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Title: The increase in fiber size in male rat gastrocnemius after chronic central leptin infusion is related to activation of insulin signaling

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Abbreviation list: Acc β , acetyl-CoA carboxylase beta; AMPK, AMP-activated protein kinase; Cpt1b, carnitine palmitoyltransferase 1b; CREB, cyclic AMP response element-binding protein; CSA, cross-sectional area; DU, densitometry units; ERK, extracellular signal-regulated kinase; Fasn, fatty acid synthase; GLUT4, glucose transporter 4; GSK3 β , glycogen synthase kinase 3 β ; IL, interleukin; IR β , insulin receptor beta chain; MFI, median fluorescent intensity; mTOR, mechanistic target of rapamycin; Myog, myogenin; NEFA, non-esterified fatty acid; PCNA, proliferating cell nuclear antigen; PEPCK, phosphoenolpyruvate carboxykinase; PI3K, phosphatidylinositol 3-kinase;

PTEN, phosphatase and tensin homolog on chromosome 10; p70S6K1, 70 kDa ribosomal protein S6 kinase 1; Rpl13a, ribosomal protein L13a; Rps18, ribosomal protein S18; Ucp2, uncoupling protein 2.

Abstract

Insulin potentiates leptin effects on muscle accrual and glucose homeostasis. However, the relationship between leptin's central effects on peripheral insulin sensitivity and the associated structural changes remain unclear. We hypothesized that central leptin infusion modifies muscle size through activation of insulin signaling. Muscle insulin signaling, enzymes of fatty acid metabolism, mitochondrial respiratory chain complexes, proliferating cell nuclear antigen (PCNA) and fiber area were analyzed in the gastrocnemius of chronic central infused (L), pair-fed (PF) and control rats. PCNA-positive nuclei, fiber area, GLUT4 and glycogen levels and activation of Akt and mechanistic target of rapamycin were increased in L, with no changes in PF. Acetyl-CoA carboxylase- β mRNA levels and non-esterified fatty acid and triglyceride content were reduced and carnitine palmitoyltransferase-1b expression and mitochondrial complexes augmented in L. These results suggest that leptin promotes an increase in muscle size associated with improved insulin signaling favored by lipid profile.

Keywords

Gastrocnemius; insulin signaling; leptin; mitochondrial respiratory chain; proliferating cell nuclear antigen

1. Introduction

Skeletal muscle represents the largest organ of the body in non-obese individuals and quantitatively is the most important target tissue for insulin in glucose metabolism. Insulin stimulation leads to the activation of phosphatidylinositol 3-kinase (PI3K), increasing glucose uptake (Dugani and Klip, 2005). An increase in insulin-stimulated glucose uptake generates a gain in skeletal muscle mass, whereas a close relationship between insulin resistance, abnormal glucose uptake and muscle atrophy has been reported (Xu et al., 2015, Rudrappa et al., 2016).

The leptin and insulin-signaling pathways are linked at several levels, such as for example insulin receptor substrates (IRS) and PI3K (Xu et al., 2005). Central leptin infusion has acute effects on the activation of insulin signaling in the soleus muscle, while PI3K inhibitors block the ability of leptin to activate Akt (Roman et al., 2010). Leptin also controls muscle glucose handling, increasing glucose turnover and uptake (Yaspelkis et al., 1999), although these effects seem to require the presence of insulin (Zierath et al., 1998).

Insulin resistance progresses together with an increase in fat mass and leptin resistance. Studies performed in the soleus muscle in aging rats showed that insulin resistance is reverted after central leptin infusion, promoting glucose uptake (De Solís et al., 2012). Additionally, leptin increases fatty acid oxidation and decreases triglyceride storage in muscle, which may partially explain the insulin-sensitizing effect of this adipokine, whereas development of leptin resistance precedes the increase in lipid uptake and accumulation that leads to insulin resistance (Dyck, 2009).

Evidence from several experimental models also implicates leptin in the regulation of skeletal muscle mass. Serum leptin concentrations were shown to positively correlate with fat-free mass compartments (Fernández-Real et al., 2000), while lower muscle mass has been linked to abnormal leptin bioavailability (Tam et al., 2016). Moreover, leptin deficiency as well as the absence of functional leptin receptors are also associated with altered myoblast rise (Arounleut et al., 2013), whereas

peripheral administration of leptin decreases atrophy-related genes (Rodríguez et al., 2015).

We hypothesized that chronic central leptin infusion would induce an increase in muscle mass due to an augment in proliferating cell nuclear antigen (PCNA) and fiber size mass. In this regard, most studies analyzing the insulin-sensitizing effects of adipokines have been performed in soleus muscle. However, no broad characterization of these effects, including the possible associated changes in muscle accrual, has ever been conducted in gastrocnemius muscle. Thus, the aim of this study was to characterize the changes in PI3K signaling mechanisms after central leptin administration and its relationship with an increase in glucose content and glycogen synthesis in gastrocnemius muscle tissue. Moreover, we determined the modifications in the expression of enzymes involved in fatty acid synthesis and oxidation, given that lipid content is inversely related to insulin sensitivity. Finally, we analyzed whether activation of the Akt/mechanistic target of rapamycin (mTOR) signaling pathway in response to leptin infusion is associated with an increase in gastrocnemius muscle accrual.

