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Exercise training decreases mitogen-activated protein kinase phosphatase-3 expression and suppresses hepatic gluconeogenesis in obese mice

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Key points summary

- When the hepatic insulin signaling is compromised, there is an inadequate suppression of gluconeogenic pathways, leading the organism to high levels of glucose.
- Studies with animals with obesity induced by high fat diet or genetically modified showed increased MKP-3 expression and MKP-3/Foxo1 association in liver, with a consequent increase in blood glucose concentration, development of insulin resistance and DM2.
- As a non-pharmacological strategy recognized and indicated for prevention and treatment of diabetes is the regular practice of physical exercise.
- In this study we demostrated that physical training is an important tool capable of reducing insulin resistance in the liver by reducing the inflammatory process, including the inhibition of MKP-3 and, therefore, suppress gluconeogenic program in obesity rats.
- The understanding of these new mechanisms by which physical training regulates glucose homeostasis has critical importance to health professionals for the understanding and prevention of diabetes.

Abstract Insulin plays an important role in the control of hepatic glucose production. Insulin resistant states are commonly associated with excessive hepatic glucose production, which contributes to both fasting hyperglycaemia and exaggerated postprandial hyperglycaemia. In this regard, increased activity of phosphatases may contribute to the dysregulation of gluconeogenesis. Mitogen-activated protein kinase phosphatase-3 (MKP-3) is a key protein involved in the control of gluconeogenesis. MKP-3-mediated dephosphorylation activates FoxO1 (a member of the forkhead family of transcription factors) and subsequently promotes its nuclear translocation and binding to the promoters of gluconeogenic genes such as phosphoenolpyruvate carboxykinase (*PEPCK*) and glucose-6-phosphatase (*G6Pase*). In this study, we investigated the effects of exercise training on the expression of MKP-3 and its interaction with FoxO1 in the livers of obese animals. We found that exercised obese mice had a lower expression of MKP-3 and FoxO1/MKP-3 association in the liver. Further, the exercise training decreased FoxO1 phosphorylation and protein levels of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and gluconeogenic enzymes (*PEPCK and G6Pase*). These molecular results were accompanied by physiological changes, including increased insulin sensitivity and reduced hyperglycaemia, which were not caused by reductions in total body mass. Similar results were also observed with oligonucleotide antisense (ASO) treatment. However, our results showed that only exercise training could reduce an obesity-induced increase in HNF-4α protein levels while ASO treatment

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alone had no effect. These findings could explain, at least in part, why additive effects of exercise training treatment and ASO treatment were not observed. Finally, the suppressive effects of exercise training on MKP-3 protein levels appear to be related, at least in part, to the reduced phosphorylation of Extracellular signal-regulated kinases (ERK) in the livers of obese mice.

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Abbreviations AUC, area under the curve; DIO, high fat diet-induced obesity mice; DIO-ASO, DIO mice treated with the MKP-3 antisense oligonucleotide; DIO-EXE, DIO mice subjected to exercise training; DIO-EXE-ASO, DIO mice subjected to exercise training and treated with the MKP-3 antisense oligonucleotide concomitantly; DIO-sense, DIO mice treated with the MKP-3 sense oligonucleotide; ERK1/2, extracellular signal-regulated kinases; FoxO1, forkhead transcription factor family; G6Pase, glucose-6-phosphatase; HFD, high fat diet; HNF-4α, hepatocyte nuclear factor 4α; ITT, insulin tolerance test; Lean, mice fed standard rodent chow; MAPK, mitogen-activated protein kinase; MKP-3, mitogen-activated protein kinase phosphatase-3; MKP-3-ASO, DIO and/or EXE were housed in individual cages and treated with the MKP-3 antisense oligonucleotide; MKP-3-sense, DIO and/or EXE were housed in individual cages and treated with the sense oligonucleotide; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1 α; PI3K, phosphatidylinositol 3-kinase; PPAR, peroxisome proliferator-activated receptor; PTT, pyruvate tolerance test; MAPK, mitogen-activated protein kinase

Introduction

The liver is a key organ in the maintenance of systemic glucose homeostasis in mammals. The liver can produce glucose by breaking down glycogen (glycogenolysis) and by *de novo* synthesis of glucose from non-carbohydrate precursors such as pyruvate, glycerol, lactate and alanine (gluconeogenesis) (Pilkis & Granner, 1992). The rate of gluconeogenesis is controlled in part by the activities of certain unidirectional enzymes, such as phosphoenolpyruvate carboxykinase (*PEPCK*), fructose-1,6-bisphosphatase and glucose-6-phosphatase (*G6Pase*) (Liao *et al.* 1998; Schmoll *et al.* 2000). In addition, factors such as gluconeogenic substrate supply and/or energy state may also control the gluconeogenic flux (Burgess *et al.* 2007). The genes encoding these proteins are powerfully controlled at the transcriptional level by key hormones, particularly insulin, glucagon, adrenaline (epinephrine) and glucocorticoids. In the fed state, the hormone insulin is considered the most important factor regulating the suppression of gluconeogenesis and hepatic glucose production (Sutherland *et al.* 1995; Schmoll *et al.* 2000; Vidal-Puig & O'Rahilly, 2001).

Insulin modulates FoxO1 activity in an Protein Kinase B (AKT) dependent manner (Puigserver *et al.* 2003*a*). In loss- and gain-of-function experiments in mice, FoxO1 has been associated with the hepatic glucose production (Pagliassotti *et al.* 2002; Puigserver *et al.* 2003). Previous studies showed that the negative modulation of insulin signal transduction through insulin phosphatidylinositol 3-kinase (PI3K)/Akt/FoxO1 is involved in gluconeogenesis (Barthel *et al.* 2001; Cintra *et al.* 2008). Insulin signalling plays an important role in controlling gluconeogenic gene expression, including *PEPCK*, which catalyses the rate-limiting step of hepatic gluconeogenesis (Sutherland *et al.* 1996). A number of observations indicate that the activation of PI3K/Akt is the major pathway involved in the modulation of hepatic genes by insulin. Thus, the block of insulin-induced PI3K activation using wortmannin and LY-294002 prevents the inhibitory effect of insulin on *PEPCK* expression (Band & Posner, 1997). Expression of a dominant negative mutant of PI3K induces the expression of *PEPCK* (Miyake *et al.* 2002).

Additionally, FoxO1 is known to increase the transcription of peroxisome proliferator-activated receptor (PPAR) γ coactivator-1 α (PGC-1α), which encodes an important amplifier for hepatic gluconeogenesis (Puigserver *et al.* 2003). Indeed, PGC-1 can powerfully stimulate the expression of key genes in the gluconeogenic pathway in the liver. This fact strongly suggests that the regulation of the transcriptional coactivator PGC-1 is of central importance in the control of gluconeogenesis by insulin and other hormones. PGC-1α robustly activates gluconeogenic gene expression through direct interactions with FoxO1 and hepatocyte nuclear factor 4α (HNF-4α) (Puigserver *et al.* 2003; Rhee *et al.* 2003). Disruption of the interaction between PGC-1α and FoxO1 by insulin represses PGC-1α-mediated gluconeogenesis (Daitoku *et al.* 2003). Previous studies also indicate that HNF-4α and FoxO1, which are transcription factors that regulate gluconeogenic gene expression (Puigserver & Spiegelman, 2003; Rhee *et al.* 2003), are both critical targets of PGC-1α coactivation.

This finely tuned metabolic process can be regulated by phosphatases. In particular, MKP-3 appears to be the key factor for the proper control of gluconeogenesis. The expression of MKP-3 is markedly elevated in the livers of obese mice, and knocking down MKP-3 expression in the livers of either lean or obese mice decreases fasting blood glucose levels (Wu *et al.* 2010).

The MKP-3 ability to promote hepatic glucose output is mediated at least partially through its ability to physically associate with and dephosphorylate FoxO1. MKP-3-mediated dephosphorylation activates FoxO1 and subsequently promotes its nuclear translocation and binding to the promoters of gluconeogenic genes, such as *PEPCK* and *G6Pase* (Wu *et al.* 2010). These results demonstrate that MKP-3 is an important regulator of hepatic gluconeogenesis and may contribute to the inappropriate activation of gluconeogenesis that occurs in type 2 diabetes.

