Excessive training impairs the insulin signal transduction in mice skeletal muscles

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Abstract

The main aim of this investigation was to verify the effects of overtraining (OT) on the insulin and inflammatory signaling pathways in mice skeletal muscles. Rodents were divided into control (CT), overtrained by downhill running (OTR/down), overtrained by uphill running (OTR/up), and overtrained by running without inclination (OTR) groups. Rotarod, incremental load, exhaustive, and grip force tests were used to evaluate performance. Thirty-six hours after the grip force test, the extensor digitorum longus (EDL) and soleus were extracted for subsequent protein analyses. The three OT protocols led to similar responses of all performance evaluation tests. The phosphorylation of insulin receptor beta (pIR β ; Tyr), protein kinase B (pAkt; Ser473), and the protein levels of plasma membrane glucose transporter-4 (GLUT4) were lower in the EDL and soleus after the OTR/down protocol and in the soleus after the OTR/up and OTR protocols. While the pIR β was lower after the OTR/up and OTR protocols, the pAkt was higher after the OTR/up in the EDL. The phosphorylation of I κ B kinase alpha and beta (pIKK α/β ; Ser180/181), stress-activated protein kinases/Jun amino-terminal kinases (pSAPK-JNK; Thr183/Tyr185), factor nuclear kappa B (pNF κ B p65; Ser536), and insulin receptor substrate 1 (pIRS1; Ser307) were higher after the OTR/down protocol, but were not altered after the two other OT protocols. In summary, these data suggest that OT may lead to skeletal muscle insulin signaling pathway impairment, regardless of the predominance of eccentric contractions, although the insulin signal pathway impairment induced in OTR/up and OTR appeared to be muscle fiber-type specific.

Key Words

- ▶ exercise
- ▶ insulin signaling
- ▶ plasma membrane glut4
- ▶ mouse

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Introduction

The beneficial effects of acute and chronic moderate intensity exercise on the glucose metabolism in skeletal muscle cells of aging, obese, and sedentary rodents may be mediated by key proteins of the insulin-dependent signaling pathway (Luciano *et al.* 2002, Ropelle *et al.* 2006, 2013, Pauli *et al.* 2008, 2010, Da Silva *et al.* 2010). The binding of insulin to its receptor activates the insulin receptor (IR) tyrosine kinase, while phosphorylating intracellular protein substrates such as the insulin receptor substrate 1 (IRS1). The tyrosine phosphorylation of IRS1 activates the phosphoinositide 3-kinase (PI3-k) by binding to the p85 subunit and activating the catalytic p110 subunit. The activation of PI3-k increases the serine phosphorylation of the protein kinase B (Akt) that contributes to various biological processes including the regulation of glucose uptake induced by the translocation of the glucose transporter-4 (GLUT4) to the plasma membrane (White & Kahn 1994, Cheatham & Kahn 1995, Virkamaki *et al.* 1999).

A growing body of evidence suggests that the phosphorylation of IRS1 at serine 307 is directly linked to the molecular mechanism responsible of the impairment of insulin signaling pathway (Hotamisligil *et al.* 1996, Virkamaki *et al.* 1999). Indeed, some inflammatory signaling proteins such as the I κ B kinase (IKK), the nuclear factor kappa B (NF κ B), the stress-activated protein kinases/Jun amino-terminal kinases (SAPK/JNK), and the tumor necrosis factor alpha (TNF α) play a fundamental role in the phosphorylation (p) of IRS1 at serine 307, impairing the insulin signal transduction (Kahn *et al.* 2006, Vichaiwong *et al.* 2009, Da Silva *et al.* 2010, Prasannarong *et al.* 2012, Ropelle *et al.* 2013). In contrast to the aforementioned beneficial effects of exercise (Luciano *et al.* 2002, Ropelle *et al.* 2006, 2013, Pauli *et al.* 2008, 2010, Da Silva *et al.* 2010), our research group verified that an overtraining (OT) protocol based on downhill running sessions decreased the pIR β (Tyr1146) and pAkt (Ser473) with concomitant upregulation of the pIKK α/β (Ser176/180), pSAPK/JNK (Thr183/Tyr185), and pIRS1 (Ser307) in skeletal muscles with different fiber type specificities (Pereira *et al.* 2014a).