2. Material and methods

2.1. Animals

Eighteen adult male Wistar rats (250 ± 10 g) were individually caged with a 12-h light/dark cycle and fed standard chow and water *ad libitum*. After an overnight fast, rats were anesthetized (0.02 ml of ketamine/100 g wt and 0.04 ml of xylazine/100 g wt) and positioned in a stereotaxic apparatus as previously reported (Burgos-Ramos et al., 2015). A cannula attached to an osmotic minipump (Alzet, Durect Corp., Cupertino, CA, USA) containing either saline or leptin was implanted into the right cerebral ventricle (-0.3 mm anteroposterior, 1.1 mm lateral from Bregma). Leptin was dissolved in saline plus 1% BSA. Rats were treated icv for 14 days with either saline with 1% BSA or leptin (12 µg/day). To discriminate the inhibitory effect of leptin on appetite, we

included a pair-fed group that received the same amount of food consumed by the leptin-treated group the day before. Food intake and body weight were measured daily. This resulted in the following groups: chronic saline with 1% BSA (control, C), pair-fed rats with chronic saline with 1% BSA (PF) and chronic leptin (L). Rats were sacrificed by decapitation at 8.00 h after a 12 h fasting period and the gastrocnemius muscle was isolated, weighed and immediately processed. Inguinal and epididymal fat pads were also isolated and weighed. Trunk blood was collected and centrifuged at 1,800 g for 10 min at 4 °C and serum was collected and stored at -80°C until serum determinations.

This study was approved by the Animal Care Committee of the University of Alcalá de Henares and complied with the Spanish RD 53/2013 pertaining to the protection of experimental animals and with the European Communities Council Directive (2010/63/EU).

2.2. Preparation of tissue lysates

Gastrocnemius muscle, including red and white portions, was homogenized on ice in lysis buffer pH 7.60 containing 10 mmol/L EDTA, 50 mmol/L sodium chloride; 1% Triton X-100, 2 mmol/L phenylmethylsulfonyl fluoride supplemented with a protease inhibitor cocktail (Complete™ Mini-EDTA free, Roche Molecular Biochemicals, Mannheim, Germany) and phosphate inhibitors (50 mmol/l sodium pyrophosphate, 0.1 mol/L sodium fluoride and 10 mmol/L sodium ortovanadate). The lysates were incubated overnight at -70°C and then centrifuged at 12,000 g for 10 min at 4°C. The supernatant was stored at -80°C until assayed. Protein concentration was determined by the method of Bradford (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.3. Western blotting

Blots were incubated with antibodies against phosphorylated and total AMP-activated protein kinase (AMPK) from Cell Signaling Technology (Danvers, MA, USA), glucose transporter 4 (GLUT4) from Millipore (Temecula, CA, USA) and the beta chain

of the insulin receptor (IR β) and phosphoenolpyruvate carboxykinase (PEPCK) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The proteins were detected by chemiluminiscence using an ECL system. Quantification of the bands was carried-out by densitometry using an ImageQuant LAS 4000 mini (GE Healthcare Life Sciences, Buckinghamshire, UK). Proteins were normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Thermo Scientific, Fremont, CA, USA).

2.4. RNA purification and real-time PCR analysis

RNA was extracted from 100 mg of gastrocnemius tissue according to the Tri-Reagent protocol (Chomczynski, 1993). The reverse transcription reaction was carried out on 2 μ g of RNA using the high capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using TaqMan PCR Master Mix and the thermocycler parameters recommended by the manufacturer. PCRs were performed in a volume of 50 μ L (Burgos-Ramos et al., 2012), containing 25 μ L of the reverse transcription reagents. TaqMan gene expression assays were used for acetyl-CoA carboxylase beta (*Accb*, Rn00588290_m1), carnitine palmitoyltransferase 1b (*Cpt1b*, Rn00682395_m1, Applied Biosystems), fatty acid synthase (*Fasn*, Rn00569117_m1; Applied Biosystems), myogenin (*Myog*, Rn00567418_m1, Applied Biosystems), ribosomal protein L13a (*Rpl13a*, Rn00821946_g1, Applied Biosystems), ribosomal protein S18 (*Rps18*, Rn01428915_g1, Applied Biosystems) and uncoupling protein 2 (*Ucp2*, Rn00571166_m1, Applied Biosystems). Relative gene expression comparison was carried-out using an invariant endogenous control; TATA-Box Binding Protein (*Tbp*, Rn01455646_m1, Applied Biosystems). The $\Delta\Delta$ CT method was used for relative quantification.