MKP-3 is a cytoplasmic phosphatase that is highly specific for dephosphorylation of the extracellular signal-regulated kinases ERK1/2 and plays a critical role in attenuating ERK1/2-mediated mitogenesis (Camps *et al.* 1998; Fjeld *et al.* 2000; Feng *et al.* 2012). MKP-3-deficient mice display enhanced basal ERK1/2 phosphorylation and increased myocyte proliferation (Maillet *et al.* 2008). Interestingly, the MEK/ERK pathway itself also plays a role in down-regulating MKP-3 expression, forming a negative feedback loop between the kinases and the phosphatases (Marchetti *et al.* 2005; Jurek *et al.* 2009).

Exercise training is known to improve insulin's actions and has significant effects on insulin signalling pathways and hepatic glucose production (De Souza *et al.* 2010; da Luz *et al.* 2011; Marinho *et al.* 2012). However, the effects of training on the expression of MKP-3 and its regulation of glucose metabolism remain unknown. Thus, the aim of this study was to investigate the effects of exercise training on the expression of MKP-3 protein as well as its association with the transcription factor FoxO1 in the livers of obese mice. In addition, we investigated the effect of training on the phosphorylation of ERK in the livers of obese mice.

Methods

Experimental animals

The experimental procedures involving mice were performed in accordance with the principles and procedures described by the National Institutes of Health *Guidelines for the Care and Use of Experimental Animals* and were approved by the University of Campinas Ethical Committee (ID 20112599-1). Male Swiss mice (15–20 g) from the University of Campinas Breeding Center were used. The mice were always housed in individual cages and subjected to a standard light–dark cycle (06.00–18.00 h/18.00–06.00 h), and the room temperature was maintained at stable levels (23 ± 2°C). After random selection, animals were fed a standard rodent chow (3.948 kcal kg^{-1}) or a high-fat diet (HFD; 5.358 kcal kg^{-1}) *ad libitum* for 16 weeks. The composition of the high-fat diet has been previously described (Pauli *et al.* 2008).

First, after the induction of obesity, the effectiveness of an antisense oligonucleotide targeting MKP-3 was assessed (Fig. 1). Then the animals were divided into six experimental groups: mice fed standard rodent chow (Lean), high fat diet-induced obesity mice (DIO), DIO mice treated with the MKP-3 antisense oligonucleotide (DIO-ASO), DIO mice treated with the MKP-3 sense oligonucleotide (DIO-sense), DIO mice subjected to exercise training (DIO-EXE) and DIO mice subjected to exercise training and treated with the MKP-3 antisense oligonucleotide concomitantly (DIO-EXE-ASO). Data obtained from DIO-sense mice (treated with a sense oligonucleotide control) were excluded from the results because they did not show appreciable differences from DIO mice.

The antisense oligonucleotide treatment

The sense and antisense (ASO) phosphorothioate oligonucleotides specific for MKP-3 (sense, 5′-TCA GGA GCT GGA TGG C-3′ and antisense, 5′-ATG ATG TTC GTG GTG TAA AGT T-3′) were selected from three unrelated pairs of oligonucleotides on the basis of their ability to block MKP-3 protein expression, as evaluated by immunoblotting total protein extracts of liver using specific anti-MKP-3 antibodies (as shown in Fig. 3*B*). The antisense oligonucleotide sequences were submitted to BLAST analyses (www.ncbi.nlm.nih.gov) and were found to match only the mouse MKP-3 coding sequence. DIO and/or EXE were housed in individual cages and treated with the sense oligonucleotide (MKP-3-sense) or with the MKP-3 antisense oligonucleotide (MKP-3-ASO). The sense and antisense MKP-3 oligonucleotides were diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA), and treatment was achieved by twice daily (08.00 h/18.00 h) I.P. injections, with 2.0 μl per dose (1.0 nmol μl^{-1}), for 5 days (see Fig. 1). The sense and antisense phosphorothioate oligonucleotides specific for MKP-3 were produced by Invitrogen Corp. (Carlsbad, CA, USA). In some experiments, in order to evaluate the efficiency of the oligonucleotides, different doses were employed as described in the Results section (dose–response experiment, Fig. 3*B*). In the experiment involving the DIO-EXE-ASO mice, the treatment with antisense oligonucleotide MKP-3 was performed simultaneously with the last five sessions of training (see Fig. 1).

Exercise training protocol

Mice were adapted to swimming for 10 min each day for 3 days to reduce water-induced stress. The exercise training was performed during the light cycle and consisted of 60 min swimming sessions, 5 days per week for 8 weeks (8–16 weeks of the experiment) in an apparatus adapted for mice (60 cm depth × 45 cm diameter cylindrical tank) containing warm water (31–32°C). The volume of exercise

training was gradually increased until the mice swam for 60 min wearing caudal dumbbells weighing 5% of their body weight, which was achieved after the eighth day of training (see Fig. 2). This exercise training protocol was previously used in other studies in our laboratory (Oliveira *et al.* 2011; Marinho *et al.* 2012). The experimental analyses were performed 36 h after the last exercise training session.

Determination of cumulative energy intake

The food intake of the animals (Lean, DIO, DIO-EXE, DIO-ASO and DIO-EXE-ASO) was evaluated every week. Thereafter, standard chow or HFD was given, and food intake was determined by measuring the difference between the weight of chow given and the weight of the chow at the end of a 24 h period. The cumulative energy intake over the 8 weeks of the experimental period was determined and expressed in kilocalories.

Insulin tolerance test and pyruvate tolerance test

Thirty-six hours after the last training session, the mice were submitted to an insulin tolerance test (ITT; 1.5U $\times$ (body weight)$^{-1}$ of insulin). Before testing, all animals were fasted for 6 h. Briefly, 1.5 IU kg^{-1} of human recombinant insulin (Humulin R) from Eli Lilly (Indianapolis, IN, USA) was injected intraperitoneally, and blood samples were collected at 0, 5, 10, 15, 20, 25 and 30 min for blood glucose determination using a glucose meter (Advantage, Boehringer Mannheim, Germany). Blood samples were obtained following a tail snip (using surgical scissors), with bleeding suppressed between samples using a compression bandage (Johnson and Johnson, São Paulo, SP, Brazil). The rate constant for blood glucose disappearance (kITT) was calculated using the formula 0.693/biological half-life ($t_{½}$). The blood glucose $t_{½}$ was calculated from the slope of the least square analysis of the plasma glucose concentration during the linear phase of decline (Bonora *et al.* 1989).

The pyruvate tolerance test (PTT) was performed to estimate gluconeogenesis as follows. Mice were starved for 16 h and then injected intraperitoneally with pyruvate (2 g kg^{-1}) dissolved in saline. Blood glucose levels were determined in the tail blood every 30 min for 2 h using a glucose meter (Advantage).

Fasting glucose and serum insulin quantification

The blood glucose level was determined by a colorimetric method using a glucose meter (Advantage). The zero time values in the insulin tolerance test were used as the fasting glucose values. Blood was collected from the cava vein. Plasma was separated by centrifugation (1100 *g*) for 15 min at 4°C and stored at −80°C until assay. A radio-immunoassay was employed to measure serum insulin as previously described (Scott *et al.* 1981).

Protein analysis by immunoprecipitation and immunoblotting

Thirty-six hours after the last training session, the mice were anaesthetized with an I.P. injection of ketamine (50 mg (kg body weight)$^{-1}$) and xylazine (20 mg (kg body weight)$^{-1}$). Ketamine and xylazine were provided by Syntec (Cotia, SP, Brazil). Moreover, before the molecular analysis experiments, the animals were fasted for 6 h. As soon as anaesthesia was assured by the loss of pedal and corneal reflexes, the abdominal cavity was opened, the portal vein was exposed, and 0.2 ml of normal saline with or without insulin (10^{-6} mol l^{-1}) was injected. After the insulin injection, hepatic tissue fragments were extracted and homogenized immediately 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 phenylmethylsulfonyl fluoride (PMSF) and 0.1 mg aprotinin ml^{-1}) at 4°C with a Polytron (MR 2100, Kinematica, Switzerland). The extracts were centrifuged at 3.900 g at 4°C (5804R, Eppendorf AG, Hamburg, Germany) for 40 min to remove insoluble material, and the supernatants were used for protein quantification, according to the Bradford method (Bradford, 1976). Equal amounts of protein were used for immunoprecipitation. The animals were killed at the end of the study by decapitation while still anaesthetized.

The immunocomplex was precipitated with Protein A–Sepharose 6MB (Pharmacia; Uppsala, Sweden) and then washed three times with 50 mM Tris (pH 7.4) containing 2 mM sodium vanadate and 0.1% Triton X-100. After this procedure, proteins were denatured by boiling in Laemmli sample buffer containing 100 mM DTT, run on SDS-PAGE, transferred to nitrocellulose membranes and blotted with specific antibodies.

