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ABSTRACT

Downhill running-based overtraining model increases the hypothalamic levels of IL-1 β , TNF- α , SOCS3 and pSAPK-JNK. The aim of the present study was to verify the effects of three overtraining protocols on the levels of BiP, pIRE-1 (Ser724), pPERK (Thr981), p α F2 (Ser52), ATF-6, GRP-94, caspase 4, caspase 12, pAKT (Ser473), pmTOR (Ser2448) and pAMPK (Thr172) proteins in the mouse hypothalamus. The mice were randomized into the control (CT), overtrained by downhill running (OTR/down), overtrained by uphill running (OTR/up) and overtrained by running without inclination (OTR) groups. After the overtraining protocols (i.e., at the end of week 8), hypothalamus was removed and used for immunoblotting. The OTR/down group exhibited increased levels of all of the analyzed endoplasmic reticulum stress markers in the hypothalamus at the end of week 8. The OTR/up and OTR groups exhibited increased levels of BiP, pIRE-1 (Ser724) and pPERK (Thr981) in the hypothalamus at the end of week 8. There were no significant differences in the levels of caspase 4, caspase 12, pAKT (Ser473), pmTOR (Ser2448) and pAMPK (Thr172) between the experimental groups at the end of week 8. In conclusion, the three overtraining protocols increased the endoplasmic reticulum stress at the end of week 8.

Keywords: overtraining protocols, overtrained by downhill running, hypothalamus, endoplasmic reticulum stress, apoptosis, mice.

INTRODUCTION

The endoplasmic reticulum (ER) is an organelle present in eukaryotic cells in which polypeptides are synthesized from messenger RNA to become mature proteins after the folding process. When disturbances occur that culminate in increased immature protein synthesis, which produces unfolded and misfolded proteins, an adaptive response known as the unfolded protein response (UPR) is triggered (Eizirik et al. 2008; da Luz et al. 2011). Physiological changes in UPR signaling generate ER stress, which is defined as an imbalance between the load and the ability to fold proteins. 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), remain inactive when they are connected to a chaperone binding protein (BiP) in the intraluminal domain (Eizirik et al. 2008; da Luz et al. 2011).

However, stress situations, including excess immature proteins, recruit chaperones that reduce their association with the membrane proteins, allowing the auto phosphorylation of IRE-1 and PERK, and the cleavage of ATF-6 (Ozcan et al. 2004; Eizirik et al. 2008; da Luz et al. 2011). IRE-1 can connect with the adaptor protein tumor necrosis factor receptor-associated factor 2 (TRAF2) to activate apoptosis signal regulating kinase 1 (ASK1) and form a ternary complex, IRE1-TRAF2-ASK1, which is responsible for the activation of c-jun-terminal kinase (JNK) and cleavage of caspase 12 (Tabas and Ron 2011). The activation of caspases, a family of proteases that cleave substrates at specific aspartate residues, is a key mechanism in the process of cell death by apoptosis (Hitomi et al. 2004). PERK phosphorylates the alpha subunit of eukaryotic translation initiation factor-2 (eIF2alpha) at serine 51. On the other hand, ATF-6 translocates to the Golgi complex, where it is cleaved, releasing an active transcription factor into the cytosol (Ron and Walter 2007).

The UPR phase may also lead to the activation or inhibition of the protein kinase B (Akt) (Matsui and Rosenzweig 2005). For instance, Akt is activated in short periods of exposure to ER stress, but is inhibited in long periods of exposure to ER stress (Kim et al. 2015). The mammalian target of rapamycin (mTOR) plays an essential role in the survival signaling mediated by Akt. The increased activation of mTOR triggers a negative feedback loop to the pathway of phosphoinositide 3-kinase (PI3K)-Akt, leading to the suppression of Akt. Increasing evidence demonstrated that the Akt/mTOR pathway is a growth factor mediating cell survival by the induction and suppression of cell death by apoptosis (Kato et al. 2012). On the other hand, the AMP-activated protein kinase (AMPK) is a negative regulator of mTOR linked to anabolic processes as well as to apoptosis and autophagy inductions (Jung and Choi et al. 2016). In the hypothalamus, the leptin and insulin signaling pathways may lead to dephosphorylation of AMPK modulating food intake and energy expenditure (Dong et al. 2010; Hsu et al. 2015).