Because other authors also reported an impairment of the insulin signaling pathway in the skeletal muscles of humans (Del Aguila *et al.* 2000) and rodents (Aoi *et al.* 2012) following one single bout of downhill running, we cannot state that our results occurred exclusively due to the OT protocol (Pereira *et al.* 2014a). Thus, to discriminate the eccentric exercise (EE) effects on the insulin signal

transduction, we compared the intramuscular responses of the pIR β (Tyr), pAkt (Ser473), plasma membrane GLUT4, pIKK α/β (Ser180/181), pSAPK/JNK (Thr183/Tyr185), pNF κ B p65 (Ser536), pIRS-1 (Ser307), and TNF α after the OT downhill running protocol to another two running OT protocols with the same intensity and volume, but one performed in uphill and the other without inclination. Based on a previous study showing that an acute concentric exercise session had no effect on insulin action (Kirwan *et al.* 1992), our hypothesis is that the other OT protocols performed in uphill or without inclination do not impair the skeletal muscle insulin signaling.

Materials and methods

Experimental animals

Male C57BL/6 mice from the Central Animal Facility of the Ribeirão Preto campus of the University of Sao Paulo (USP) were kept in individual cages with controlled temperature ($22 \pm 2^\circ\text{C}$) on a 12 h light:12 h darkness inverted cycle with food (Purina chow, Curitiba, Paraná, Brazil) and water available *ad libitum*. The experimental procedures were performed in accordance with the Brazilian College of Animal Experimentation (<http://www.cobea.org.br>) and were approved by the Ethics Committee of the University of Sao Paulo (ID 14.1.873.53.0). Eight-week-old C57BL/6 mice were divided into four groups (i.e. $n=10$ mice/group): control (CT; sedentary mice), overtrained by downhill running (OTR/down; performed the OT protocol based on downhill running), overtrained by uphill running (OTR/up; performed the OT protocol based on uphill running), and overtrained by running without inclination (OTR; performed the OT protocol based on running without inclination). The CT, OTR/down, OTR/up, and OTR mice were manipulated and/or trained in a dark room between 06:00h and 08:00h (Pereira *et al.* 2012, 2014a,b, 2015a,b, da Rocha *et al.* 2015a,b).

Incremental load test (ILT)

Mice were first adapted to the treadmill running (INSIGHT, Ribeirão Preto, São Paulo, Brazil) for 5 days, for 10 min.day⁻¹ at 3 m.min⁻¹. Then, rodents performed the ILT with an initial intensity of 6 m.min⁻¹ at 0% with increasing increments of 3 m.min⁻¹ every 3 min until exhaustion, as defined by the time at which the mice touched the end of treadmill five times in 1 min. Mice were

encouraged using physical prodding (Pereira *et al.* 2012, 2014a,b, 2015a,b, da Rocha *et al.* 2015a,b). If mice became exhausted without completing the stage, the exhaustion velocity (EV; m.min⁻¹) was corrected according to Kuipers and coworkers (Kuipers *et al.* 1985). The EV of mice was used to determine the intensity of the OT protocols.

Overtraining protocols based on downhill running, uphill running, and running without inclination

The 8-week OT protocols based on downhill running, uphill running, and running without inclination were performed as described previously (da Rocha *et al.* 2015a,b, Pereira *et al.* 2015a,b), and each experimental week consisted of 5 days of training followed by 2 days of recovery. During the first 4 weeks of the OT protocols (i.e. first stage), the intensity was maintained at 60% of EV and the volume was gradually increased to 60 min per day in the 4th week. In this first stage, rodents ran at a grade of 0%. In the 5th 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 6th week of the OT protocols, the intensity was increased to 70% of EV. In the 7th week of the OT protocols, the intensity and volume

were increased to 75% of EV and 75 min, respectively. In the 8th week of the OT protocols, the number of daily sessions was doubled. The rest interval between daily sessions during the 8th week was 4 h.