Antibodies used for immunoblotting and immunoprecipitation were anti-PEPCK (P-16) (sc-28477, goat polyclonal), anti-G6Pase (G-20) (sc-33839, goat polyclonal), anti-PGC-1α (H-300) (sc-13067, rabbit polyclonal), anti-HNF-4α (C-19) (sc-6556, goat polyclonal), anti-MKP-3 (F-12) (sc-377070, mouse monoclonal), anti-phospho-ERK1/2 (sc-16982, goat polyclonal), anti-β-actin (N-21) (sc-130656, rabbit polyclonal) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-phospho-FoxO1 (Ser^{256}) (no. 9461, rabbit polyclonal) and anti-FoxO1 (no. 2880, rabbit monoclonal) (Cell Signaling Technology, MA, USA). Chemiluminescence detection was performed with horseradish peroxidase-conjugated secondary antibodies (Thermo Scientific, Rockford, IL, USA). Visualization of protein bands was performed by exposure of membranes to RX-films. The original membrane was stripped and reblotted with β-actin as a loading control. Band

intensities were quantified by optical densitometry (Scion Image software, Scion Corp., Frederick, MD, USA).

Nuclear extraction

To characterize the expression and subcellular localization of FoxO1, a subcellular fractionation protocol was employed, as previously described (Prada *et al.* 2006). Liver fragments from mice of all groups (except DIO-sense) were minced and homogenized in 2 volumes of STE buffer (0.32 M sucrose, 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1 mM DTT, 100 mM sodium orthovanadate, 1 mM PMSF and 0.1 mg aprotinin ml^{-1}) at 4°C with a Polytron homogenizer. The homogenates were centrifuged (1000 *g*, 25 min, 4°C) and suspended in Triton buffer (1% Triton X-100, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 200 mM EDTA, 10 mM sodium orthovanadate, 1 mM PMSF, 100 mM NaF, 100 mM sodium pyrophosphate and 0.1 mg ml^{-1} aprotinin), kept on ice for 30 min, and centrifuged (15,000 *g*, 30 min, 4°C) to obtain the nuclear fraction. The samples (1 mg) were used for immunoprecipitation with FoxO1 antibody and Protein A–Sepharose 6MB (Amersham Pharmacia Biotech United Kingdom Ltd. (Buckinghamshire, UK)). After the addition of Laemmli buffer with 100 mM DTT, samples were heated in a boiling water bath for 5 min, and aliquots (100 μg of protein) were subjected to SDS-PAGE and Western blotting with anti-body against IRS-2 (anti-IRS-2); alternatively, anti-histone antibodies were used to demonstrate the efficiency of the method.

Immunohistochemistry

For immunohistochemistry analyses, six mice were anaesthetized with an intraperitoneal injection of ketamine (50 mg (kg body weight)$^{-1}$) and xylazine (20 mg (kg body weight)$^{-1}$). As soon as anaesthesia was assured by the loss of pedal and corneal reflexes, the chest was opened and a small incision made at the rear end of the left ventricle using iris scissors. Then a catheter was introduced into the left ventricle reaching the lumen of the aorta. With scissors, an opening was made in the right atrium and immediately an infusion pump was activated, where the catheter had been previously linked. Saline solution was perfused (100–200 ml) via the pump. The fluid and liver there seemed clearer, with no color paraformaldehyde (100–200 ml) was perfused to fix the liver and then samples were extracted. Due to blood loss during perfusion, the animals died. While this procedure occurred, the animals were always under anaesthesia effects.

Samples from liver obtained from mice were examined to determine the expression and tissue distribution of the MKP-3 protein. Hydrated 4 μm sections of paraformaldehyde-fixed, paraffin-embedded tissue were transferred and coated with poly-L-lysine and fixed in methanol for 10 min. The sections were washed twice in 0.01 M phosphate-buffered saline (PBS), pH 7.4 and incubated in PBS with 1% bovine serum albumin (BSA, w/v) for 30 min to block non-specific background. Upon BSA removal, the sections were treated with primary antibody to MKP-3 (Santa Cruz Biotechnology, Inc.) at a 1:100 dilution in PBS/BSA overnight at 4°C. After washing in PBS, slides were covered with a 1:200 dilution of fluorescein isothiocyanate (FITC)-conjugated secondary donkey anti-rabbit IgG (Santa Cruz Biotechnology, Inc.) in PBS for 120 min. The sections were subsequently washed in PBS and incubated for 10 min with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA), prepared according to the manufacturer's instructions. Analysis and documentation of results were performed using a Leica FW 4500 B microscope.

Statistical analysis

All numerical results are expressed as the means ± SEM of the indicated number of experiments. The results of blots are presented as direct comparisons of bands (according to the area and intensity) in autoradiographs and were quantified by optical densitometry. Statistical analysis was performed using the ANOVA test with the Bonferroni post test. Significance was established at the $P < 0.05$ level.

Results

Changes in the protein levels of MKP-3 in response to varying doses of an antisense oligonucleotide

MKP-3 levels in the livers of obese mice (DIO) were significantly increased (Fig. 3*A*) when compared with lean mice. Treatment of DIO mice for 5 days with an antisense oligonucleotide to MKP-3 produced a dose-dependent inhibition of MKP-3 expression in liver (Fig. 3*B*). Based on the dose–response experiment, we selected a twice daily dose of 2.0 nM for use in the remainder of the experiments. This dose induced a reduction of 91 ± 4% in MKP-3 expression after 5 days.

Effects of exercise training on physiological parameters

In our analysis of physiological parameters, we observed that the DIO group showed higher cumulative energy intake in 8 weeks, total body mass, epididymal fat, fasting glucose and fasting insulin levels compared to the lean group (Fig. 4*A*–*E*, respectively). The exercised mice showed a slight reduction in total body weight and epididymal fat mass, but without significant difference in comparison with the DIO mice (Fig. 4*B* and *C*, respectively). However, when compared with DIO mice,

exercised mice (DIO-EXE) had lower blood glucose levels (Fig. 4*D*) and fasting insulin (Fig. 4*E*). Food intake increased in exercised animals at the beginning of the exercise training protocol (first 3 weeks). After this period, food intake was normalized (data not shown). However, in the analysis of cumulative food intake during 8 weeks, the DIO-EXE group was found to consume similar amounts of energy compared to the DIO group (Fig. 4*A*).

Next, we examined the glucose reduction during the ITT. The DIO mice presented lower glucose reductions compared to the lean mice (Fig. 4*F*). However, swimming exercise training partially restored the insulin sensitivity; in fact, their insulin sensitivity values were higher than those of the DIO mice and lower than those of lean mice. The group treated with the antisense oligonucleotide to MKP-3 (DIO-ASO) had physiological parameters similar to those found in the exercised group (Fig. 4*A*–*F*).

Additionally, we evaluated the combined effect of training with MKP-3 antisense oligonucleotide treatment on these physiological parameters. There were no additive effects of exercise training with the antisense oligonucleotide treatment (DIO-EXE-ASO; Fig. 4*A*–*F*).

Exercise training reduces MKP-3 protein levels and disrupts the association between MKP-3 and FoxO1

Thirty-six hours after the last session of exercise training, the MKP-3 protein levels and the association between the MKP-3 and FoxO1 proteins were assessed. MKP-3 protein levels in the livers of exercised mice (DIO-EXE) were significantly decreased when compared with those in the DIO mice. Treatment of DIO mice with an antisense oligonucleotide to MKP-3 produced a decrease in MKP-3 protein levels similar to that observed in lean animals (Fig. 5*A*).

Increases in the association of FoxO1 and MKP-3 were found in DIO mice compared with the lean group (Fig. 5*B*). Interestingly, exercise training decreased the association between the FoxO1 and MKP-3 proteins. Treatment with the antisense oligonucleotide targeting MKP-3 (DIO-ASO) significantly reduced the association of FoxO1 with MKP-3 compared with that observed in the DIO mice, with values similar to those in the lean group (Fig. 5*B*, upper panel). The FoxO1 protein levels did not differ between groups (Fig. 5*B*, lower panel).

In addition, the mice were injected (I.V.) with insulin, and liver tissue samples were taken to estimate FoxO1 phosphorylation and nuclear FoxO1 content (Fig. 5*C* and *D*, respectively). Phosphorylation of FoxO1 induced by insulin was reduced in the livers of obese mice (DIO) compared to the lean group (Lean). Swimming exercise training significantly increased phosphorylated (p)FoxO1 in the liver compared to that in DIO mice. Treatment with antisense oligonucleotide targeting MKP-3 increased insulin-induced phosphorylation of FoxO1 to levels similar to those observed in the lean mice (Fig. 5*C*, upper panel). The protein levels of FoxO1 showed no difference between the groups (Fig. 5*C*, lower panel).

