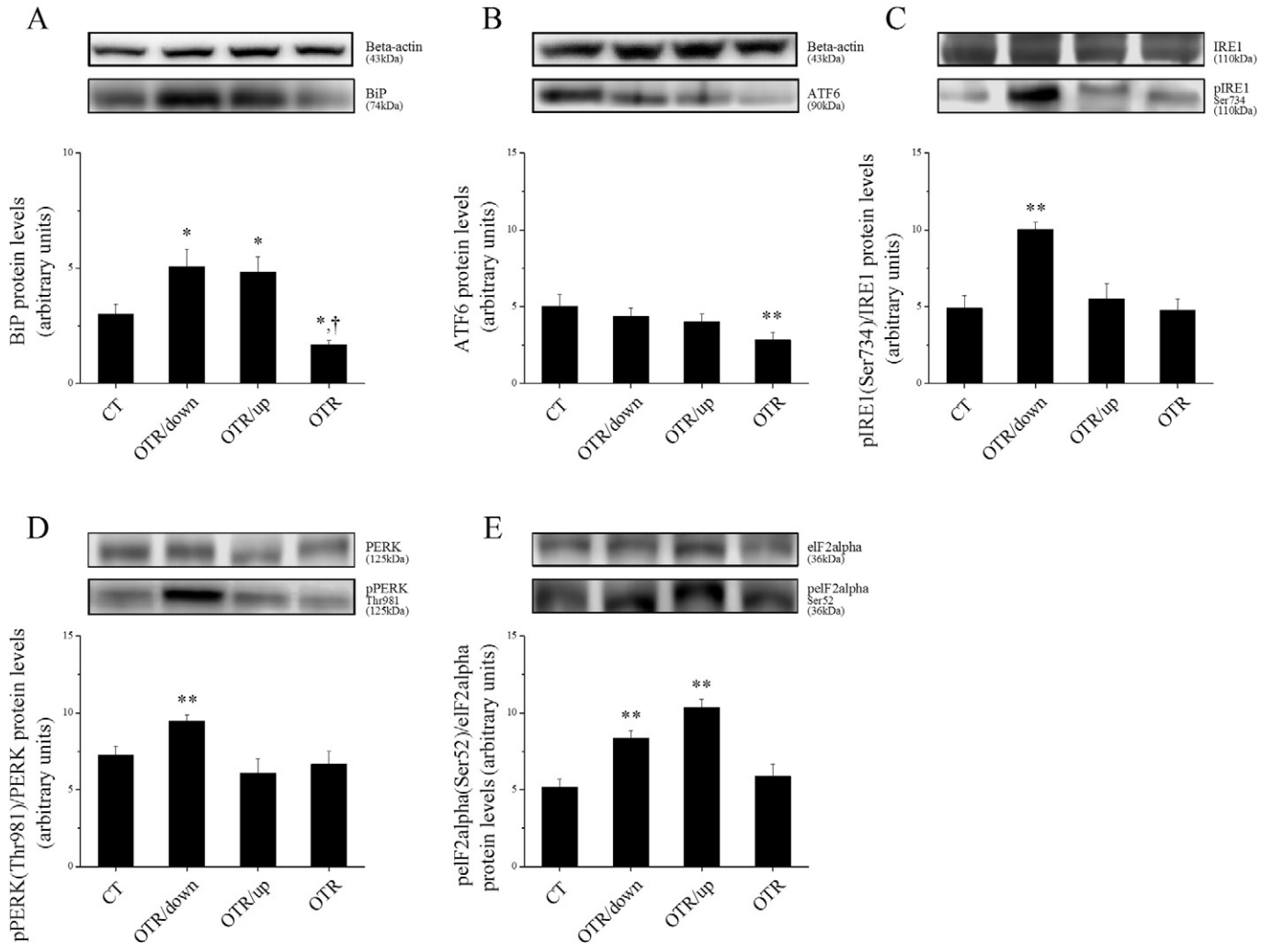

Fig. 2. Responses (arbitrary units) of BiP and its respective beta-actin (A), ATF-6 and its respective beta-actin (B), pIRE1 (Ser734)/IRE1 (C), pPERK (Thr981)/PERK (D) and peIF2alpha (Ser52)/eIF2alpha (E) measured at the end of week 10 in EDL for the experimental groups. Data correspond to means ± SE of n = 6 mice. CT: sedentary mice; OTR/down: overtrained by downhill running; OTR/up: overtrained by uphill running; OTR: overtrained by running without inclination. *P < 0.05 vs. the CT group. **P < 0.05 vs. all experimental groups. †P < 0.05 vs. the OTR/down group.

The extracts were centrifuged (9900 *g*) for 40 min at 4 °C to remove the insoluble material, and the supernatants of these homogenates were used for protein quantification using the Bradford method [3]. Proteins were denatured by boiling in Laemmli sample buffer containing 100 mM DTT, run on SDS-PAGE gel and transferred to nitrocellulose membranes (GE Healthcare, Hybond ECL, RPN303D). The amount of protein employed for the immunoblotting analysis was 150 μg for both skeletal muscle samples. The transfer efficiency to nitrocellulose membranes was verified by brief staining of the blots with Ponceau red stain. These membranes were then blocked with Tris-buffered saline (TBS) containing 5% BSA and 0.1% Tween-20, for 1 h, at 4 °C.

The antibodies used for immunoblotting overnight at 4 °C were BiP (SC33757; 1:750), beta-actin (SC69879; 1: 750), PERK (SC13073; 1: 750), pPERK (Thr981; SC32577; 1: 750), eIF2alpha (SC11386; 1: 750) and peIF2alpha (Ser52; SC101670; 1: 750) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); ATF-6 (NBP1-40,256; 1:1000) from Novus Biologicals (Littleton, CA, USA); IRE1 (AB37073; 1:1000) and pIRE1 (Ser724; AB104157; 1:1000) from Abcam (Cambridge, UK). After washed with TBS containing 0.1% tween-20, all membranes were incubated for 1 h at 4 °C with the appropriate secondary antibody conjugated to horseradish peroxidase. The specific immunoreactive bands were detected by chemiluminescence (GE Healthcare, ECL Plus Western Blotting Detection System, RPN2132). The images were acquired by the C-DiGit™ Blot Scanner (LI-COR^R, Lincoln, Nebraska, USA) and quantified using the software Image Studio for C-DiGit Blot Scanner.

2.6. Statistical analysis

Results are expressed as the means ± standard error (SE). According to Shapiro–Wilk's W-test, the data were normally distributed and homogeneity was confirmed by Levene's test. Therefore, the repeated-measures analysis of variance (ANOVA) was used to examine the effects of the experimental groups on the analyzed performance parameters, body weight and food intake. Planned comparisons were used to analyze the present data. For each parameter, values within groups were compared between the experimental weeks, in addition to comparisons of values between the groups in any given experimental week. The effects of the experimental groups on the molecular parameters were evaluated by the ANOVA one-way. When repeated measures and/or one-way ANOVA indicated significance, Bonferroni's post hoc test was performed. All statistical analyses were two-sided and the significance level was set at $P < 0.05$. Statistical analyses were performed using STATISTICA 8.0 computer software (StatSoft®, Tulsa, OK).

