Introduction

High-intensity training sessions during a training program are widely utilized to maximize athletes’ performance in different sports. However, these training sessions need to comprise an adequate recovery period for the achievement of better athletes’ performance. When the athlete is submitted to an overtraining (OT) period, the time destined to recovery is not respected, the nonfunctional overreaching (NFOR) – defined as a drop of performance that can be reversed after weeks or months of recuperation – may occur.

Recently, da Rocha et al. verified that mice in NFOR, induced by an OT protocol based on downhill running sessions, presented an improvement of the hepatic insulin signaling pathway when compared to other two OT protocols with the same external load (i.e., the product between intensity and volume of training), but performed in uphill and without inclination. Also, these mice showed increased levels of hepatic glycogen even with high contents of tribbles-like protein 3 (TRB3), an Akt inhibitor. These contradictory results may be better elucidated with the investigation of other proteins that are involved in this pathway modulation, such as the adaptor proteins APPL1 and APPL2.

APPL1 and APPL2 are homologous proteins that bind to diverse groups of trans-membrane receptors or signaling proteins. APPL1 acts on the adiponectin signaling cascade, communicating signals of the adiponectin receptors (AdipoR1 and AdipoR2) to downstream targets through direct interaction with the NH2-terminal region. According to Ryu and coworkers, APPL1 also interacts directly to the insulin receptor beta (IRbeta). Also, these authors verified that the high APPL1 expression increased the interaction between IRbeta and insulin receptor substrate-1 (IRS-1), as well as Akt activation. On the other hand, APPL1 suppression impaired Akt activation and glucose transporter type 4 (GLUT4) translocation to the membrane when stimulated by insulin in L6 myocytes and 3T3-L1 adipocytes.

Although the physiological role of APPL2 is not fully understood it is interesting to note that only APPL1, and not APPL2, interacts directly with Akt. Wang et al. demonstrated that under basal conditions, APPL2 interacts with AdipoR1 and competes with APPL1, negatively regulating adiponectin signaling in muscle cells. Therefore, APPL2 has an inhibitory role in the insulin sensitizing effects, blocking the adiponectin-signaling pathway.
effect in Akt activation stimulated by insulin. Interestingly, APPL2 also seems to have an involvement in macrophages inflammatory response, suppressing it via the toll-like receptor 4 (TLR4)\textsuperscript{11}.

Regarding their physiological importance and capacity to modulate the aforementioned molecular pathways, our first aim was to verify the effects of the OT protocol based on downhill running sessions on the hepatic contents of APPL1 and APPL2. Although the other two OT protocols performed in uphill and without inclination led to similar decrements in the analyzed performance parameters\textsuperscript{3,12,13}, they induced particular modulations of the hepatic insulin signaling pathway\textsuperscript{3}. Thus, we also evaluated their effects on the hepatic protein levels of APPL1 and APPL2.

Methods

Experimental Animals

Male C57BL/6 mice from the Central Animal Facility of the Ribeirão Preto campus of the University of São Paulo (USP) were maintained in individual cages with controlled temperature (22±2°C) on a 12:12-h light-dark inverted cycle (light: 6 PM to 6 AM, dark: 6 AM to 6 PM) with food (Purina chow) and water ad libitum. The experimental procedures were performed by the Brazilian College of Animal Experimentation and were approved by the Ethics Committee of the University of São Paulo (ID 14.1.873.53.0). Eight-week-old C57BL/6 mice were divided into four groups: control (CT; sedentary mice; n = 8), overtrained by downhill running (OTR/down; performed the OT protocol based on downhill running; n = 8), overtrained by uphill running (OTR/up; performed the OT protocol based on uphill running; n = 8) and overtrained by running without inclination (OTR; performed the OT protocol based on running without inclination; n = 8). The CT, OTR/down, OTR/up and OTR mice were manipulated and/or trained in a dark room between 6 to 8 am.

Overtraining protocols and performance evaluations

The eight-week OT protocols based on downhill running, uphill running and running without inclination were performed as previously described\textsuperscript{13}, and each experimental week consisted of five days of training followed by two days of recovery. During the first four weeks of the OT protocols (i.e. first stage), the intensity was maintained at 60% of the exhaustion velocity (EV) that was obtained by the incremental load test (ILT), and the volume was gradually increased to 60min per day in the fourth week. In this first stage, rodents ran at a grade of 0%. In the fifth week of the OT protocols, the intensity and volume were maintained, but the rodents ran at a grade of – 14% (OTR/down), 14% (OTR/up) or 0% (OTR).

These running grades were maintained until the end of the OT protocols. In the sixth week of the OT protocols, the intensity was increased to 70% of EV. In the seventh week of the OT protocols, the intensity and volume were increased to 75% of EV and 75min, respectively. In the eighth week of the OT protocols, the number of daily sessions was doubled. The resting interval between daily sessions during the eighth week was 4h.

