Original article (short paper)

Hypothalamic endoplasmic reticulum stress of overtrained mice after recovery.

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Abstract — Aims: knowing the relationship between endoplasmic reticulum (ER) stress and inflammation and based on the fact that downhill running-based overtraining (OT) model increases hypothalamus levels of some pro-inflammatory cytokines, we verified the effects of three OT protocols on the levels of BiP, pIRE-1 (Ser734), pPERK (Thr981), pelF2alpha (Ser52), ATF-6 and GRP-94 proteins in the mouse hypothalamus after two weeks of recovery. Methods: the mice were randomized into control (CT), overtrained by downhill running (OTR/down), overtrained by uphill running (OTR/up) and overtrained by running without inclination (OTR) groups. After 2-week total recovery period (i.e., week 10), hypothalamus was removed and used for immunoblotting. Results: the OTR/down group exhibited high levels of BiP and ATF6. The other OT protocols showed higher levels of pPERK (Th981) and pelf-2alpha (Ser52) when compared with the CT group. Conclusion: the current results suggest that after a 2-week total recovery period, the overtrained groups increased partially their ER stress protein levels, but without hypothalamic inflammation, which characterizes a physiological condition related to an adaptation mechanism.

Keywords: ER stress; overtraining protocols, hypothalamus, inflammation.

Introduction

The correct functioning of the endoplasmic reticulum (ER) is essential for cell survival. However, some disturbances that culminate in increased immature protein synthesis, which produces unfolded and misfolded proteins, may lead to an adaptive response known as the unfolded protein response (UPR)[1,2]. An imbalance between the load and the ability to fold proteins is defined as the ER stress, occurring physiological changes in UPR signaling. Three proteins associated with the ER membrane, inositol-requiring protein-1 (IRE-1), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor-6 (ATF-6) are monitored when reticular function and UPR signaling are analyzed. These proteins are connected to a chaperone binding protein (BiP) in the intraluminal domain remaining inactive. However, stress situations, including excessive immature proteins, recruit chaperones that reduce their association with the membrane proteins, allowing the autophosphorylation of IRE-1 and PERK and the cleavage of ATF-6[1-3]. IRE-1 can form a ternary complex, IRE1-TRAF2-ASK1, because it connects with the adaptor protein tumor necrosis factor receptor-associated factor 2 (TRAF2) to activate apoptosis signal regulating kinase 1 (ASK1). This complex is responsible for the activation of c-jun-terminal kinase (JNK). PERK phosphorylates the alpha subunit of eukaryotic translation initiation factor-2 (elF2alpha) at serine 51. Moreover, ATF-6 moves to the Golgi complex, where it is cleaved, liberating an active transcription factor into the cytosol[4-7].

The heat shock protein 90 kDa beta member 1 (GRP-94) is linked with the folding and/or assembly of secreted and membrane proteins; likewise, this protein plays a central role in controlling the production of growth factors such as insulin-like growth factor (mature IGF), which are crucial for the cell growth, differentiation and the ER stress response[8]. Although all of these mechanisms are aimed to protect the cell, the malfunction of these processes, which reduces the production of malformed protein, stimulates apoptosis[4-7]. It is known that nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) can be triggered in the UPR by suppressing the transcription of kappa B (IkB) inhibitor, thus increasing the levels of inflammatory mediators such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-alpha)[5,6].

Knowing that low body weight gain and food intake are related to exhaustive training and overtraining[10-12] Pereira et al.[13]

evaluated the effects of three different running overtraining (OT) protocols performed in downhill, uphill and without inclination on the inflammatory pathway in the mice hypothalamus. They observed that the increased levels of interleukin 1 beta (IL-1beta), TNF-alpha, suppressor of cytokine signaling 3 (SOCS3) and pSAPK-JNK in the hypothalamus were linked with reduced food intake in response to excessive eccentric exercise (downhill running) in mice[13]. Remarkably, hypothalamic responses were different after two other running protocols with the same external load (intensity versus volume) that were performed uphill and without inclination. In addition, after a 2-week total recovery period, all OT groups exhibited increased IL-10 levels. The OTR/down group also reversed the hypothalamic inflammation, with normalization of the body weight and food intake. Knowing the relationship between ER stress and inflammation[14-17], here we tested the effects of these three OT protocols[13] on the levels of the BiP, pIRE-1 (Ser734), pPERK (Thr981), pelF2alpha (Ser52), ATF-6 and GRP-94 proteins in mice hypothalamus after a 2-week total recovery. It is well established that these OT protocols are linked to a nonfunctional overreaching (NFOR) state[13,18] which is well-defined as a decrement or stagnation in performance that may be reestablished after weeks or months of recovery and may be associated to psychological and hormonal disturbances[11]