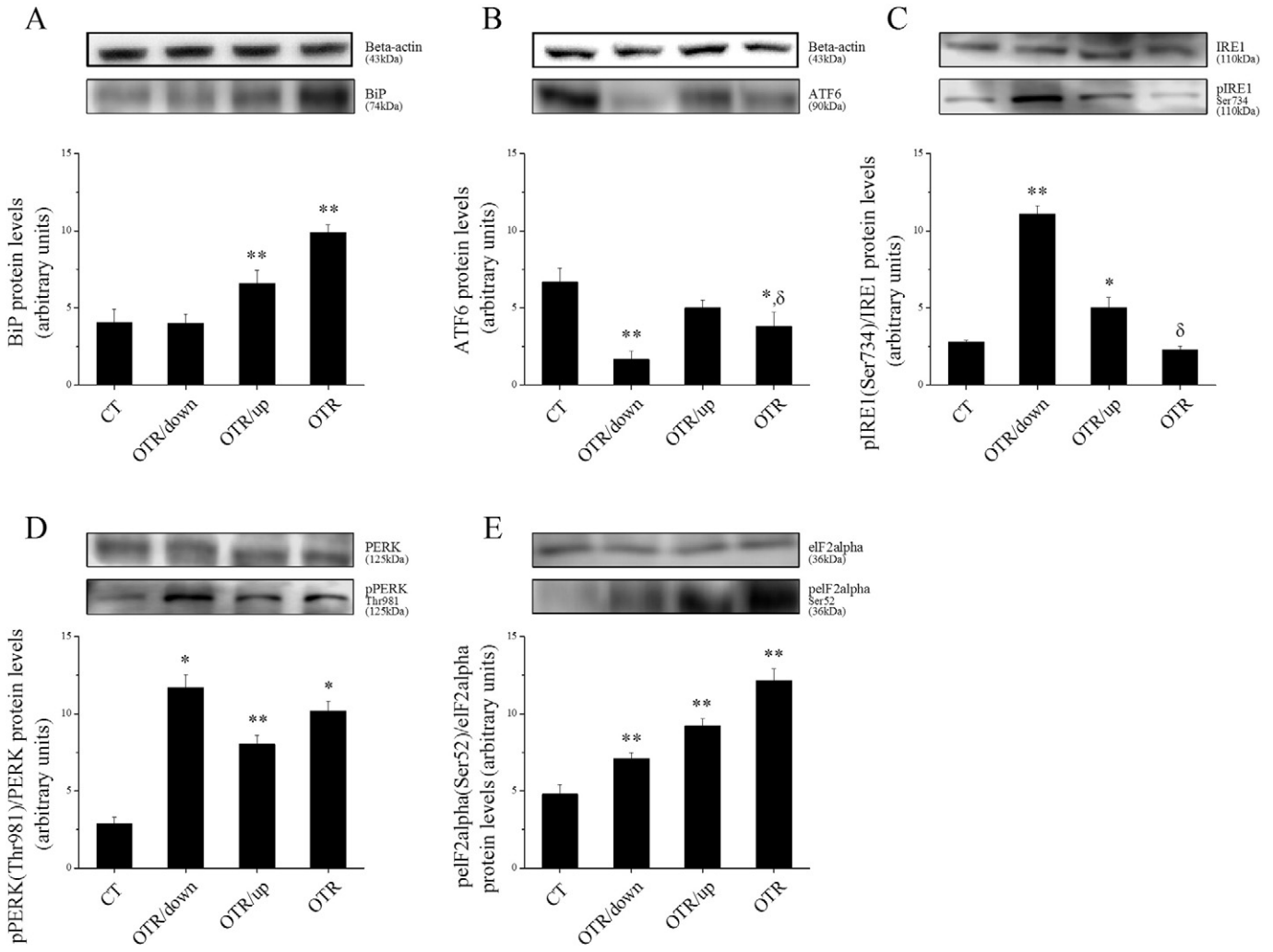

Fig. 3. Responses (arbitrary units) of BiP and its respective beta-actin (A), ATF-6 and its respective beta-actin (B), pIRE1 (Ser734)/IRE1 (C), pPERK (Thr981)/PERK (D) and peIF2alpha (Ser52)/eIF2alpha (E) measured at the end of week 8 in soleus for the experimental groups. Data correspond to means ± SE of n = 6 mice. CT: sedentary mice; OTR/down: overtrained by downhill running; OTR/up: overtrained by uphill running; OTR: overtrained by running without inclination. *P < 0.05 vs. the CT group. **P < 0.05 vs. all experimental groups. $^{\delta}$P < 0.05 vs. the OTR/up group.