The performance evaluations were made on week 0 and 48 h after the last sessions of the OT protocols at the end of week 8 and consisted of a rotarod test\textsuperscript{12,14,15}, the ILT\textsuperscript{3,12,14,16-18}, an exhaustive test\textsuperscript{3,12,14,16-18} and a grip force test\textsuperscript{12,14,16-18}. The description of these tests and their results were recently published\textsuperscript{7} using the same sample as the current study.

Liver Extraction

Mice were anesthetized at the end of week 8. After an overnight fast (12h), rodents were anesthetized with an intraperitoneal (i.p.) injection of 2-2-2 tribromoethanol 2.5% (10–20μL.g\textsuperscript{-1}). As soon as the effect of anesthesia was confirmed by the loss of pedal reflexes, the abdominal cavity was opened and each mouse liver was removed. Subsequently, the livers were quickly stored at – 80 ° C for later analysis of the contents of APPL1 and APPL2 by the immunoblotting technique.

Immunoblotting technique

The liver samples were homogenized in extraction buffer (1% de Triton X-100, 100 mM Tris, pH 7.4, containing 100 mM sodium pyrophosphate, sodium fluoride 100 mM, EDTA 10 mM, 10 mM sodium vanadate, 2 mM de PMSF and 0.1 mg.ml\textsuperscript{-1} aprotinin) at 4°C with a Polytrom PTA 20S generator (Brinkmann Instruments model PT 10/35), operated at a maximum speed of 30s. The extracts were centrifuged (9900g) for 40 minutes at 4°C to remove insoluble material, and the supernatants of these homogenates were used for protein quantitation using the Bradford method\textsuperscript{20}.

Proteins were denatured by boiling in Laemmli\textsuperscript{21} sample buffer containing 100mM DTT, run on SDS-PAGE gel and transferred to nitrocellulose membranes (GE Healthcare, Hybond ECL, RPN303D). The efficiency of transfer to nitrocellulose membranes was verified by the brief staining of bands with Ponceau. These membranes were blocked with Tris-buffered saline (TBS) containing 5% BSA and 0.1% Tween-20 for 1 hour at 4°C. Subsequently, the membranes were incubated in 1:1000 dilution overnight with a specific antibody anti-APPL1 (SC-67402) and anti-APPL2 (SC-67403) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sixteen hours after the incubation with the specific primary antibody, the membranes were washed 5 times (5min each time) with TBS containing 0.1% Tween-20. Then, all the membranes were incubated for 1 hour at 4°C with secondary antibody conjugated with a horseradish peroxidase. The specific immunoreactive bands were detected by chemiluminescence (GE Healthcare, ECL Plus Western Blotting Detection System, RPN2132). The images
were acquired using the C-DiGit™ Blot Scanner (LI-COR®, Lincoln, Nebraska, USA) and quantified using the software Image Studio for C-DiGit Blot Scanner.

**Statistical analysis**

The results are expressed as mean ± 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 verify the effects of the experimental groups on the hepatic contents of APPL1 and APPL2. When one-way ANOVA indicated statistical significance, a Bonferroni’s post hoc test was used. When Bonferroni’s post hoc indicated tendency (P<0.1), Cohen’s d test was used to measure the effect size. Statistical analysis was performed using STATISTICA 8.0 computer software (StatSoft1, Tulsa, OK) and the significance level was set at p<0.05.

**Results**

The protein levels of APPL1 were significantly higher in the OTR/down and OTR/up groups compared to the CT group. Also the OTR group presented a tendency (p=0.071) to increase APPL1 compared to the CT group (Figure 1A). Regarding APPL2, OTR/up group increased its protein levels compared to the CT group, and OTR/down group presented a tendency (p=0.058) to increase APPL2 compared to the CT group (Figure 1B).

![Figure 1. Protein levels (arbitrary units) of APPL1 (A) and APPL2 (B). Data correspond to the mean ± SE of 8 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 group.](image-url)
Regarding tendency values, we have calculated the effect size values by Cohen’s test. The result for APPL1 was Cohen’s $d=1.469139$ when OTR was compared to CT. The result for APPL2 was Cohen’s $d=1.414457$ for APPL2 when OTR/down was compared to CT. Both Cohen’s $d$ results were classified as a strong effect (i.e., larger than 0.8).

**Discussion**

The main findings of the present investigation were: a) In general, the OT protocols increased the hepatic APPL1 protein levels; b) OTR/up group also presented high values of hepatic APPL2 protein levels. Taken together, these results indicated that APPL1 could be part of the molecular mechanism that contributes to the glucose homeostasis in the liver of overtrained mice. Da Rocha et al. using the same liver samples of the current investigation verified that mice from the OTR/down group improved some initial proteins of the hepatic insulin signaling pathway, but upregulated the TRB3 and its association with Akt, implying a limitation on this downstream point of the insulin signaling pathway. The authors also observed high levels of hepatic glycogen, which leads to the hypothesis that another molecule could be responsible for this result especially in the OTR/down and OTR/up groups.