Hypothalamus is the main energy regulation center inside the central nervous system (CNS) and is responsible for fundamental homeostatic functions such as thermogenesis and control feeding (Morton et al. 2006). Elevated hypothalamic contents of tumor necrosis factor alpha (TNF-alpha) are linked to food intake restriction and proinflammatory signaling activation, which involves I κ B kinase (IKK), stress-activated protein kinases/Jun amino terminal kinases (SAPK-JNK) and the suppressor of cytokine signaling 3 (SOCS3). Recently, Pereira et al. (Pereira et al. 2015a) demonstrated that an eccentric exercise (EE)-based overtraining (OT) model increased temporarily the hypothalamic levels of interleukin 1 beta (IL-1beta), TNF-alpha, SOCS3 and phosphorylation (p) of SAPK-JNK with concomitant reductions in the body weight and food intake. Probably, due to the singular characteristics of EE such as the lengthening of the muscle-tendon complex, unique strategies of activation by the CNS,

and the ability to achieve high force levels with reduced oxygen consumption (Hody et al. 2013), the hypothalamic responses were different after two other running OT protocols with the same external load (i.e., intensity versus volume) that were performed uphill and without inclination (Pereira, da Rocha et al. 2015a).

The OT may be defined as a process of intensified training that leads to functional overreaching (FOR), nonfunctional overreaching (NFOR), or OT syndrome (OTS). The OT protocols used by our research group (Pereira et al. 2015a; Pereira et al. 2015b; Pereira et al. 2015c; da Rocha et al. 2016) led to the NFOR state, which is defined as a performance decrement that may be reversed after weeks or months of recovery and may be related to psychological and hormonal disturbances (Meeusen et al. 2013). Based on the crosstalk between ER stress and inflammation (Urano et al. 2000; Zhang et al. 2011) and knowing that OT is linked to ER stress in mice skeletal muscles (Pereira et al. 2015b), herein we verified the effects of three OT models (Pereira et al. 2015a) on the levels of the BiP, pIRE-1 (Ser724), pPERK (Thr981), p $\text{eIF2}\alpha$ (Ser52), ATF-6, GRP-94, caspase 4, caspase 12, pAKT (Ser473), pmTOR (Ser2448) and pAMPK (Thr172) proteins in the mouse hypothalamus. Considering our previous findings (Pereira et al. 2015a), we hypothesize that only the EE-based OT model will modulate the mentioned proteins.

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 maintained in individual cages with a controlled temperature ($22\pm 2^\circ\text{C}$) on a 12:12-h inverted light-dark cycle (light: 6 PM to 6 AM, dark: 6 AM to 6 PM), with food (Purina chow) and water available *ad libitum*. The experimental procedures were approved by

the Ethics Committee of USP (ID 14.1.873.53.0). Mice were randomized into control (CT; sedentary mice; n=12), overtrained by downhill running (OTR/down; performed the OT protocol while running downhill; n=12), overtrained by uphill running (OTR/up; performed the OT protocol while running uphill; n=12) and overtrained by running without inclination (OTR; performed the OT protocol while running without inclination; n=12) groups. The experimental groups were manipulated and/or overtrained in a dark room between 6 to 8 AM (Pereira et al. 2012; Pereira et al. 2015a; Pereira et al. 2015b; da Rocha et al. 2016).

Running OT protocols and performance evaluations

The animals were adapted to the treadmill running for 5 days at 10 min.day⁻¹ and 3 m.min⁻¹. Each week of the downhill, uphill and no inclination OT running protocols consisted of 5 days of training, followed by 2 days of recovery, and was applied as previously described (Pereira et al. 2015a; Pereira et al. 2015b; da Rocha et al. 2016). The performance evaluations were performed at week 0 and 48 h after the last sessions of the OT protocols at the end of week 8. The detailed description of the performance tests and their results were published recently (Pereira et al. 2015a) using the same sample as the present study.

Previous investigations from our research group (Pereira et al. 2015a; Pereira et al. 2015c) showed that these OT protocols led to similar reductions in the following performance parameters: exhaustion velocity, time to exhaustion, rotarod and grip force. In addition, we verified that mice from the OTR/down, OTR/up and OTR groups did not restore their performance, even after 2 weeks of total recovery (Pereira et al. 2015a). Because FOR performance restoration occurs after days of recovery (Meeusen et al. 2013), we can state that these OT protocols led to NFOR. It is important to point out

that the performance decrement is the only accepted parameter in the literature to characterize the FOR, NFOR, and OTS states (Meeusen et al. 2013).