3. Results

3.1. Performance parameters, body weight and food intake

Table 1 shows the responses of the incremental load test ($m \cdot min^{-1}$) and exhaustive test (s) to CT, OTR/down, OTR/up and OTR groups at weeks 0, 8 and 10. Considering the incremental load test, all the experimental groups decreased their performances at the end of weeks 8 and 10 compared to week 0. The OTR/down, OTR/up and OTR groups presented lower performance levels compared to the CT group at the end of weeks 8 and 10. The OTR/down protocol decreased its performance at the end of week 10 compared to the end of week 8. The OTR/down group presented lower performance levels compared to the OTR/up group at the end of weeks 0 and 8. Considering the exhaustive test, the OTR/down, OTR/up and OTR groups presented lower performance levels at the end of weeks 8 and 10 compared to their own week 0, and to the CT group at the end of weeks 8 and 10. At the end of week 10, the OTR/down group presented lower performance levels compared to the OTR/up group.

Table 2 shows that all experimental groups increased their body weighs at the end of weeks 8 and 10 compared to week 0. The OTR/down, OTR/up and OTR groups presented lower body weights compared to the CT group at the end of weeks 0 and 8. At the end of week 8, the OTR/down group presented lower body weight compared to the OTR group. At the end of week 10, the OTR/down group presented lower body weight compared to the CT and OTR/up groups. The OTR/up protocol increased its body weight at the end of week 10 compared to the end of week 8. Regarding the food intake, the CT and OTR/up groups increased this metabolic parameter at the end of week 8 compared to week 0. At the end of week 8, the OTR/down group presented lower food intake compared to the other experimental groups, and the OTR group presented lower food intake compared to the OTR/up group. At the end of week 10, the OTR/down and OTR groups presented lower food intake compared to the CT group. The OTR/up protocol increased the food intake compared to its own week 0 and to the OTR/down and OTR groups at the end of week 10. Finally, the OTR protocol decreased this metabolic parameter compared to its own week 8.

3.2. EDL endoplasmic reticulum stress signaling

At the end of week 8, the OTR/down protocol increased the BiP, ATF-6, pIRE1 (Ser734) and peIF2alpha (Ser52) levels compared to the CT, OTR/up and OTR groups (Fig. 1A, B, C and E). Fig. 1A shows that the OTR/up protocol decreased the BiP levels compared to the CT group.