The results regarding FoxO1 protein levels in the nucleus of the liver cells are presented in Fig. 5*D*. DIO mice had a higher amount of insulin-induced FoxO1 nuclear translocation than lean mice. Exercise training partially reversed this effect. In response to treatment with the antisense oligonucleotide to MKP-3 (DIO-ASO), there was a significant reduction in the nuclear content of insulin-induced FoxO1 in hepatocytes compared to that in the DIO and DIO-EXE groups. There was no additive effect in the group of animals treated with ASO and exercised concomitantly (Fig. 5*A*–*D*).

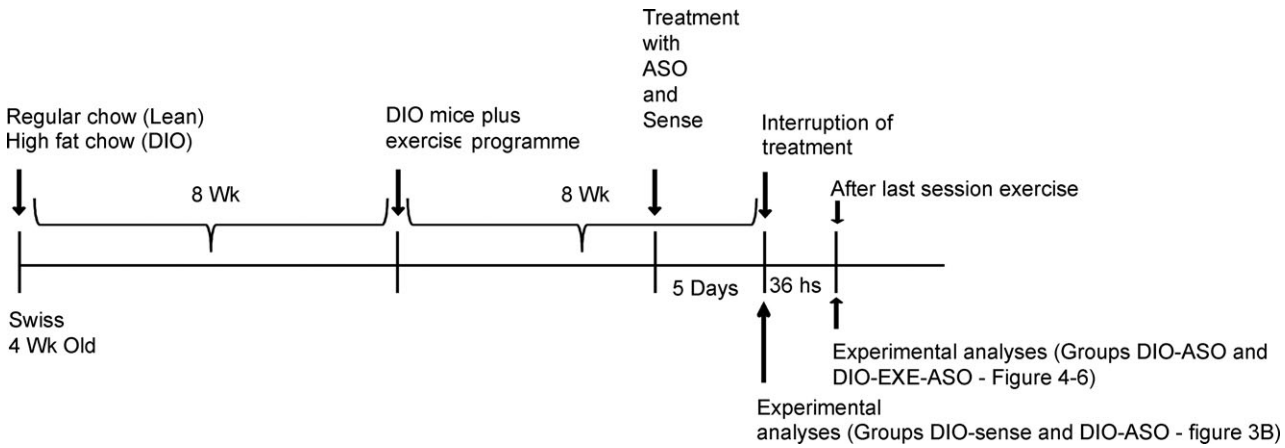

Figure 1. Treatment with ASO
Schematic diagram of treatment with the antisense oligonucleotide targeting MKP-3.

Exercise training reduces the levels of PGC-1α, HNF-4α and gluconeogenic enzymes

In this study, we also evaluated the effect of exercise training on the levels of proteins involved in the transcription of gluconeogenic genes. Obese animals had increased PGC-1α protein levels in their livers compared to lean animals. Training reduced the hepatic expression of PGC-1α compared with that observed in DIO animals. The decrease of the PGC-1α content in the liver of the DIO-EXE group was only partial, once the levels were higher than those in the lean group. A similar effect was observed in the DIO-ASO group (Fig. 6*A*).

The obese mice had a significant increase in HNF-4α protein levels when compared to the control group. Exercise training reduced the content of HNF-4α compared to that observed in sedentary obese animals. The HNF-4α levels observed in exercised animals were higher than those in the lean group. The animals in the DIO-ASO group showed no change in the expression of HNF-4α, and the values were similar to those obtained in obese mice (DIO) (Fig. 6*B*).

We analysed the expression of gluconeogenic enzymes in the hepatic tissue and observed that in obese mice (DIO) the expression of *PEPCK* and *G6Pase* were significantly increased when compared with the lean mice (Fig. 6*C* and *D*, respectively). *PEPCK* and *G6Pase* levels in the livers of DIO-EXE mice were reduced compared to those observed in DIO mice. The levels of *G6Pase* and *PEPCK* in the livers of the group treated with the antisense oligonucleotide to MKP-3 were similar to those observed in the DIO-EXE group (Fig. 6*C* and *D*, respectively). There was no additive effect in the group of animals treated with ASO and exercised concomitantly (Fig. 6*C* and *D*).

Finally, in order to determine the role of MKP-3 in modulating gluconeogenesis *in vivo*, we also examined the blood glucose levels in mice following intraperitoneal injection of pyruvate in a separate set of experiments (Fig. 6*E*). As shown in Fig. 6*E* and *F*, the glycaemic curve and the area under the curve (AUC) were higher in the DIO mice than in the other groups. Additionally, the pyruvate tolerance test showed that DIO mice had a pronounced increase in blood glucose until 90 min after injection. After this time, there was a slight decline in blood glucose (Fig. 6*E*). However, the glucose curves for DIO-EXE, DIO-ASO and DIO-EXE-ASO mice were similar, showing an increase in glucose levels until 60 min after injection and then a decline in values until the end of the test.

Effects of exercise training on ERK1/2 phosphorylation

In this study, we determined the effect of exercise training on the phosphorylation of ERK1/2 in the livers of obese mice. The DIO mice increased the phosphorylation of ERK1/2 compared to the lean group. There was a significant reduction in the phosphorylation of ERK1/2 in the EXE group 36 h after the last exercise training session compared to that observed in DIO mice (Fig. 7*A*). These results were accompanied by reduced levels of MKP-3 in the livers of exercised mice (Fig. 7*B*).

Next, we performed immunohistochemical analyses to evaluate the localization of MKP-3 in obese mice (Fig. 7*C*–*E*). Immunohistochemistry with an MKP-3-specific antibody revealed that obesity increased

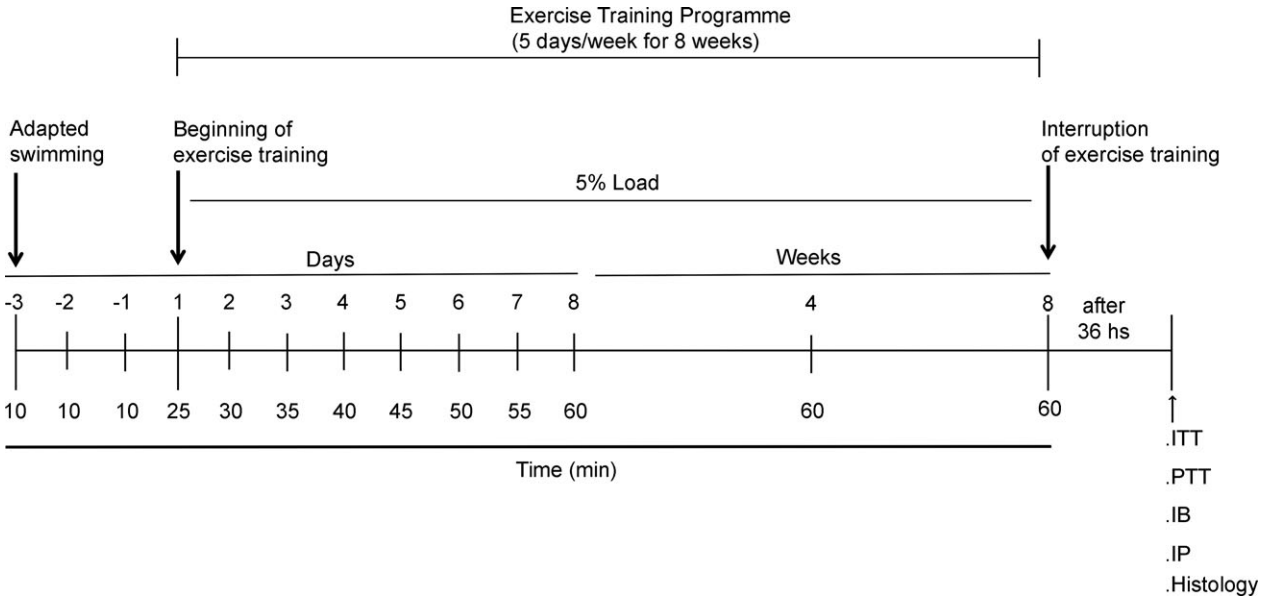

Figure 2. Schematic diagram of exercise training programme

the MKP-3 content and that exercise training was able to decrease the MKP-3 protein levels (Fig. 7*E*).

