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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.