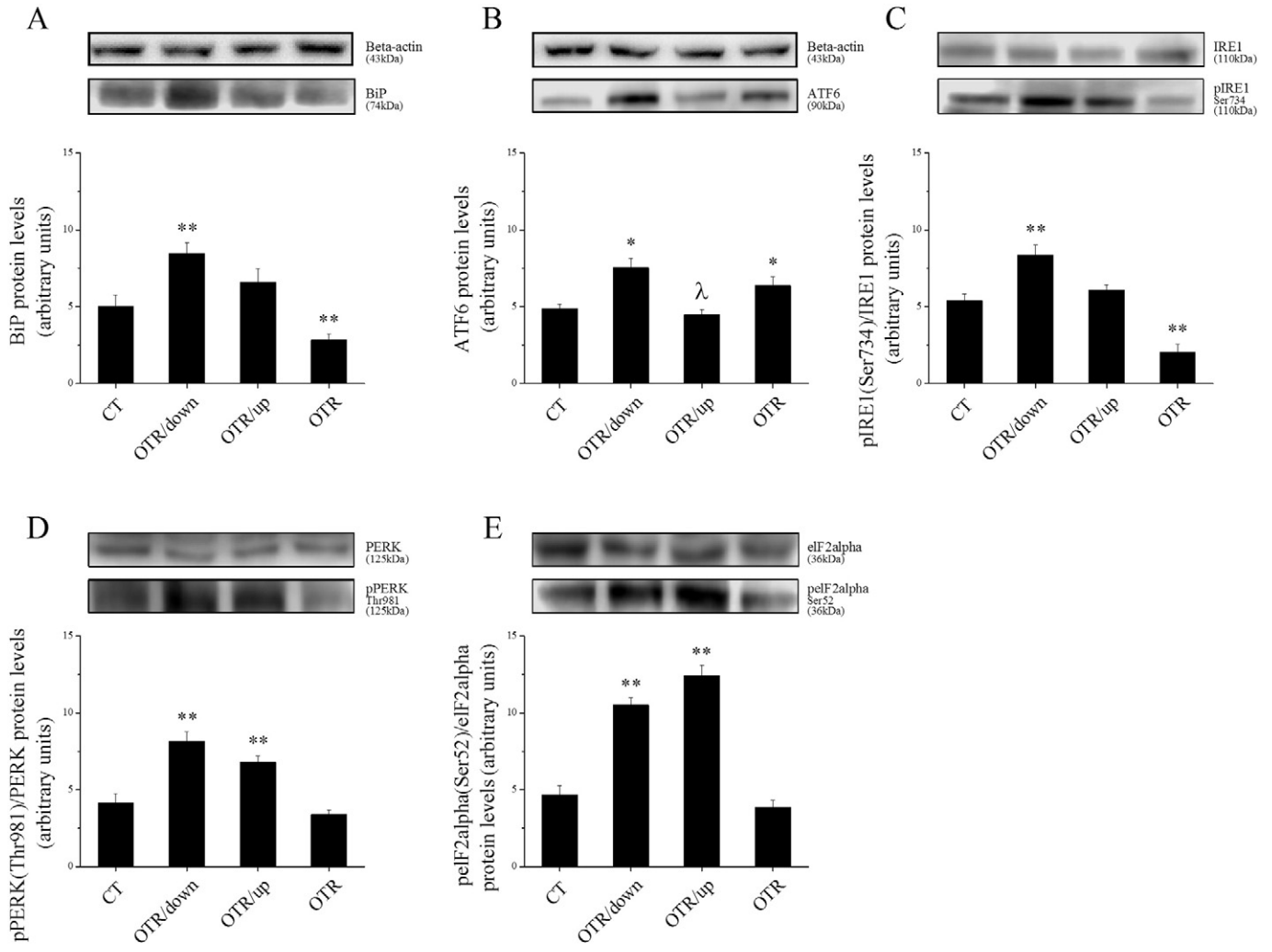

Fig. 4. Responses (arbitrary units) of BiP and its respective beta-actin (A), ATF-6 and its respective beta-actin (B), pIRE1 (Ser734)/IRE1 (C), pPERK (Thr981)/PERK (D) and peIF2alpha (Ser52)/eIF2alpha (E) measured at the end of week 10 in soleus for the experimental groups. Data correspond to means ± SE of n = 6 mice. CT: sedentary mice; OTR/down: overtrained by downhill running; OTR/up: overtrained by uphill running; OTR: overtrained by running without inclination. *P < 0.05 vs. the CT group. **P < 0.05 vs. all experimental groups. ^P < 0.05 vs. the OTR/down and OTR groups.

The OTR/down and OTR/up protocols increased the pPERK (Thr981) levels compared to the CT group (Fig. 1D). After the 2-week total recovery period, Fig. 2A shows that the OTR/down and OTR/up protocols increased the BiP levels compared to the CT group. On the other hand, the OTR protocol decreased the BiP levels compared to the CT and OTR/down groups. Fig. 2B shows that the OTR protocol decreased the ATF-6 levels compared to the CT, OTR/down and OTR/up groups. The OTR/down protocol increased the pIRE1 (Ser734) and pPERK (Thr981) levels compared to all groups (Fig. 2C and 2D). While the OTR/down protocol increased the peIF2alpha (Ser52) levels compared to the CT and OTR groups, the OTR/up increased the phosphorylation of this protein compared to all experimental groups (Fig. 2E).