Discussion

Insulin resistance plays a central role in metabolic control because the defects in intracellular insulin signalling in the liver impair adequate suppression of gluconeogenesis, leading to increased glucose in both the fed and fasting states. Inactivation of FoxO1 is the major mechanism by which insulin represses gluconeogenesis. However, MKP-3-mediated dephosphorylation of FoxO1 at Ser^{256} promotes its nuclear translocation and subsequent recruitment to the promoters of key gluconeogenic genes (Wu *et al.* 2010; Jiao *et al.* 2012). Here, we showed that expression of MKP-3 is markedly increased in the livers of diet-induced obese mice. However, our data demonstrated that exercise training was sufficient to reduce the MKP-3 protein levels in liver. In parallel, we observed that exercised mice showed reductions in the interaction between MKP-3 and FoxO1, improving the action of insulin in liver and reducing hyperglycaemia. We also determined that training reduces hepatic MKP-3 protein levels and this may be related to ERK1/2. These data are important because the inhibition of hepatic gluconeogenesis is a potential target therapy to combat diabetic hyperglycaemia. Thus, our study provides substantial evidence that exercise training can help the hepatic insulin signalling restoration in liver and, therefore, it is important to counteract the disturbance of glucose homeostasis induced by obesity, including changes in hepatic MKP-3 protein levels.

MKP-3 is a dual-specificity phosphatase that plays an important role in glucose metabolism (Jiao *et al.* 2012). The expression of this protein is elevated in both genetically (*ob*/*ob*) and diet-induced obese mice (Wu *et al.* 2010). Previous studies indicate that MKP-3 most likely increases transcription of gluconeogenic gene expression through activation of FoxO1 (Wu *et al.* 2010; Jiao *et al.* 2012). Interestingly, the reduction of MKP-3 expression in experimental obesity models alleviated hyperglycaemia (Wu *et al.* 2010). Therefore, the elevation of hepatic MKP-3 expression in the insulin-resistant state may contribute to the inappropriate activation of gluconeogenesis in type 2 diabetes. *In vitro* experiments and immunoblot analyses have demonstrated that MKP-3 dephosphorylates FoxO1, preventing its phosphorylation and translocation from the nucleus to the cytoplasm after stimulation with insulin (Wu *et al.* 2010). Moreover, the overexpression of MKP-3 resulted in insulin resistance and increased hepatic glucose production (Wu *et al.* 2010). Conversely, in the same study, the authors demonstrated that lean mice with reduced hepatic MKP-3 expression had a significantly decreased gluconeogenic capacity in response to a challenge with a gluconeogenic precursor (pyruvate).

Reduction of MKP-3 expression in the livers of obese mice (MKP-3 knockdown in DIO mice) decreased blood glucose levels in both fed and fasted states without affecting body weight (Wu *et al.* 2010). Plasma insulin levels were lower in DIO mice with reduced hepatic MKP-3 expression. Additionally, DIO mice with reduced hepatic MKP-3 expression had an impaired response to a pyruvate challenge (Wu *et al.* 2010). Corroborating with these

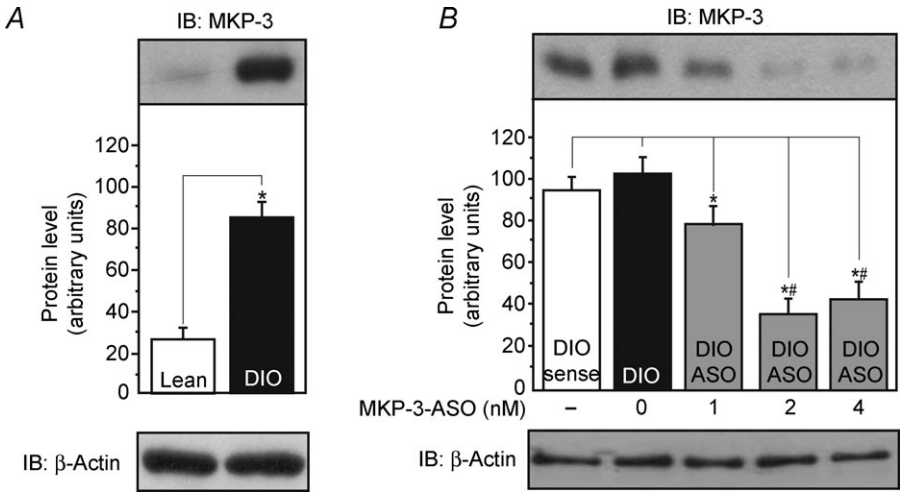

Figure 3. MKP-3 protein levels in the livers of obese mice treated with various doses of the antisense oligonucleotide targeting MKP-3
A, hepatic MKP-3 protein levels in lean and diet-obesity mice (DIO, $n = 5$). *B*, MKP-3 inhibition assay was assessed in a dose–response experiment with sense and antisense oligonucleotides to MKP-3 ($n = 5$). Representative blots are shown. Data were expressed as the means ± SEM of five mice. $^*P < 0.05$ *vs.* the lean group (chow) and $\#P < 0.05$ *vs.* the obese group (DIO).

genetic strategies, our data demonstrated that swimming training was sufficient to reduce MKP-3 protein levels and the FoxO1/MKP-3 interaction in the hepatic tissue of obese rodents. It is important to note that exercise training reduced MKP-3 protein levels and partially restored the insulin action without changing the body weight or adiposity. The favourable effects of exercise training on insulin signalling pathways have also been demonstrated in previous studies (Ropelle *et al.* 2009; De Souza *et al.* 2010; da Luz *et al.* 2011; Oliveira *et al.* 2011; Marinho *et al.* 2012; de Moura *et al.* 2013); however, the molecular mechanisms responsible for the improvement in insulin sensitivity in response to exercise training are not yet fully known. Here, we demonstrated that exercise training can restore insulin signalling in liver and this seems to be related to the MKP-3 expression reduction. In this respect, future studies in the presence of MKP-3 (overexpression) can help to establish the necessity of MKP-3 for exercise training effects.

To explore the molecular mechanism underlying the suppression of gluconeogenesis by exercise training, we also examined the protein levels of PGC-1α, a key gluconeogenic transcription coactivator, in the liver. Under normal, *ad libitum*-fed conditions, the expression of PGC-1α is relatively low in the liver compared with other tissues that rely on aerobic metabolism for ATP production (Puigserver & Spiegelman, 2003). Conversely, the expression of PGC-1α and hepatic glucose output are both elevated in several models of diabetes and insulin signalling deficiency (De Souza *et al.* 2010). Systemic glucose tolerance and insulin sensitivity have