Performance evaluations

The performance evaluations of the experimental groups were performed on week 0 and 48 h after the last sessions of the OT protocols at the end of weeks 4 and 8, and consisted of the rotarod test (Turgeman *et al.* 2008, Pereira *et al.* 2015a, da Rocha *et al.* 2015b), the ILT (Pereira *et al.* 2012, 2014a,b, 2015a,b, da Rocha *et al.* 2015a,b), the exhaustive test (Pereira *et al.* 2012, 2014a,b, 2015a,b, da Rocha *et al.* 2015a,b), and the grip force test (Anderson *et al.* 2004, da Rocha *et al.* 2015a,b, Pereira *et al.* 2015a). On week 0, the experimental groups performed the ILT without inclination. However, at the end of weeks 4 and 8, the CT and OTR performed the ILT without inclination, the OTR/down performed the ILT in downhill running, and the OTR/up performed the ILT in uphill running (da Rocha *et al.* 2015a,b, Pereira *et al.* 2015a,b).

Rotarod test

Mice were placed one at a time on the rotarod treadmill (INSIGHT, Ribeirão Preto, São Paulo, Brazil) with an initial

Figure 1
Variation in mean time on rotarod (%) between week 0 and week 8 (A), variation in exhaustion velocity (%) between week 4 and week 8 (B), and variations in time to exhaustion (%) (C) and grip force (%) (D) between week 0 and week 8 for the experimental groups. Data correspond to mean $\pm$ S.E.M. of $n = 10$ 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 the OTR/down group; ‡ $P < 0.05$ vs the OTR/up group; †† $P < 0.05$ vs the OTR group.

intensity of 1 r.p.m. and a final intensity of 40 r.p.m. that was reached 300 s later. Mice performed three consecutive trials and the mean time that each rodent was able to stay on the top of the rotarod treadmill was recorded (Turgeman *et al.* 2008). Four hours after the rotarod test, mice performed the ILT (Pereira *et al.* 2015a, da Rocha *et al.* 2015a).

Exhaustive test

Twenty-four hours after the ILT, mice ran at 36 m.min⁻¹ with 8% treadmill grade until exhaustion, which was determined when mice touched the end of treadmill five times in 1 min. Mice were encouraged using physical prodding. This value was recorded as the time to exhaustion (da Rocha *et al.* 2015a,b, Pereira *et al.* 2015a,b).

Grip force test

Four hours after the exhaustive test, mice performed the grip force test (da Rocha *et al.* 2015a,b, Pereira *et al.* 2015a). The researcher gently held each mouse by the tail and allowed it to grasp the horizontally positioned

metal bar of the Grip Strength System (Avs Projetos, São Carlos, São Paulo, Brazil) with the hindpaws. Each mouse performed three trials for adaptation and three trials for force measurement. The highest force value that was applied to the metal bar was recorded as the peak tension (N) and was used as a performance parameter (Anderson *et al.* 2004).

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

At the end of week 8, 24 h after the last sessions of the OT protocols, mice were injected intraperitoneally with glucose (2 g.kg⁻¹) after a fast period of 6 h. Blood samples from tails were collected at 0, 15, 30, 60, and 120 min to measure blood glucose concentrations using a glucometer (Accu-chek; Roche Diagnostics) (Bradley *et al.* 2008). For the ITT, fed mice were injected intraperitoneally with human recombinant insulin (1.5 U.kg⁻¹, Eli Lilly). Blood samples were collected at 0, 5, 10, 15, 20, 25, and 30 min to measure blood glucose concentrations using the previously mentioned glucometer. For the GTT and ITT, areas under the curves

Figure 2

Blood glucose (% baseline) for GTT (A); blood glucose (mg.dL⁻¹) responses during 120 min after an intraperitoneal injection of glucose (2 g.kg⁻¹) (B); the area under the curve (AUC; mg.dL⁻¹ x min) for the blood glucose responses during 120 min after an intraperitoneal injection of glucose (2 g.kg⁻¹) (C); blood glucose (% baseline) for ITT (D); blood glucose (mg.dL⁻¹) responses during 30 min after an intraperitoneal injection of insulin (1.5 U.kg⁻¹) (E); the area under the curve (AUC; mg.dL⁻¹ x min) for the blood glucose responses during 30 min after an intraperitoneal injection of insulin (1.5 U.kg⁻¹) (F). Data correspond to mean $\pm$ S.E.M. of $n = 10$ mice. CT, sedentary mice; OTR/down, overtrained by downhill running; OTR/up, overtrained by uphill running; OTR, overtrained by running without inclination; GTT, glucose tolerance test; ITT, insulin tolerance test. * $P < 0.05$ vs the CT group.