Materials and Methods

Experimental animals

Eight-week-old male C57BL/6 mice were provided by the Central Animal Facility of the Ribeirão Preto campus of the University of Sao Paulo (USP) and were randomized into the control (CT; sedentary mice; n=6), overtrained by downhill running (OTR/down; performed the OT protocol while running downhill; n=6), overtrained by uphill running (OTR/up; performed the OT protocol while running uphill; n=6) and overtrained by running without inclination (OTR; performed the OT protocol while running without inclination; n=6) groups. The animals were maintained in individual cages with controlled temperature (22±2°C) on a 12:12-h inverted light-dark cycle (light: 6 pm to 6 am, dark: 6 am to 6 pm), with water and food (Purina chow) available *ad libitum*. The experimental procedures were approved by the Ethics Committee of USP (ID 14.1.873.53.0) and the experimental groups were manipulated and/or overtrained in a dark room between 6 to 8 am[13,18-22].

Incremental load test (ILT)

Once being adapted to the treadmill running (INSIGHT®, Ribeirão Preto, São Paulo, Brazil) for 5 days at 10 min.day^{-1}and 3 m.min^{-1}, the rodents were submitted to the ILT at an initial intensity of 6 m.min^{-1} at 0%, with gradual increments of 3 m.min^{-1} every 3 min until exhaustion, which was demarcated as the point when the rodents touched the end of treadmill 5 times in 1 min. Throughout the ILT, mice were stimulated using physical prodding and, when they became exhausted without completing the stage, the exhaustion velocity (EV; m.min^{-1}) was corrected according to the method of Kuipers et al.[23]. The EV reached by each animal was used to establish the intensity of the OT protocols.

Running OT protocols and performance evaluations

Each week of the downhill, uphill and without inclination OT running protocols consisted of 5 days of training, followed by 2 days of recovery, and were applied as previously described[13,18-22]. The performance evaluations were done at week 0 and 48 h after the last sessions of the OT protocols at the end of week 10 and consisted of a rotarod test[13,22,24], the ILT [13,18-22], an exhaustive test[13,18-22] and a grip force test[13,18-22,25]. The description of these tests and their results were made available recently[13], using the same sample as this study.

Hypothalamus extractions and immunoblotting analysis

Thirty-six hours after de grip force test completed after the 2-week total recovery period[13], the rodents were anaesthetized, after a fasting period of 12h[13,26,27], with an intraperitoneal (i.p.) injection of 2-2-2 tribromoethanol 2.5% (10-20 µL.g^{-1}). Immediately after the anesthesia was confirmed by the loss of pedal reflexes, the hypothalamus was 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.mL^{-1}aprotinin) at 4°C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35, KINEMATICA AG, Lucerne, Switzerland) operated at the maximum speed for 30 s.

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[28]. The proteins were denatured by boiling in Laemmli sample buffer containing 100 mM DTT, separated on an SDS-PAGE gel and transferred to nitrocellulose membranes (GE Healthcare, Hybond ECL, RPN303D). The efficiency of the transfer to the nitrocellulose membranes was verified by briefly staining the blots with Ponceau red. These membranes were then blocked for 1 hour at 4°C with Tris-buffered saline (TBS) containing 5% BSA and 0.1% Tween-20.

The antibodies used for immunoblotting overnight at 4°C were BiP (SC33757), beta-actin (SC69879), PERK (SC13073), pPERK (Thr981; SC32577), eIF2alpha (SC11386), peIF2alpha (Ser52; SC101670) and GRP-94 (SC11402) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); IRE1 (AB37073) and pIRE-1 (Ser724; AB104157) from Abcam (Cambridge, UK); and ATF-6 (NBP1-40256) from Novus Biologicals (Littleton, CA, USA). After washing with TBS containing 0.1% Tween-20, all membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 hour at 4°C. The precise immunoreactive bands were detected by chemiluminescence (GE Healthcare, ECL Plus Western Blotting Detection System, RPN2132). The images were developed

by the C-DiGit™ Blot Scanner (LI-COR®, Lincoln, Nebraska, USA) and measured using the Image Studio software for the C-DiGit Blot Scanner.