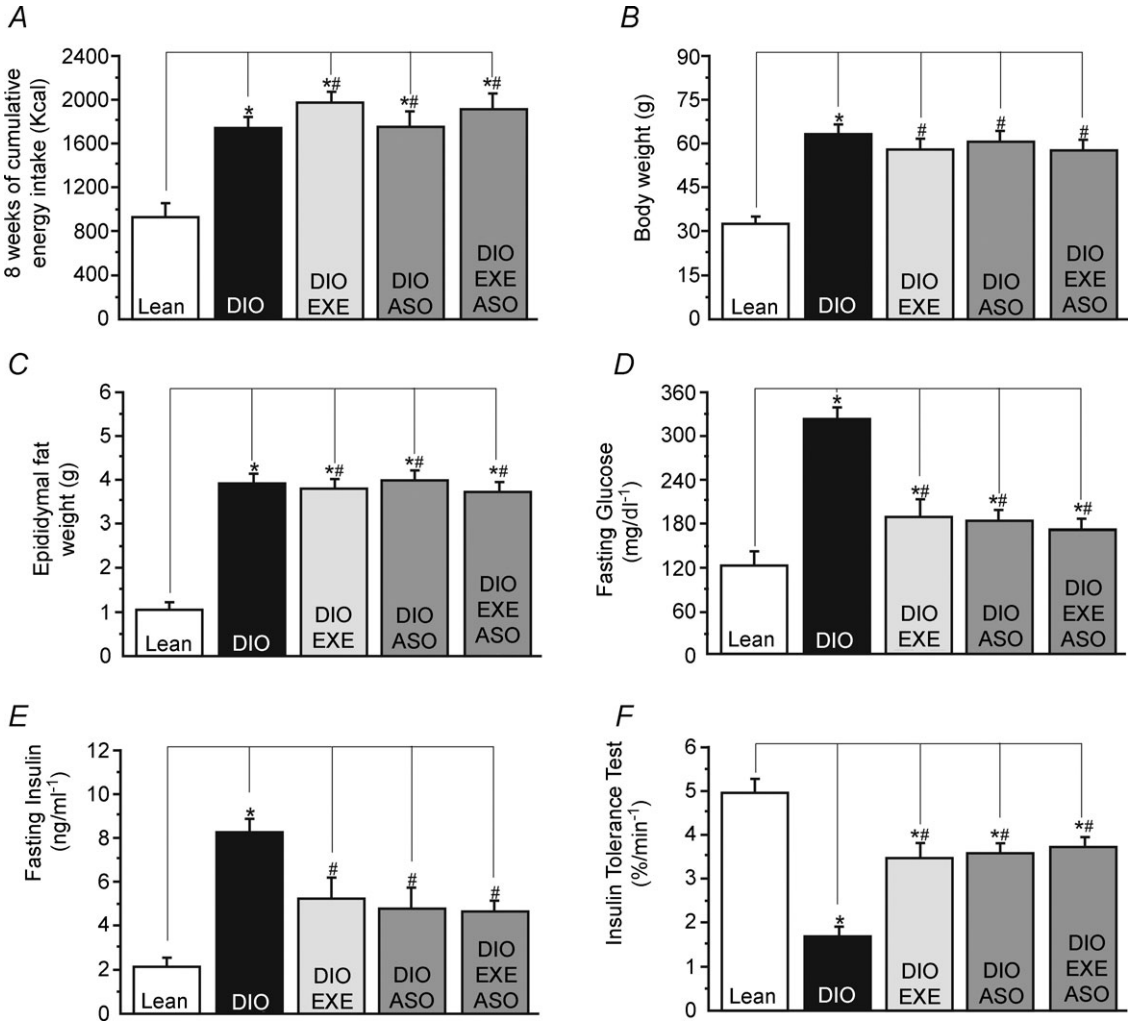

Figure 4. Physiological parameters of groups of mice used in the study
Lean, lean mice; DIO, obese mice; DIO-EXE, exercised obese mice; DIO-ASO, obese mice treated with the antisense oligonucleotide targeting MKP-3; DIO-EXE-ASO, obese mice exercised and treated with the antisense oligonucleotide targeting MKP-3 concomitantly. *A*, cumulative caloric intake ($n = 8$); *B*, body mass ($n = 8$); *C*, epididymal fat ($n = 8$); *D*, fasting glucose ($n = 8$); *E*, fasting insulin ($n = 8$); *F*, insulin tolerance test (ITT) ($n = 8$). Bars represent the means ± SEM of eight mice. $^*P < 0.05$ *vs.* the lean group (chow) and $\#P < 0.05$ *vs.* the obese group (DIO).

been characterized in PGC-1α-deficient mice; in fact, the liver-specific overexpression and knockdown strategies indicate that PGC-1α, when activated, drives hepatic glucose production (Yoon *et al.* 2001; Lin *et al.* 2003). Additionally, FoxO1 acts as a transcription factor in various cell types. Specifically in the liver, this molecule is responsible for increased transcription of PGC-1α, which is an important amplifier of hepatic gluconeogenesis (Puigserver & Spiegelman, 2003).

In an elegant study, Wu and colleagues showed that PGC-1α mRNA levels were markedly increased by MKP-3 overexpression, and this induction was prevented when cells were transduced with a PGC-1α short hairpin (sh)RNA adenovirus (Wu *et al.* 2010). Moreover, the increases in *PEPCK* and *G6Pase* mRNA levels induced by MKP-3 overexpression were completely abolished when PGC-1α was knocked down. In addition, PGC-1α knockdown completely abolished the MKP-3-induced increase in glucose output in rat primary hepatocytes. These results indicate that MKP-3-induced gluconeogenesis requires PGC-1α. Previously, we demonstrated that exercise training improves

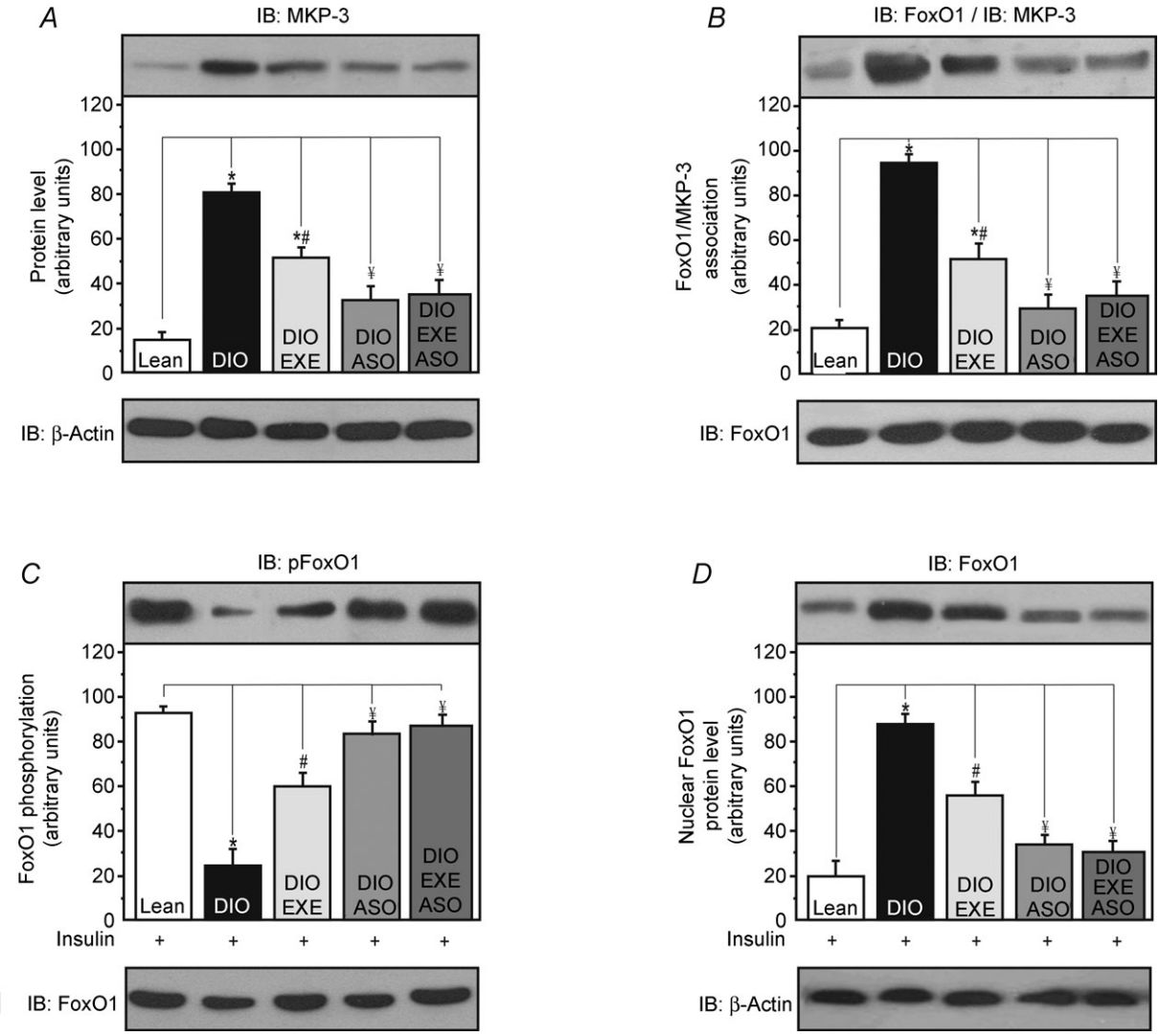

Figure 5. Protein levels of FoxO1 and FoxO1/MKP-3 association in the livers of groups of mice used in this study

Abbreviations as in Fig. 4. *A*, liver samples were immunoblotted (IB) with an anti-MKP-3 antibody and an anti-β-actin antibody ($n = 5$, upper and lower panels, respectively); *B*, immunoprecipitation (IP) and immunoblot (IB) assays were performed to evaluate the FoxO1/MKP-3 association and FoxO1 protein levels in hepatic samples from various groups of mice ($n = 5$, upper and lower panels, respectively); *C*, liver samples were immunoblotted (IB) with anti-phospho-FoxO1 and anti-FoxO1 antibodies ($n = 5$, upper and lower panels, respectively); *D*, nuclear extraction assay was performed to evaluate FoxO1 protein levels in hepatic samples from various groups of animals ($n = 5$). Data were expressed as the means ± SEM of five mice. $^*P < 0.05$ *vs.* the lean group (chow), $\#P < 0.05$ *vs.* the obese group (DIO) and $¥P < 0.05$ *vs.* the exercised obese (DIO-EXE).