Hypothalamus extractions and immunoblotting analysis

Mice were anaesthetized 36 h after the grip force test that was performed at the end of the OT protocols (i.e., at the end of week 8; n=6) (Pereira et al. 2015a). After a fasting period of 6 h (Pereira et al. 2015a), rodents were anaesthetized with an intraperitoneal (i.p.) injection of 2-2-2 tribromoethanol 2.5% (10-20 $\mu\text{L}\cdot\text{g}^{-1}$). As soon as 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 $\text{mg}\cdot\text{mL}^{-1}$ 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 insoluble material, and the supernatants of these homogenates were used for protein quantification using the Bradford method. 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 transfer efficiency 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); ATF-6 (NBP1-40256) from Novus Biologicals (Littleton, CA, USA); and caspase 4 (CST 44505), caspase 12 (CST 2202S), Akt (CST 9272S), pAkt (Ser473; CST 4060S), mTOR (CST 2972S), pmTOR (Ser2448; CST 29715), AMPK (CST 2532S) and pAMPK (Thr172; CST 2531S) from Cell Signaling Technology (Beverly, MA, 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 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®, Lincoln, Nebraska, USA) and quantified using the Image Studio software for the C-DiGit Blot Scanner.

Statistical analysis

The results are expressed as means $\pm$ standard error (SE). According to the Shapiro-Wilk *W*-test, the data were normally distributed and homogeneity was confirmed by Levene's test. Therefore, one-way analysis of variance (ANOVA) was used to examine the effects of the experimental groups on the protein levels in the hypothalamus. When the 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 $P < 0.05$. The statistical analyses were performed using the STATISTICA 8.0 computer software (StatSoft®, Tulsa, OK).

RESULTS

Figure 1A shows that BiP levels for the CT group (5.5 ± 0.7 arbitrary units) were significantly lower compared to the OTR/down (10.8 ± 0.7 arbitrary units), OTR/up (11.9 ± 0.7 arbitrary units) and OTR groups (8.7 ± 0.2 arbitrary units). In addition, BiP levels for the OTR group were significantly lower compared to the OTR/down and OTR

groups. Figure 1B shows that pIRE-1 (Ser724) levels for the CT group (5.5 ± 0.5 arbitrary units) were significantly lower compared to the OTR/down (8.3 ± 0.8 arbitrary units), OTR/up (7.9 ± 0.6 arbitrary units) and OTR groups (7.8 ± 0.5 arbitrary units). Figure 1C shows that pPERK (Thr981) levels for the CT group (2.6 ± 0.3 arbitrary units) were significantly lower compared to the OTR/down (8.4 ± 0.5 arbitrary units), OTR/up (6.2 ± 0.5 arbitrary units) and OTR groups (4.5 ± 0.6 arbitrary units). In addition, pPERK (Thr981) levels for the OTR/down group were significantly higher compared to the OTR/up and OTR groups.

Figures 1D and 1E show that pelf2alpha (Ser52) and ATF-6 for the OTR/down group (8.3 ± 1.0 and 8.5 ± 0.6 arbitrary units, respectively) were significantly higher compared to the CT (6.5 ± 0.5 and 5.2 ± 0.3 arbitrary units, respectively), OTR/up (6.2 ± 0.7 and 5.0 ± 0.4 arbitrary units, respectively) and OTR groups (6.7 ± 0.5 and 5.6 ± 0.6 arbitrary units, respectively). Figure 1F shows that GRP-94 levels for the CT group (6.0 ± 0.3 arbitrary units) were significantly lower compared to the OTR/down (11.5 ± 0.5 arbitrary units), OTR/up (11.8 ± 0.4 arbitrary units) and OTR groups (8.7 ± 0.5 arbitrary units). In addition, GRP-94 levels for the OTR group were significantly lower compared to the OTR/down and OTR/up groups. There were no significant differences in the protein levels of caspase 4, caspase 12, pAKT (Ser473), pmTOR (Ser2448) and pAMPK (Thr172) between the experimental groups (Figure 1G, 1H, 2A, 2B and 2C, respectively).

DISCUSSION

The main findings of this study are 1) the OTR/down group exhibited increased levels of all of the analyzed ER stress markers in the hypothalamus at the end of week 8; 2) The OTR/up and OTR groups exhibited increased levels of BiP, pIRE-1 (Ser724) and pPERK (Thr981) in the hypothalamus at the end of week 8. The present results

suggest that the three OT protocols increased the ER stress at the end of week 8. However, the changes in these proteins were most prevalent in the OTR/down group.