3.3. Soleus endoplasmic reticulum stress signaling

At the end of week 8, while the OTR/up protocol increased the BiP levels compared to the CT and OTR/down groups, the OTR protocol increased this protein expression compared to all experimental groups (Fig. 3A). Fig. 3B shows that the OTR/down protocol decreased the ATF-6 levels compared to all experimental groups. In addition, the OTR protocol decreased this protein expression compared to the CT and OTR/up groups (Fig. 3B). While the OTR/down protocol increased the pIRE1 (Ser734) levels compared to all experimental groups, the OTR/up protocol increased the phosphorylation of this protein compared to the CT group. In addition, the OTR protocol decreased the pIRE1 (Ser734) levels compared to the OTR/up group (Fig. 3C). Fig. 3D shows that the OTR/down, OTR/up and OTR protocols increased the pPERK (Thr981) levels compared to the CT group. In addition, the OTR/up protocol decreased the phosphorylation of this protein compared to the OTR/down and OTR groups. Fig. 3E shows that the OTR/down, OTR/up and OTR protocols increased the peIF2alpha (Ser52) levels compared to the CT group, and the OTR/up protocol increased the phosphorylation of this protein compared to the OTR/down group. In addition, the OTR protocol increased the peIF2alpha (Ser52) levels compared to the OTR/down and OTR/up groups.

After the 2-week total recovery period, the OTR/down protocol increased the BiP, pIRE1 (Ser734) and pPERK (Thr981) levels compared to all experimental groups (Fig. 4A, 4C and 4D). Fig. 4A shows that the OTR protocol decreased the BiP levels compared to the CT and OTR/up groups. The OTR/down and OTR protocols increased the ATF-6 levels compared to the CT and OTR/up groups (Fig. 4B). Fig. 4C shows that the OTR protocol decreased the pIRE1 (Ser734) levels compared to the CT and OTR/up groups. The OTR/up protocol increased the pPERK (Thr981) levels compared to the CT and OTR groups (Fig. 4D). While

the OTR/down protocol increased the peIF2alpha (Ser52) levels compared to the CT and OTR groups, the OTR/up protocol increased the phosphorylation of this protein compared to all groups (Fig. 4E).

4. Discussion

The main findings of the present investigation are: a) In general, the OT protocols led to similar responses of the performance parameters as well as of the body weight and food intake; b) For both skeletal muscle types, the OTR/down protocol increased the most of the ER stress proteins, which were not normalized after the total recovery period; c) Only for soleus muscle, the other two OT protocols up-regulated the BiP, pPERK and peIF2alpha levels, which were partially normalized after the total recovery period. Taken together, the current results confirmed our hypothesis showing that the OTR/down protocol is linked to an up-regulation of the skeletal muscle ER stress proteins. Because these proteins were not normalized after the 2-week total recovery period, we suggest a possible pathological condition of ER stress in both skeletal muscle types.

The responses of the exhaustion velocity and time to exhaustion were similar between the OT models measured at weeks 0, 8 and 10, and partially reproduced our recently published data [25]. The newest result of this study is that rodents from the different OT protocols did not improve their performance after the 2-week total recovery period. Because the performance repair occurs after at most 14 days of recovery in the FOR state, we consider that these OT protocols induced the NFOR state, which is defined as a decrement or stagnation in performance that may be restored after weeks or months of recovery and may be linked to psychological and hormonal disturbances [22]. Although this lack of performance repair was previously observed for the OTR/down protocol [24], herein we verified similar results for the OTR/up and OTR groups.