Statistical analysis

The results are expressed as means ± standard error (SE). According to the Shapiro-Wilk *W*-test, data were normally distributed and homogeneity was confirmed by Levene's test. Hence, one-way analysis of variance (ANOVA) was used to test the effects of the experimental groups on the protein levels in the hypothalamus. After the one-way ANOVA indicated significance, Bonferroni's post hoc test was performed. All statistical analyses were two-sided and the significance level was established at $P < 0.05$. The statistical analyses were performed using the STATISTICA 8.0 computer software (StatSoft®, Tulsa, OK).

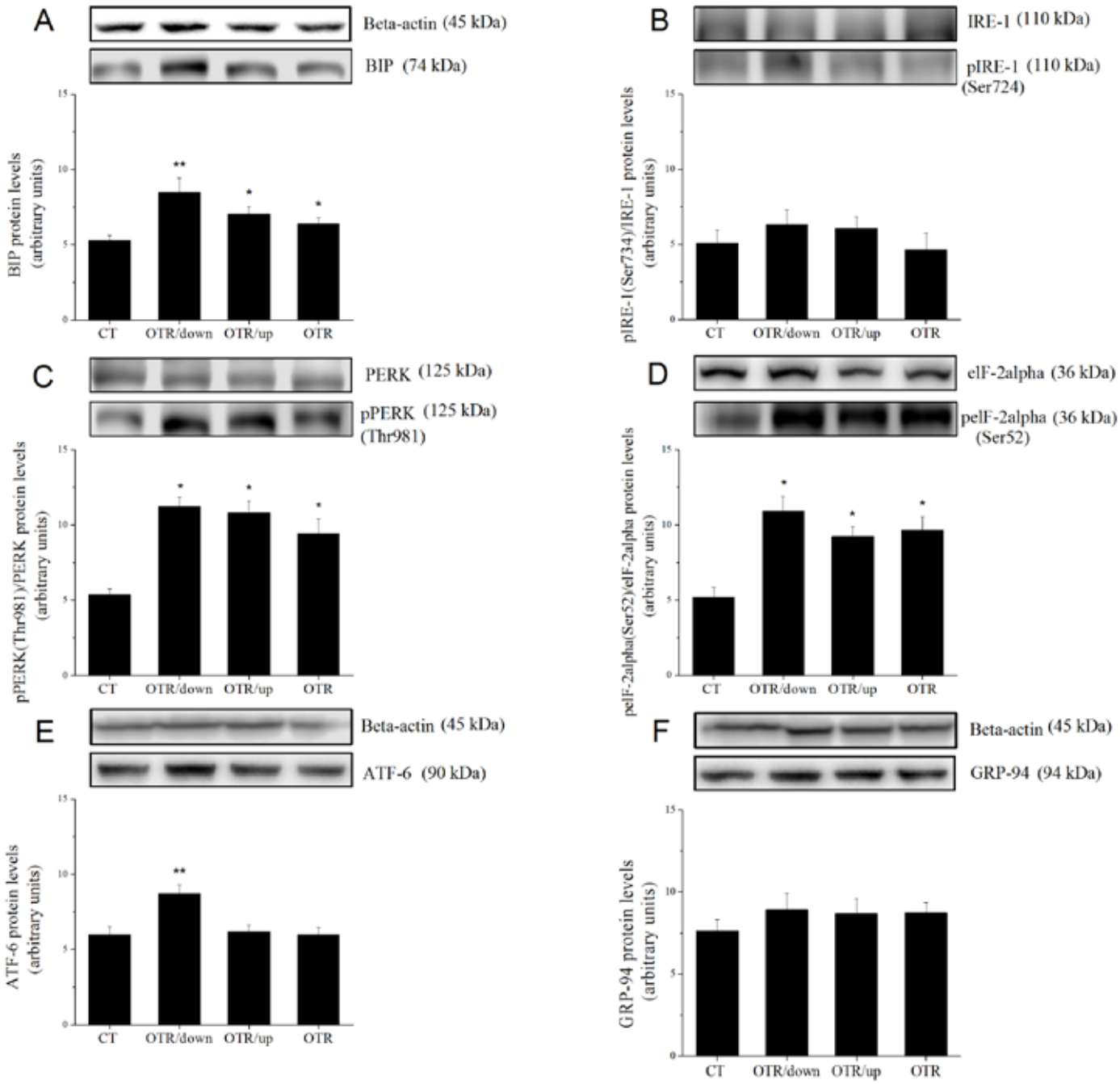

Figure 1. The responses (arbitrary units) of BIP relative to beta-actin (Figure 1A), pIRE1 (Ser734)/IRE1 (Figure 1B), pPERK (Thr981)/PERK (Figure 1C), and peIF2alpha (Ser52)/eIF2alpha (Figure 1D); ATF-6 relative beta-actin (Figure 1E); and GRP-94 relative to beta actin (Figure 1F) were measured in the hypothalamus of the experimental groups at the end of 10 weeks. Data correspond to the 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. CT. **P < 0.05 vs. all groups.