Figure 3

Protein levels (arbitrary units) of pIRβ (Tyr) and IRβ (A), pAkt (Ser473), Akt and β-actin (B), plasma membrane GLUT4, total GLUT4, and β-actin (C) in EDL for the experimental groups. The blot for IRβ was performed on total protein EDL extracts. Data correspond to mean $\pm$ s.e.m. of $n=8-10$ mice (i.e. $n=4-5$ mice without insulin stimulation and $n=4-5$ mice with insulin stimulation). 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 the OTR/up group; *** $P<0.05$ vs the OTR group.

(AUC) were calculated using the trapezoidal principle (da Rocha *et al.* 2015b).

Skeletal muscle extraction, immunoprecipitation, and immunoblotting analyses

Mice were anaesthetized 36 h after the grip force test (i.e. at the end of week 8). After a fasting period of 6 h, rodents were anaesthetized with an intraperitoneal (i.p.) injection

of 2-2-2 tribromoethanol 2.5% (10–20 μ L.g⁻¹). As soon as the effect of anaesthesia was confirmed by the loss of pedal reflexes, the abdominal cavity was opened, the portal vein was exposed, and saline with and without human recombinant insulin (10 U.kg⁻¹, Eli Lilly) was injected. At 90 s after saline or saline with human recombinant insulin injection (Ropelle *et al.* 2006, Pauli *et al.* 2008), each extensor digitorum longus (EDL) and soleus were removed and homogenized in an extraction buffer (1% Triton

Figure 4

Protein levels (arbitrary units) of pIKKα/β (Ser180/181), IKKβ, and β-actin (A); pSAPK-JNK (Thr183/Tyr185), SAPK-JNK, and β-actin (B); pNFκB p65 (Ser536), NFκB p65, and β-actin (C); pIRS1 (Ser307), IRS1, and β-actin (D); and TNFα and β-actin (E) in EDL for the experimental groups. Data correspond to mean $\pm$ s.e.m. of $n=5$ 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 the OTR/up group; *** $P<0.05$ vs the OTR group.

Figure 5

Protein levels (arbitrary units) of pIR β (Tyr) and IR β (A); pAkt (Ser473), Akt, and β -actin (B); and plasma membrane GLUT4, total GLUT4, and β -actin (C) in soleus for the experimental groups. The blot for IR β was performed on total protein soleus extracts. Data correspond to mean $\pm$ S.E.M. of $n=8-10$ mice (i.e. $n=4-5$ mice without insulin stimulation and $n=4-5$ mice with insulin stimulation). 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 the OTR/up group; *** $P < 0.05$ vs the OTR group.

X-100, 100mM Tris, pH 7.4, containing 100mM sodium pyrophosphate, 100mM sodium fluoride, 10mM EDTA, 10mM sodium vanadate, 2mM PMSF, and 0.1 mg.mL⁻¹ aprotinin) at 4°C with a Polytron PTA 20S generator (Brinkmann Instruments model PT 10/35), operated at maximum speed for 30s. The extracts were centrifuged (9900g) for 40min at 4°C to remove insoluble material, and the supernatants were used for protein quantification using the Bradford method as described previously (Pereira *et al.* 2014a,b, 2015a,b, da Rocha *et al.* 2015a,b).

Equal amounts of protein were used for immunoprecipitation with 10 μ L of the insulin IR β (SC20739) from Santa Cruz Biotechnology. The immunocomplex was precipitated with protein A-Sepharose 6MB (Pharmacia) and washed three times with 50mM Tris (pH 7.4) containing 2mM sodium vanadate and 0.1% Triton X-100. Proteins were denatured by boiling in a Laemmli sample buffer containing 100mM DTT, run on SDS-PAGE gel and transferred to nitrocellulose membranes (GE Healthcare, Hybond ECL, RPN303D). The transfer efficiency to nitrocellulose membranes was verified by brief staining of the blots with a 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.