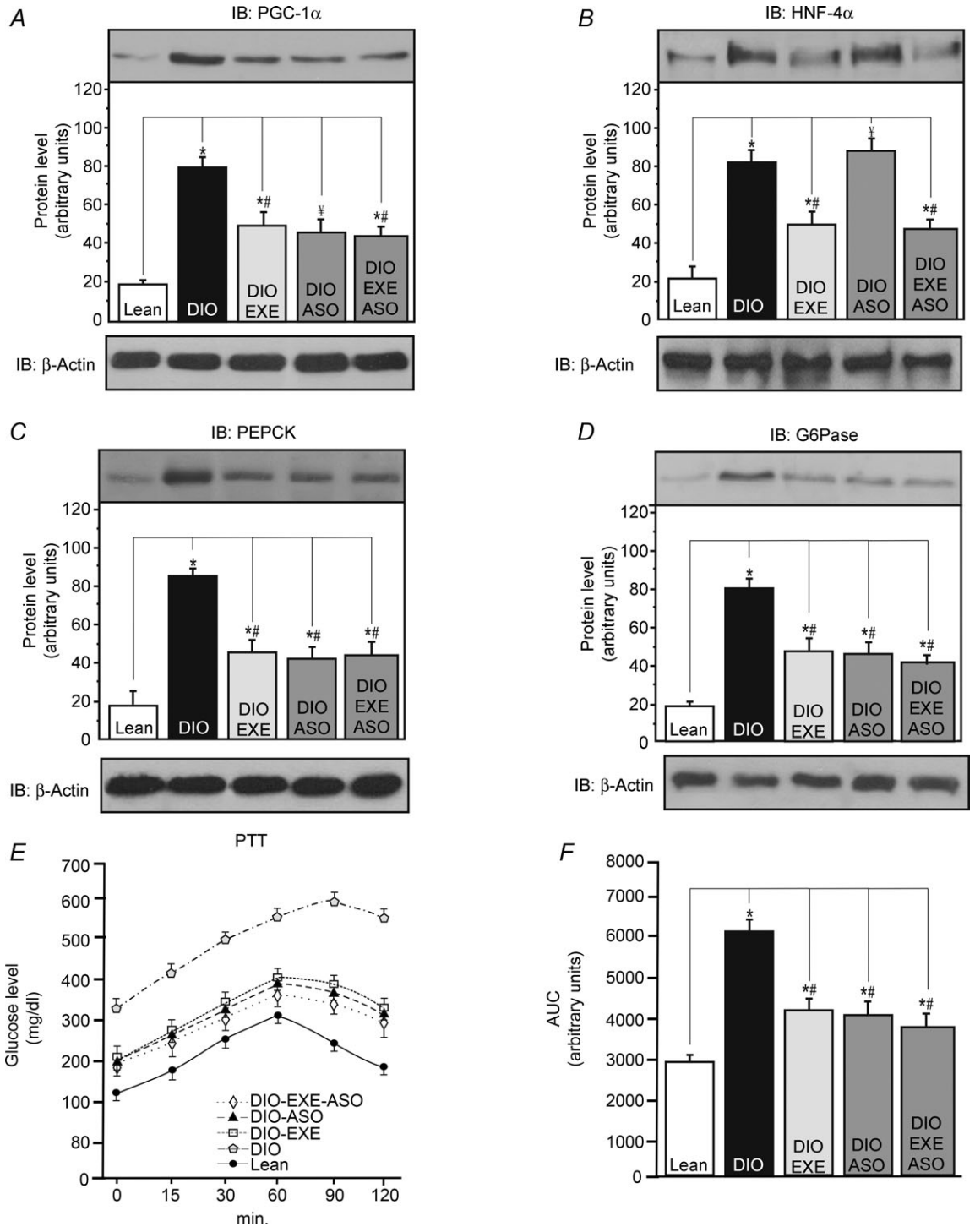

Figure 6. Exercise training reduces the levels of PGC-1α, HNF-4α and gluconeogenic enzymes in the liver of obese mice

Proteins levels of PGC-1α, HNF-4α, *PEPCK* and *G6Pase* in hepatic tissues from the different study groups were evaluated by immunoblot (IB). *A*, samples of livers were immunoblotted (IB) with antibodies to PGC-1α and β-actin ($n = 5$, upper and lower panels, respectively). *B*, liver samples were immunoblotted (IB) with antibodies to HNF-4α and β-actin ($n = 5$, upper and lower panels, respectively). *C*, liver samples were immunoblotted (IB) with antibodies to *PEPCK* and β-actin ($n = 5$, upper and lower panels, respectively). *D*, liver samples were immunoblotted (IB) with antibodies to *G6Pase* and β-actin ($n = 5$, upper and lower panels, respectively). Blood glucose levels during the pyruvate tolerance test (PTT, $n = 8$; *E*) and area under curve (AUC; *F*). Data were expressed as the means ± SEM of five or eight mice. $^*P < 0.05$ *vs.* the lean group (chow) and $\#P < 0.05$ *vs*. the obese group (DIO).

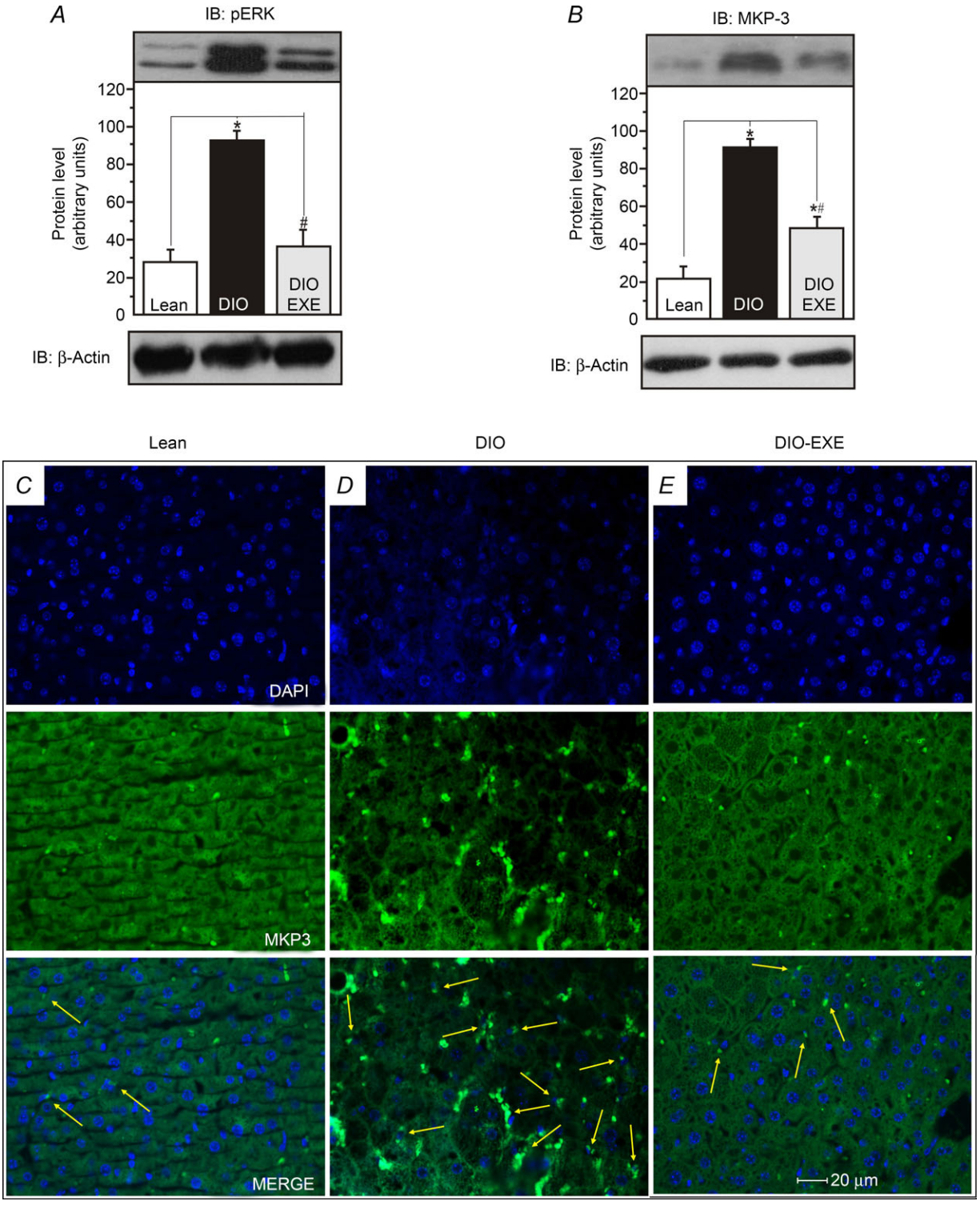

Figure 7. Exercise training reduces hepatic ERK phosphorylation and MKP-3 protein levels in obese mice