Rayavarapu et al. (Rayavarapu et al. 2012) hypothesized that the physical exercise-induced ER stress in skeletal muscle is an adaptive mechanism that becomes pathological in the presence of excess ER stress, because there is a cross-talk with the mitochondria that activates the inflammatory pathways and cell death (i.e., necrosis, autophagy and apoptosis). Recently, we verified that the OTR/down protocol increased the ER stress in mice skeletal muscles. Because most of the proteins were not normalized after a total recovery period, we considered that the OTR/down model-induced skeletal muscle ER stress may be associated with a pathological condition. Interestingly, the OTR/up and OTR protocols increased the levels of BiP, pPERK and p $\text{eIF2}\alpha$ only in the soleus sample. Because the OTR/up group kept the elevated contents of pPERK and p $\text{eIF2}\alpha$ after the recovery period, we also suggested a possible pathological condition of the ER stress in this particular skeletal muscle sample (Pereira et al. 2015b).

Regarding the effects of these OT models on hypothalamus, Pereira et al. (Pereira et al. 2015a) used the same sample as this study and observed that the OTR/down group exhibited elevated protein levels of IL-1 β , TNF- α , pSAPK-JNK and SOCS3 at the end of week 8. In addition, the serum levels of IL-1 β and IL-6 were increased after the OTR/down, OTR/up and OTR protocols compared to the CT group. Finally, the OTR/down and OTR/up groups presented higher serum levels of TNF- α compared to the CT and OTR groups (Pereira et al. 2015a). TNF- α causes stress in the ER, with subsequent activation of pIRE-1, pPERK and ATF-6 (Ron and Walter. 2007). Denis and coworkers (Denis et al. 2010) verified that animals receiving an acute dose of TNF- α (i.e., 300 μl , 10^{-8} M) increased JNK

phosphorylation in the hypothalamus after 60 min. After 6 h of the administration of this dose, there was also an increase in the expression of BiP, pIRE-1, pPERK and pelf2alpha.

Therefore, the current upregulation of the analyzed ER stress proteins observed in the OTR/down group at the end of week 8 may be justified by their high levels of proinflammatory cytokines in the hypothalamus and serum (Pereira et al. 2015a). In addition, the increased levels of BiP, pIRE-1 (Ser724) and pPERK (Thr981) observed in the hypothalamus of the OTR/up and OTR groups at the end of week 8 may be partially explained by their high serum levels of proinflammatory cytokines (Pereira et al. 2015a). The formation of the IRE1-TRAF2 complex appears to be essential for the activation of both JNK and NF- κ B in response to ER stress, which results in the production of proinflammatory cytokines (Ron and Walter. 2007).

The increased levels of pIRE-1 in the OTR/down group at the end of week 8 likely contributed to the increased pSAPK-JNK levels that were previously observed in this group (Pereira et al. 2015a). In addition, the UPR can promote the activation of NF- κ B through PERK-eIF2alpha (Ron and Walter. 2007), where the phosphorylation status of PERK and, hence, its target protein, eIF2alpha, is a key indicator of the presence of ER stress. This is in agreement with the findings of this study, as both proteins were increased at 8 weeks in the OTR/down group. The mechanism by which cytokines cause ER stress is not yet elucidated, but it is suggested that cytokines would activate calcium release from the ER and the accumulation of reactive oxygen species, thus interfering with the mitochondrial metabolism and protein assembly (Zhang et al. 2011).

Besides the activation of JNK, the IRE1-TRAF2 complex is responsible for the cleavage of caspase 12 (Tabas and Ron. 2011). Although we observed upregulation of the pIRE-1 after the OT protocols at the end of week 8, the protein levels of caspase 4

and 12 were not different between the experimental groups. These data could be justified by a possible lower activation of TRAF2 that would culminate in the absence of the IRE1-TRAF2 complex. However, this hypothesis should be addressed in future studies. In accordance, Cragle and Baldini (Cragle and Baldini. 2014) verified that hypothalamic neurons exposed to high concentrations of palmitate presented increased levels of ER stress proteins (i.e., XBP1 and eIF2alpha) without upregulation of caspases and CHOP, which was considered by the authors as a moderate ER stress.