Antibodies used for immunoblotting overnight at 4°C were phospho-tyrosine (SC8954S), IR β (SC20739), IRS1 (SC560), phospho-IRS1 (Ser307; SC33956), IKK β (SC34674), phospho-IKK α/β (Ser180/181; SC23470R), GLUT4 (SC53566), and β -actin (SC69879) from Santa Cruz Biotechnology; Akt (CELL9272S), phospho-Akt (Ser473; CELL4058S), SAPK-JNK (CELL9252S), and phospho-SAPK-JNK (Thr183/185; CELL9251S) from Cell Signaling Technology; and TNF α (AB9635) from Abcam. After washing with TBS containing 0.1% Tween 20, all membranes were

incubated for 1 h at 4°C with secondary antibody conjugated with horseradish peroxidase. The specific immunoreactive bands were detected by chemiluminescence (GE Healthcare, ECL Plus Western Blotting Detection System, RPN2132). Images were acquired by the C-DiGit Blot Scanner (LI-COR, Lincoln, NE, USA) and quantified using the software Image Studio for C-DiGit Blot Scanner.

Subcellular fractionation

As described previously (Mizukami *et al.* 1997, Gasparetti *et al.* 2003), the EDL and soleus samples were removed and homogenized in 2 volumes of STE buffer (0.32M sucrose, 20mM Tris-HCl/pH 7.4, 2mM EDTA, 1mM DTT, 100mM sodium fluoride, 100mM sodium pyrophosphate, 10mM sodium orthovanadate, 1mM PMSF, 0.1mg aprotinin mL⁻¹) at 4°C with a Polytron PTA 20S generator. The homogenates were centrifuged (1000g, 25 min, 4°C), the obtained pellets were washed once with STE buffer (1000g, 10min, 4°C), and suspended in Triton buffer (1% TritonX-100, 20mM Tris-HCl/pH 7.4, 150mM NaCl, 200mM EDTA, 10mM sodium orthovanadate, 1mM PMSF, 100mM NaF, 100mM sodium pyrophosphate, 0.1mg aprotinin mL⁻¹), maintained on ice for 30 min and centrifuged (15,000g, 30 min, 4°C) to obtain the nuclear fraction. The supernatants were centrifuged (100,000g, 60 min, 4°C) to obtain the cytosol fraction, and the pellets were suspended in STE buffer plus 1% Nonidet P-40, maintained on ice for 20min and centrifuged (100,000g, 20min) to obtain the membrane fraction. The plasma membrane and nuclear fractions were treated with Laemmli buffer with 100mM dithiothreitol, heated in a boiling water bath for 5 min, and aliquots were subjected to the previously described immunoblotting with the

following antibodies: GLUT4 (SC53566) for plasma membrane aliquots, and NFκB p65 (SC372) and phospho-NFκB p65 (Ser536; SC33020) for nuclear aliquots.

Statistical analysis

Results are presented as the mean ± S.E.M. According to Shapiro–Wilk's *W*-test, the data were normally distributed and homogeneity of variances was confirmed by Levene's test. Therefore, a one-way ANOVA was used to examine the effects of the OT protocols. When one-way ANOVA indicated statistical 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, USA).

Results

Performance parameters, glucose tolerance test (GTT), and insulin tolerance test (ITT)

Figure 1A shows that the variation in mean time on rotarod from week 0 to week 8 was higher in the OTR/down group

compared with the CT, OTR/up and OTR groups. In addition, the OTR/up and OTR groups presented higher variations in mean time on rotarod from week 0 to week 8 compared with the CT group. The variation in exhaustion velocity from week 4 to week 8 (Fig. 1B), and the variations in time to exhaustion (Fig. 1C) and grip force (Fig. 1D) from week 0 to week 8 were higher in the OTR/down, OTR/up, and OTR groups as compared with the CT group. In addition, the OTR/up group presented lower variation in the exhaustion velocity from week 4 to week 8 as compared with the OTR/down and OTR groups (Fig. 1B). Figure 2A shows the blood glucose (% baseline) for GTT, Fig. 2B shows the blood glucose (mg.dL⁻¹) responses during the GTT, and Fig. 2C shows that the AUC for the GTT was lower in the OTR/down, OTR/up, and OTR groups as compared with the CT group. Figure 2D shows the blood glucose (% baseline) for ITT, Fig. 2E shows the blood glucose (mg.dL⁻¹) responses during the ITT, and Fig. 2F shows that the AUC for the ITT was not different between the experimental groups.