A, liver samples were immunoblotted (IB) with antibodies to pERK and β-actin ($n = 5$, upper and lower panels, respectively). *B*, liver samples were immunoblotted (IB) with antibodies to MKP-3 and β-actin ($n = 5$, upper and lower panels, respectively). *C–E*, confocal microscopy was performed to evaluate the localization of MKP-3 (green) in the livers of lean mice (*C*), obese mice (*D*) and exercised mice (*E*), with $\times 200$ magnification (scale bar, 20 μm). Yellow arrows indicate MKP-3-positive cells. Data were expressed as the means ± SEM of five mice. $^*P < 0.05$ *vs.* the lean group (chow) and $\#P < 0.05$ *vs.* the obese group (DIO).

insulin signalling and reduces PGC-1α protein levels in the livers of obese mice (Ropelle *et al.* 2009; Marinho *et al.* 2012). In the present study, we demonstrated that PGC-1α protein levels are decreased in livers of obese mice subjected to an exercise training protocol or to treatment with an antisense oligonucleotide targeting MKP-3.

Decreases in the levels of PGC-1α in the liver can also reduce the expression of HNF-4α and the transcription of *PEPCK* and *G6Pase*. A study showed that HNF-4α has an important role in regulating gluconeogenesis and glycolysis in response to fasting and feeding (Hirota *et al.* 2008). It is currently recognized that FoxO1 plays a key role in switching between glycolysis and gluconeogenesis, and HNF-4α is an indispensable component of this switching mechanism. Here, we demonstrated that HNF-4α protein levels in liver were increased significantly in DIO mice compared to lean mice. It was observed that the exercised mice, but not obese rodents, treated with an antisense oligonucleotide to MKP-3 demonstrated a significant reduction in the levels of HNF-4α compared to DIO mice. However, our results showed that exercise training could only induce a partial reduction in obesity-induced increase in HNF-4α protein levels while ASO treatment alone had no effect. These findings could explain, at least in part, why additive effects of exercise training treatment and ASO treatment were not observed in the present study. In this regard, it is known that HNF-4α is required for the proper control of the expression of many genes, especially in the liver (e.g. *FoxO1*, *C/EBP*, *PPARα*, *LRX*, *COUP*), and is associated with a number of critical metabolic pathways (e.g. glycolysis, gluconeogenesis, ureagenesis, fatty acid metabolism, bile acid synthesis, apolipoproteins and hepatocyte differentiation; Gonzalez, 2008). Training induces changes in these metabolic processes that may partially explain the reduction in the levels of HNF-4α found in the exercised animals, but not in mice treated with oligonucleotide antisense. HNF-4α also regulates the constitutive expression of target genes encoding enzymes, transporters and even other nuclear receptors, and accounts for, in large part, the liver-specific expression of these genes (Gonzalez, 2008). Additionally, it should be noted that many other HNF-4α target genes are not co-activated by PGC-1α (Rhee *et al.* 2003; Hirota *et al.* 2008). The mechanism underlying this specificity is not known, but it may be due to the presence of other specific sequences near the HNF-4α binding site.

In addition to HNF-4α, PGC-1α also regulates a number of other transcription factors such as FoxO1, glucocorticoid receptor, PPARα and PPARγ (peroxisome proliferator-activated receptors) in liver and other tissues and is a critical component of other important metabolic consequences of the fasting response including fatty acid β-oxidation and ketogenesis. However, unlike gluconeogenesis, these processes do not depend on the HNF-4α (Rhee *et al.* 2003). In a interesting investigation, Rhee and colleagues demonstrated that the presence of HNF-4α is absolutely required for PGC-1α-induced *PEPCK* and *G6Pase* gene expression (Rhee *et al.* 2003). The results obtained in our study show that obese animals presented significant increases in the protein level of PGC-1α and HNF-4α and these responses were accompanied by increases in *PEPCK* and *G6Pase* levels in liver. These results are consistent with other studies that examined mice with severe insulin resistance (Lima *et al.* 2009; Wu *et al.* 2010). In the same vein, Heled and colleagues showed that exercise enhances hepatic insulin signalling and inhibits the activity of *PEPCK* in animals prone to diabetes (*Psammomys obesus*, also known as desert rats; Heled *et al.* 2004). According to these data, the results obtained in the present study regarding the pyruvate tolerance test and analysis of protein involved in gluconeogenesis suggest increased insulin hepatic sensitivity in exercised mice compared with DIO mice. In this regard, it is notable that the effects of exercise training were similar to those found with treatment with antisense oligonucleotide. In light of these findings, it seems evident that exercise training plays an important role in inhibiting gluconeogenesis in obese rodents and has effects on FoxO1, PGC-1α and HNF-4α in the liver.

Furthermore, our findings suggest that the reduction in the expression of MKP-3 after exercise training may be related to ERK. Both ERK-1 and ERK-2 are part of the mitogen-activated protein kinase (MAPK) subfamily. Activation of the MAPK signalling pathway is a known mechanism that may induce insulin resistance through increases in Insulin Substrate Receptor 1 Ser^{307} phosphorylation. A previous study by our research group showed that obese mice have increased phosphorylation of ERK1/2, which may contribute to the induction of insulin resistance in peripheral tissues (Oliveira *et al.* 2011). MKP-3 specifically inactivates ERK, and in turn, ERK causes the activation of MKP-3. It has been observed that the interaction between ERK and MKP-3 occurs physically, allowing a reciprocal cross-regulation of the activity of these proteins (Fjeld *et al.* 2000). In our study, we observed that the levels of ERK phosphorylation were higher in obese mice compared to those in lean mice. Interestingly, exercise training significantly reduced the phosphorylation of ERK, and this was accompanied by reduced expression of MKP-3 (as shown by immunoblot and immunohistochemical analyses) and reduced association between FoxO1 and MKP-3. Conversely, other studies in the literature have observed that MAPK activity was increased during exhaustive acute exercise (Ryder *et al.* 2000; Chambers *et al.* 2009), but the exercise protocol and the evaluated tissue type may explain these differences. Furthermore, the exercise-induced increase in MAPK phosphorylation rapidly decreases upon the cessation of exercise and is completely restored to resting levels at 60 min after exercise (Widegren *et al.* 1998). Oliveira

and colleagues also found a significant decrease in the ERK protein levels in the liver of obese Wistar rats with a swimming exercise protocol equivalent to that used in the present study (Oliveira *et al.* 2011). The decline in ERK phosphorylation was observed 24 and 36 h after the last exercise training session.

In conclusion, the results of this study demonstrated that exercise training markedly decreased both MKP-3 protein levels and the association between FoxO1 and MKP-3 in the livers of obese mice. In addition, exercised obese mice had higher levels of phosphorylated FoxO1 and lower levels of PGC-1α and HNF-4α proteins, with consequent reductions in the expression of gluconeogenic enzymes (*PEPCK* and *G6Pase*). These findings were accompanied by physiological changes, including increased insulin sensitivity and reduced hyperglycaemia in exercised obese mice, with similar results to those obtained with oligonucleotide antisense treatment. However, additive effects were not observed in concomitant intervention (i.e. exercise training plus oligonucleotide antisense treatment). Finally, the suppressive effects of exercise training on MKP-3 protein levels may be related to the decreased phosphorylation of ERK in the livers of obese mice.

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Additional information

Competing interests

The authors declare that there are no conflicts of interest regarding the present study.

Author contributions

L.S.S.P., J.R.P. and E.R.R. had the overall responsibilities of the experiment design, data collect and statistical analysis, and wrote the manuscript. E.C.C.R., C.T.deS., D.E.C., A.S.R.daS., B.deA.R., L.P.deM., R.M., V.deO. and C.K.; The experiments were conducted at School of Applied Science, University of Campinas (UNICAMP), Brazil; Laboratory of Exercise Biochemistry and Physiology, Health Science Unit, Universidade do Extremo Sul Catarinense, Brazil, and São Paulo University State (UNESP), Brazil. All the authors have read and approved the final manuscript.

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