At week 8, the other OT groups (i.e., OTR/up and OTR) exhibited a significant increase in the levels of the ER stress proteins, but without hypothalamic inflammation (Pereira et al. 2015a). These data reinforce the role of physical exercise as a protective mechanism against the production of misfolded or unfolded proteins (Kim et al. 2010; Rayavarapu et al. 2012). To date, no study has investigated the responses of the GRP-94 protein in the hypothalamus to physical exercise; however, it is known that IGF-1 increases the reserves of this chaperone in the ER to reduce the signs of stress (Eletto et al. 2010). Thus, further studies are needed to verify the relationship between IGF-1 and the GRP-94 chaperone in animals subjected to the OTR/down, OTR/up and OTR protocols.

Herein, we did not observe significant differences for the protein levels of pAkt (Ser473) between the experimental groups at the end of week 8. Although the activation or inhibition of Akt is dependent of the period of exposure to ER stress (Kim et al. 2015), the inactivation of the PI3K-Akt pathway is linked to the apoptotic phase of the UPR (Petrovski et al. 2011). This last statement corroborates the current results, since the OT models did not modulate the protein levels of caspase 4 and 12. Besides the upregulation of all of the ER stress proteins at the end of week 8, the OTR/down protocol also led to food intake reduction (Pereira et al. 2015a). Because AMPK

activation is linked to hyperphagia (Melo et al. 2014) and ER stress attenuation (Dong et al. 2010), we expected to find out a downregulation of the hypothalamic levels of pAMPK (Thr172) in this specific OT group. Considering that proteins and their targets are phosphorylated at different times (Kholodenko. 2006), future studies should investigate the time-course of caspase 4, caspase 12, pAkt (Ser473), pmTOR (Ser2448) and pAMPK (Thr172) after the OTR/down protocol.

Because the OTR/down group exhibited transitory hypothalamic inflammation with concomitant reductions in body weight and food intake (Pereira et al. 2015a) as well as central and peripheral ER stress, we consider these data negative. In addition, based on the relationship between hypothalamic inflammation, ER stress and apoptosis (Williams. 2012), we hypothesize that the continuity of the OTR/down protocol would induce hypothalamic apoptosis. On the other hand, the OTR/up and OTR protocols led to hypothalamic ER stress without inflammation or significant changes in body weight and food intake (Pereira et al. 2015a). These findings corroborate the hypothesis of Rayavarapu et al. (Rayavarapu et al. 2012) and may be considered a physiological adaptive mechanism. It is important to point out that the use of the OTR/up and OTR protocols was essential to discriminate the effects of the EE-induced inflammation on the hypothalamus (Pereira et al. 2015a).

In summary, we conclude that the ER stress induced by the OTR/down protocol at the end of week 8 is associated with the adaptation phase of the UPR, since there is no evidence of apoptosis (Rayavarapu et al. 2012). Because the OTR/down group also exhibited hypothalamic inflammation in this period (Pereira et al. 2015a), we consider that its ER stress was more intense compared to the OTR/up and OTR groups. Figure 3 summarizes the molecular relationships between ER stress and inflammation in the mouse hypothalamus in response to the OT protocols at the end of week 8.

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Conflict of interest statement

The authors declare no conflicts of interest.

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Figure 1. The responses (arbitrary units) of BIP relative to beta-actin (Figure 1A), pIRE1 (Ser724)/IRE1 (Figure 1B), pPERK (Thr981)/PERK (Figure 1C), peIF2alpha (Ser52)/eIF2alpha (Figure 1D), ATF-6 relative to beta-actin (Figure 1E), GRP-94 relative to beta actin (Figure 1F), caspase 4 relative to beta-actin (Figure 1G) and caspase 12 relative to cleaved caspase 12 (Figure 1H) were measured in the hypothalamus of the experimental groups at the end of 8 weeks. The data correspond to the means $\pm$ 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. all groups. $^{\Phi}P < 0.05$ vs. OTR/down and OTR/up.

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Figure 2. The responses (arbitrary units) of pAKT (Ser473)/AKT (Figure 2A), pmTOR (Ser2448)/mTOR (Figure 2B), and pAMPK (Thr172)/AMPK (Figure 2C) were measured in the hypothalamus of the experimental groups at the end of 8 weeks. The data correspond to the means $\pm$ 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.

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Figure 3. Schematic model of the molecular relationships between ER stress and inflammation in the mouse hypothalamus, in response to the OTR protocols at the end of week 8

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215x279mm (300 x 300 DPI)

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