Insulin and inflammatory signaling pathways in EDL

Figure 3A shows that tyrosine phosphorylation of IRβ after insulin injection was lower in the OTR/down, OTR/up,

Figure 6

Protein levels (arbitrary units) of pIKKα/β (Ser180/181), IKKβ, and β-actin (A); pSAPK-JNK (Thr183/Tyr185), SAPK-JNK, and β-actin (B); pNFκB p65 (Ser536), NFκB p65, and β-actin (C); pIRS-1 (Ser307), IRS-1, and β-actin (D); and TNFα and β-actin (E) in soleus for the experimental groups. Data correspond to mean ± S.E.M. of $n = 5$ 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 the OTR/up group; *** $P < 0.05$ vs the OTR group.

and OTR groups than in the CT group. In addition, tyrosine phosphorylation of IR β after insulin injection was lower in the OTR/down group than in the OTR/up and OTR groups. The serine phosphorylation of Akt after insulin injection was lower in the OTR/down group than in the CT, OTR/up, and OTR groups. In addition, serine phosphorylation of Akt after insulin injection was higher in the OTR/up group than in the OTR group (Fig. 3B). Figure 3C shows that the plasma membrane GLUT4 after insulin injection in the OTR/down group was lower than in the CT group. The pIKK α/β (Fig. 4A), pSAPK-JNK (Fig. 4B), pNF κ B p65 (Fig. 4C), and pIRS1 (Fig. 4D) levels were higher in the OTR/down group than in the CT, OTR/up, and OTR groups. Figure 4E shows that the TNF α levels were higher in the OTR/down and OTR/up groups than in the CT and OTR/up groups. In addition, the TNF α level was higher in the CT group than in the OTR/up group.

Insulin and inflammatory signaling pathways in soleus

Figure 5A shows that tyrosine phosphorylation of IR β after insulin injection was lower in the OTR/down, OTR/up, and OTR groups than in the CT group. In addition, tyrosine phosphorylation of IR β after insulin injection was lower in the OTR/down group than in the OTR/up and OTR groups. The serine phosphorylation of Akt after insulin injection was lower in the OTR/down, OTR/up, and OTR groups than in the CT group (Fig. 5B). Figure 5C shows that the plasma membrane GLUT4 after insulin injection in the OTR/down, OTR/up, and OTR groups was lower than in the CT group. The pIKK α/β (Fig. 6A), pSAPK-JNK (Fig. 6B), pNF κ B p65 (Fig. 6C), and pIRS1 (Fig. 6D) levels were higher in the OTR/down group than in the CT, OTR/up, and OTR groups. In addition, the pSAPK/JNK levels were lower in the OTR group than in the CT and OTR/up groups. Figure 6E shows that the TNF α levels were higher in the OTR/down, OTR/up, and OTR groups than in the CT group.

Discussion

The present investigation compared the effects of different OT protocols on the insulin and inflammatory signaling pathways in mouse skeletal muscles. First, we found that the three OT protocols led to similar responses of rotarod, exhaustion velocity, time to exhaustion, grip force, and AUC for GTT and ITT. Second, independently from the fiber type specificity, the OTR/down protocol

decreased the muscle protein contents of pIR β , pAkt and plasma membrane GLUT4 with a concomitant increase in pIKK α/β , pSAPK/JNK, pNF κ B p65, pIRS1, and TNF α . The OTR/up and OTR protocols decreased the EDL protein contents of pIR β , and the soleus protein contents of pIR β , pAkt, and plasma membrane GLUT4 without a concomitant increase in pIKK α/β , pSAPK/JNK, pNF κ B p65, and pIRS1 in both skeletal muscle samples. Finally, the OTR/up (i.e. in soleus) and OTR (i.e. in EDL and soleus) protocols increased the TNF α levels. Taken together, differently from the OTR/down protocol, our data showed that the OTR/up and OTR protocols only led to insulin signaling pathway impairment in soleus samples. Interestingly, these results were not linked to the increase in all analyzed inflammatory proteins.