The lower body weight of the OT groups compared to the CT group at the end of week 8 may be related to the hypermetabolism and proteolysis under persistent workloads [14,25] and reinforces that the decrease of this metabolic parameter is one of the classic symptoms of OT [1]. Interestingly, after the recovery period, only the OTR/down group kept the low levels of body weight compared to the CT group. The food intake reduction is also considered a symptom of exhaustive training and OT [1,21]; however, only the OTR/down group was different from the CT group at the end of week 8. In contrast, the OTR/up group presented higher food intake compared to the other experimental groups, which reinforces that the uphill running demands more energy than the downhill running [4–6]. After the recovery period, while the OTR/down and OTR/up groups kept the food intake levels observed at the end of week 8, the OTR protocol diminished this metabolic parameter. This last result corroborates Kaiyala and coworkers [16] showing that the acutely decreased energy expenditure diminishes food intake.

Regarding the skeletal muscle ER stress, the OTR/down protocol up-regulated the most of the analyzed proteins in EDL and soleus at the end of week 8. Because the chronic increase of some pro-inflammatory cytokines induces ER stress [36], we consider these data may be partially explained by the high levels of IL-6 and TNF-alpha that were previously verified in both skeletal muscle types after the OTR/down protocol [27]. In addition, the current elevated levels of pIRE1 probably contributed for the previously observed increase of JNK after the OTR/down protocol [26]. Wu and coworkers [33] verified skeletal muscle UPR activation after one bout of exhaustive treadmill running; however, they showed that trained mice presented less activation or even repression of some proteins compared to untrained rodents after challenged with equal distance treadmill running. The authors concluded that the moderate training adapts UPR and protects skeletal muscle from future exercise stress.

In agreement, Kim and coworkers [19] verified that a 5-week high intensity training period decreased the ER stress in rat gastrocnemius muscle. According to Rayavarapu and coworkers [28], the moderate exercise-induced skeletal muscle ER stress is considered an adaptive mechanism that becomes pathological in situations in which an uncontrolled ER stress leads to a crosstalk with inflammatory activation. Based on the previously mentioned investigations [19,26–28,33], we suggest that the OTR/down protocol-induced skeletal muscle ER stress may be linked to a pathological condition. This hypothesis is reinforced by the high levels of most ER stress proteins for OTR/down group in EDL and soleus muscles even after the 2-week total recovery period.

Except for the BiP and pPERK levels of the OTR/up group, the other ER stress proteins measured in EDL at the end of week 8 were not altered in response to OTR/up and OTR protocols. In order to justify these data, we consider two hypotheses. The first one reinforces the previously mentioned investigations [19,33] and suggests a positive adaptation of UPR in EDL after these OT protocols. Our second hypothesis is that the muscle recruitment pattern during running performed in uphill and without inclination may be different compared to downhill running. Therefore, further investigations should verify the electromyographic activity of EDL during these different OT protocols to test the preceding theory.

Interestingly, after the recovery period, the OTR/up group displayed higher levels of peIF2alpha compared to the CT, OTR/down and OTR groups in EDL. Even a small phosphorylation of this protein in Ser51 drastically reduces the eukaryotic initiation factor 2B (eIF2B) activity [23], which actively participates in the protein synthesis of the skeletal muscle [9]. Regarding the soleus results at the end of week 8, the OTR/up and OTR groups presented high levels of BiP, pPERK and peIF2alpha, which indicate UPR activation to deal with the exercise demands of these OT protocols. Even after the 2-week total recovery period, the OTR/up group displayed high levels of pPERK and peIF2alpha suggesting a possible pathological condition of ER stress for this skeletal muscle sample [28].

5. Conclusion

In summary, we confirmed our hypothesis showing that the OTR/down protocol increased the most of the ER stress proteins in both skeletal muscle types. Because most of these proteins were not normalized after the 2-week total recovery period, we suggest that the OTR/down protocol-induced skeletal muscle ER stress may be linked to a pathological condition. Even performed with the same external load, the effects of the OTR/up and OTR protocols on the ER stress proteins occurred basically on soleus muscle. Because the OTR/up group kept the high levels of pPERK and peIF2alpha after the total recovery period, we also consider a possible pathological condition of the ER stress in this specific skeletal muscle type.

Conflict of interest statement

The authors declare no conflicts of interest.

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