Results

At the end of week 10, the OTR/down group exhibited significantly higher levels of the BiP and ATF-6 proteins compared to the CT, OTR/up and OTR groups (Figure 1A and 1E, respectively). In addition, the OTR/up and OTR groups also showed significantly higher levels of the BiP protein compared to the CT group (Figure 1A). There was no significant difference in the levels of the pIRE-1 (Ser734) and GRP-94 proteins between the experimental groups (Figure 1B and 1F, respectively). The levels of the pPERK (Thr981) and pelF2alpha (Ser52) proteins were significantly higher in the OT groups compared to the CT group (Figure 1C and 1D, respectively).

Discussion

The main findings of this study are: 1) The OTR/down group exhibited high levels of BiP and ATF6; 2) The other two OT protocols showed higher levels of pPERK (Th981) and pelf-2alpha (Ser52) when compared with the CT group. The present results suggest that the three OT protocols had partial increased of the protein levels after 2 weeks of full recovery. However, the changes in these proteins were most prevalent in the OTR/down group.

The activation of UPR components suggests that a change in the normal function of the ER contributes to the progression of various diseases such as obesity, ischemic myocardial injury, fatty liver and diabetes mellitus[3,5]. ER stress is also associated with inflammation, oxidative stress and damage signaling insulin in different organs[3,29,30]; however, regular physical exercise has shown positive effects in reducing both inflammation and high levels of ER stress proteins[29]. Da Luz et al.[1] demonstrated that animals receiving high-fat diet and submitted to swimming training reduced both inflammatory proteins (i.e., JNK, IkB, NF-kB) and ER stress (i.e., PERK phosphorylation and elF2alpha) in adipose and hepatic tissues[1].

In the study performed by Kim et al.[31], C57BL/6 mice receiving low-fat (LFD) or high-fat diet (HFD) had free access to running voluntary exercise for three weeks. The authors found that voluntary exercise increased the hypothalamic levels of ATF-6 and pelF2alpha in the LFD group and the hypothalamic levels of ATF-6, pelF2alpha and BiP in the HFD group (31). However, Rodrigues et al.[32] found that obese rats submitted to an acute swimming exercise session showed reduced phosphorylation of PERK and CHOP levels in the hypothalamus[32].

Pereira et al.[13] used the same hypothalamic samples of this study and found that the OTR/down, OTR/up e OTR groups exhibited high levels of IL-10 at the end of the recovery period. The authors concluded that after recovery, the upregulation of IL-10 observed in the OTR/down group was partially responsible for the hypothalamic inflammation reversion and for the concomitant normalization of body weight and food intake[13]. Rayavarapu et al.[33] theorized that the physical exercise-induced ER stress on the skeletal muscle is an adaptive mechanism that becomes pathological when the ER stress is excessive, because there is a cross-talk with the mitochondria that starts the inflammatory and cell death pathways (i.e., necrosis, autophagy and apoptosis)[33].

While the aids of moderate physical exercise are well known in the scientific literature, the divergent results[29,31] could be attributed to a protective mechanism against the production of unfolded and misfolded proteins. That because during the adaptation phase of the UPR occurs the transcription of genes involved in increasing cellular capacity to degrade and clew proteins, besides the inhibition of mRNA translation. However, when stress is prolonged and intense, the UPR activates the signaling pathways involved in cell death[34]. The BiP participates in the folding or re-folding polypeptide chains and acts as a sensor detecting the accumulation of unfolded proteins, which could explain its increase in the present study[34]. These data reinforce the role of adaptive response to the physical exercise as a protective mechanism against the production of misfolded or unfolded proteins.

Conclusion

After a 2-week total recovery period, the overtrained groups had partial increase of the ER stress protein levels, but without hypothalamic inflammation[13], which characterizes a physiological condition related to the adaptation mechanism[33].

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Acknowledgments

The present work received financial support from the São Paulo Research Foundation (FAPESP; process numbers 2013/19985-7, 2013/20591-3, 2014/25459-9, 2015/08013-0 and 2015/13275-3).

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Manuscript received on August 25, 2016
Manuscript accepted on October 18, 2016