The effects of the three OT protocols on the performance parameters are partially in agreement with our recently published data (da Rocha *et al.* 2015a,b). However, here, we verified that the OTR/up group presented a lower change in the exhaustion velocity as compared with the other OT protocols. In addition, differently from Rocha's investigation (da Rocha *et al.* 2015b), we showed that the OTR protocol also reduced the rotarod performance as compared with the CT group. These findings indicate that the application of the same OT protocols in different batches of C57BL/6 mice may induce minimal differences of adaptation.

In the current investigation, we observed that the overtrained mice improved their glucose tolerance even with reduced muscle insulin signaling. Previously, Pereira and coworkers (Pereira *et al.* 2014a) verified muscle insulin signal transduction impairment after OTR/down without significant differences in ITT and hypothesized that other tissues such as untrained skeletal muscles, liver, and heart may not present inhibition of this pathway, playing an important role in the maintenance of glucose homeostasis. Indeed, da Rocha and coworkers (2015b) showed that OTR/down and OTR/up models upregulated the phosphorylation and inhibition of glycogen synthase kinase 3 beta (GSK3 β), increasing hepatic glycogen deposition. In accordance with other studies (Zisman *et al.* 2000, Kotani *et al.* 2004), the authors concluded that liver acted as a compensatory organ when skeletal muscle presents insulin signaling impairment (da Rocha *et al.* 2015b).

However, future investigations should evaluate the effects of the current OT protocols on the insulin signaling pathway of other skeletal muscle samples and cardiomyocytes in order to verify their role in the blood glucose homeostasis. In addition, previous studies performed by

our research group observed that diet-induced obese Wistar rats secreted high levels of insulin to compensate the insulin resistance, which keeps the glucose levels similar to the control animals (Pauli *et al.* 2008, Da Silva *et al.* 2010). Although this physiological mechanism of compensation may be considered in order to explain the apparent discrepancy between improved glucose tolerance and reduced muscle insulin signaling in overtrained C57BL/6 mice, further experiments should measure the insulin levels of the OT groups to test this hypothesis.

The responses of pIR β , pAkt, pIKK α/β , pSAPK-JNK, pIRS1, and TNF α to the OTR/down protocol for both skeletal muscle samples are in agreement with the previous findings published by Pereira and coworkers (Pereira *et al.* 2014a,b). However, recently da Rocha and coworkers (2015a) verified that the increase in pIRS1 (Ser307) after the OTR/down protocol occurred only in the EDL sample, which implies that different batches of C57BL/6 mice may present minimal differences of adaptation. On the other hand, the current data reinforce that the imbalance between chronic sessions of EE and adequate recovery periods leads to skeletal muscle insulin signaling pathway impairment. In accordance with other studies about the EE effects on the insulin pathway (Del Aguila *et al.* 1999, 2000, Kirwan & del Aguila 2003, Pereira *et al.* 2014a), we also showed that the defect in the signal transduction of this hormone was related to the increase in pIRS-1 at serine 307. Although Pereira and coworkers (2014a) had shown that this increase occurred concomitantly with the increase in pIKK α/β and pSAPK-JNK, this is the first investigation demonstrating that the nuclear content of pNF κ B p65 is also upregulated in response to excessive EE.

The increase in the NF κ B, a proinflammatory transcription factor, impairs the insulin signaling pathway in skeletal muscle cells (Kim *et al.* 2001) and stimulates the expression of the interleukin-6 (IL6) and tumor necrosis factor alpha (TNF α), which were upregulated after the OTR/down protocol (Pereira *et al.* 2014b). Another novelty of this study concerning the OTR/down protocol was the measurement of the plasma membrane GLUT4 content. Fam and coworkers (2012) showed that the deletion of the skeletal muscle GLUT4 on the pure C57BL6/J background strain did not impair the whole-body glucose disposal. These authors, among others (Ryder *et al.* 1999, Charron *et al.* 2005, Fam *et al.* 2012), considered that other unidentified GLUTs were upregulated in an attempt to compensate the lack of GLUT4. Probably, the previous hypothesis justifies the fact that even with lower content of plasma membrane

GLUT4 in EDL and soleus, the OTR/down group did not exhibit lower glycogen concentrations when compared with the CT group (Pereira *et al.* 2014b).