It is known that APPL1 can limit the interaction between Akt and TRB3 in mouse liver tissue, which is accompanied by an increase in the membrane translocation of APPL1, Akt activation, and enhancement in response to insulin stimulation. Also, adiponectin also stimulates APPL1 in mouse hepatocyte cells with activation of another class of proteins, the p38 mitogen-activated protein kinases (p38MAPK), which activates glucose transporters protein. The APPL1 overexpression was sufficient to enhance GLUT4 membrane translocation to a level comparable with that induced by adiponectin. Therefore, considering the increased values of liver glycogen concentration previously related for OTR/down and OTR/up groups and the current significant elevation of APPL1 in these same groups, we consider that APPL1 plays a major role in the hepatic glucose homeostasis of overtrained mice.

Studies using depletion of APPLs also revealed their regulation of signaling for glucose metabolism and inflammatory responses by the interaction with adiponectin receptors, phosphorylating its downstream targets: AMP-activated protein kinase (AMPK), GLUT4, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC1-alfa), and FoxO1. Da Rocha et al. demonstrated that the phosphorylation levels of FoxO1 and GSK-3beta are increased in the OT groups after insulin stimulation. Also, consistent evidence shows the potential role of insulin-mediated APPL1, resulting in a markedly enhanced GSK-3beta phosphorylation and increased glycogen storage in the mouse liver. APPL1 downregulation is associated with GSK-3beta phosphorylation in liver of obese mice under fasting conditions. However, in response to endurance exercise training, these obese mice increased APPL1 expression, Akt/APPL1 association, and GSK-3beta phosphorylation, which corroborates our consideration above.

Recently, Pereira et al. demonstrated that the currently used OT protocols impaired the insulin signaling pathway in skeletal muscles with different fiber type specificities, but did not induce significant differences in the insulin tolerance test (ITT). The authors concluded that other tissues such as liver played a major role in the maintenance of glucose homeostasis. It is known that APPL1 also plays a key role in the regulation of glucose metabolism by mediating the adiponectin signaling. In fact, the adiponectin-stimulated activation of AMPK can reduce the glucose levels in vivo, being considered as an anti-diabetic strategy. Thus, we may hypothesize that the current high levels of hepatic APPL1 (i.e., in the OTR/down and OTR/up groups) and its interaction with adiponectin contributed to the maintenance of glucose homeostasis in these OT groups.

The interaction between APPL1 and adiponectin may also be linked to an anti-inflammatory effect. Previously, our research group showed that the NFOR induced by the current OT protocols led to low-grade chronic inflammation state. Also, the OTR/down group increased the hepatic levels of interleukin-10 (IL-10), a classical anti-inflammatory cytokine. Although the anti-inflammatory effects of adiponectin are not well documented, it is known that this adipocyte-specific protein can increase the expression of anti-inflammatory mediators such as the IL-10. Finally, APPL1 is necessary for the anti-inflammatory and cytoprotective effects induced by adiponectin.

Regarding APPL2, it is known that this protein can negatively modulate the adiponectin signaling by direct connection with AdipoR1 and/or AdipoR2 via BAR domain, thereby preventing the APPL1 interaction with the Adipo receptors. On the other hand, adiponectin modulates the dissociation of the APPL1/APPL2 heterodimers, which can also be triggered by insulin. Interestingly, Cheng et al. demonstrated that APPL2 inhibits insulin-stimulated glucose uptake in skeletal muscle. In this study, only the OTR/up group increased the hepatic levels of APPL2, which may be linked to the increased phosphorylation of the insulin receptor substrate 1 (IRS-1) at serine 307, a molecular marker directly related to insulin signaling pathway impairment.

**Conclusion**

In summary, the OTR/down and OTR/up protocols increased the protein levels of APPL1, but only the OTR/up group increased the protein levels of APPL2 in liver. These results suggest a possible molecular pathway that potentiates hepatic glucose uptake in overtrained mice. Finally, figure 2 summarizes the current findings. In future investigations, we will perform an experiment silencing APPL1 and APPL2 to confirm their contributions to hepatic adaptations in response to these OT models.
Hepatic APPL1 and overtraining

Figure 2. A schematic figure representing the possible mechanisms by which APPL1 and APPL2 act in the liver of overtrained mice. The overtraining protocols based on downhill and uphill running upregulated the protein levels of APPL1, which can potentiate the action of Akt. The higher the activation of Akt, the higher is the phosphorylation and inactivation of GSK-3 beta, activating glycogen synthase and increasing hepatic glycogen storage. The OTR/up group also upregulated APPL2, which may decrease insulin sensitization. It is important to highlight that the protein levels of Akt and GSK-3 beta, as well as the glycogen contents of the OTR/down, OTR/up and OTR groups, were recently published using the same liver samples as the current investigation.

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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).

Corresponding author

Adelino S. R. da Silva. Address: Avenida Bandeirantes, 3900, Monte Alegre, Ribeirão Preto, São Paulo, Brasil.

Email: adelinosanchez@usp.br

Manuscript received on August 30, 2016
Manuscript accepted on October 07, 2016

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