Because OTR/down group displayed lower pIR β in both skeletal muscle samples, we cannot rule out the possibility that this specific OT model damaged capillaries to muscles, which results in a reduction in insulin delivery. The skeletal muscle insulin signal transduction impairment in response to acute and chronic downhill running sessions is well described in the literature (Del Aguila *et al.* 2000, Aoi *et al.* 2012, Pereira *et al.* 2014a); however, to this date, this is the first study describing the effects of two other OT protocols performed without the predominance of eccentric muscular contractions on the insulin signaling pathway. Although these OT protocols diminished the pIR β , one of them (i.e. OTR/up) increased the pAkt in the EDL. This skeletal muscle sample is predominantly composed of type II fibers (Armstrong & Phelps 1984). In addition, Ferreira and coworkers (2007) showed that 60% of the EV measured in the ILT represented the intensity corresponding to the maximal lactate steady state (MLSS), which was considered the gold standard protocol in identifying the metabolic aerobic/anaerobic transition point during exercise (Billat *et al.* 2003, Da Silva *et al.* 2010). Because only 2 weeks of these OT protocols were performed above the MLSS intensity, our hypothesis was that the EDL was more recruited during this period allowing the positive adaptation of the pAkt.

The significant increase in pAkt (Ser473) without previous upregulation of pIR in response to chronic exercise is not novel (Luciano *et al.* 2002); however, so far, the molecular mechanisms responsible of this phenomenon remain unknown. Lee and coworkers (2009) verified that the global deletion of the Rho kinase 1 (ROCK1), a serine/threonine protein kinase identified as a GTP-Rho-binding protein (Matsui *et al.* 1996), impaired the skeletal muscle insulin signaling. In addition, *in vitro* studies using skeletal muscle cells demonstrated that the increase in the glucose transport stimulated by insulin was decreased with the inhibition of the endogenous ROCK1 expression (Chun *et al.* 2012). Indeed, ROCK1 is able to phosphorylate IRS1 on serine 632/635, leading to serine phosphorylation of Akt and improving the glucose uptake by muscle tissue (Furukawa *et al.* 2005). Thus, as a next step, our research laboratory will investigate the effects of the present OT protocols on the ROCK1 metabolism.

Regarding the soleus samples, the OTR/up and OTR protocols decreased the protein levels of pIR β , pAkt, and

Figure 7

Proposed molecular mechanisms by which the OT models lead to skeletal muscle insulin signaling pathway impairment.

plasma membrane GLUT4. Because this muscle is predominantly composed of type I fibers (Armstrong & Phelps 1984), we considered that the soleus was more recruited than the EDL during the 8 weeks of the OT protocols. However, further investigations should verify the electromyographic activity of these skeletal muscles during the different OT protocols to test our preceding hypothesis. Differently from the OTR/down group, the impairment of the insulin signaling pathway in the soleus after the OTR/up and OTR protocols did not occur with concomitant increase in pIKK α / β , pSAPK/JNK, pNF κ B p65, and pIRS1. Therefore, we consider that the decrease in pAkt and plasma membrane GLUT4 in these groups occurred mainly due to the reduced tyrosine kinase phosphorylation of the IR β , which may be partially explained by the current increase in their TNF α levels (Hotamisligil *et al.* 1994, Hotamisligil 2006, Gautam *et al.* 2014). In addition, we observed that the OTR/up protocol increased the TNF α serum levels (Pereira *et al.* 2015a).

In conclusion, the present findings testified partially our initial hypothesis showing that the OT protocols performed in uphill or without inclination did not impair the insulin signaling pathway in the EDL samples. Indeed, even with the reduction in the pIR β , the pAkt was upregulated after the OTR/up protocol in this skeletal muscle. However, the soleus samples presented insulin signal transduction impairment after the OTR/up and OTR

protocols. These data suggest that OT may lead to skeletal muscle insulin signaling pathway impairment, regardless of the predominance of eccentric contractions, although the insulin signal pathway impairment induced in OTR/up and OTR appeared to be muscle fiber type specific. Finally, Fig. 7 summarizes the molecular mechanisms by which the OT models lead to skeletal muscle insulin signaling pathway impairment.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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