2.5. Multiplexed bead immunoassay

Phosphorylated and total protein levels of Akt, cAMP response element-binding protein (CREB), extracellular signal-regulated kinase (ERK)1/2; glycogen synthase kinase 3 β (GSK3 β), mTOR, phosphatase and tensin homolog on chromosome 10 (PTEN) and 70 kDa ribosomal protein S6 kinase 1 (p70S6K1) were measured in duplicate by multiplexed bead immunoassays (Bio-Rad Laboratories, Madrid, Spain and Merck Millipore, Darmstadt, Germany) as reported (Khan et al., 2004). Briefly, magnetic beads conjugated to the appropriate antibodies and tissue lysates (50 μ l each) were incubated for 18 hours at 4°C. Afterwards, wells were washed using a magnetic separation block (Millipore) and antibody conjugated to biotin (25 μ l) was added. After incubation for 30 min at room temperature, beads were incubated during 30 min with 50 μ l streptavidin conjugated to phycoerythrin. A minimum of 50 beads per parameter were analyzed in the Bio-Plex suspension array system 200 (Bio-Rad). Raw data (median fluorescence intensity, MFI) were analyzed with the Bio-Plex Manager Software 4.1 (Bio-Rad Laboratories). Mean intra- and inter-assay coefficients of variation were 8.3% and 11.9%, respectively.

2.6. Measurement of muscle glucose and glycogen

An intramuscular portion of gastrocnemius, containing red and white muscle, was homogenized in three volumes of perchloric acid, neutralized with potassium carbonate (Niewoehner and Nuttall, 1988) and glucose measured by an enzymatic method from Sigma-Aldrich (GAGO-20). Briefly, glucose is oxidized to gluconic acid by glucose oxidase and obtained hydrogen peroxide reacts with o-dianisidine. Oxidized o-dianisidine reacts with sulfuric acid to form a more stable colored product measured at 540 nm.

For quantification of glycogen, muscle samples were processed as previously reported (Burgos-Ramos et al., 2015). Briefly, muscle samples were incubated with two volumes of potassium hydroxide 30% at 100°C for 15 min. After centrifugation at 2,000 g for 5 min, the supernatants were precipitated with sodium sulfate plus ethanol and

then cooled on ice for 15 min. Glycogen pellets were obtained by centrifugation at 2,000 *g* for 5 min and hydrolyzed with 1 ml sulfuric acid 5N and boiled for 45 min. After neutralization with sodium hydroxide, glucose concentrations were determined by the same technique.

2.7. Determination of non-esterified fatty acid and triglyceride levels. Concentrations of non-esterified fatty acids (NEFA) and triglycerides were determined by using commercially available kits (Wako Chemicals, Neuss, Germany and Spinreact, St. Esteve de Bas, Spain), following the manufacturer's instructions. Samples were run in duplicate and the average coefficients of variation were lower than 10%. For determination of muscle concentration of these metabolites, an extraction of tissue was performed following the method of Folch et al. (1957).

2.8. Histological Analysis

Histological analysis was performed as previously reported (Sáinz et al., 2009). Sections (5 μ m) of formalin-fixed paraffin-embedded muscle were dewaxed with xylene and rehydrated. Endogenous peroxidase activity was quenched using 3% H₂O₂ (Sigma) in methanol for 20 min at RT, and washed 3 times with ethanol. Sections were immersed in 10 mmol/L citrate buffer (pH 6.00) and heated for 10 min. After cooling, sections were blocked for 30 min at RT in a chamber with 5% goat serum (Sigma-Aldrich) in TBS and incubated with mouse monoclonal PCNA antibody (Dako Cytomation, Glostrup, Denmark) diluted 1:200 in TBS with 5% goat serum (Sigma) (1:100) in a humidified chamber overnight at 4°C. After washing with TBS, sections were incubated with horseradish peroxidase-conjugated secondary antibodies diluted in TBS with 5% BSA (1:100) for 60 min at RT. Localization of the antigen-antibody binding antibodies was performed by adding diaminobenzidine/H₂O₂ solution (Sigma). The reaction was stopped and contrasted with Harris hematoxylin solution (Sigma). Sections were dehydrated with increasing concentrations of ethanol and xylol, mounted

in DePeX (Panreac, Barcelona, Spain), and viewed with an optic microscope (Axiovert 40 CFL, Zeiss, Gottingen, Germany). Fiber size was determined by measuring the cross-sectional area (CSA) of gastrocnemius muscle fibers (Sáinz et al., 2009); using the software AxioVision Release 4.6.3 (Zeiss, Göttingen, Germany). Positive myonuclei for PCNA were calculated from four randomly selected microscope fields (magnification 200X). Mean value was expressed as positive myonuclei number per 100 cells (Rodríguez et al., 2015).

2.9. Mitochondrial respiratory chain array panel

A customized PrimePCR screening panel, containing genes involved in mitochondrial respiratory chain, was carried out to analyze the gene expression levels in samples from gastrocnemius (Bio-Rad Laboratories Inc.). RNA integrity was assessed using Agilent's 2100 Bioanalyzer. The six samples of each experimental group had an RNA integrity number (RIN) > 8, so they were suitable for assay. The plate was formed by a total of 68 genes from which 27 genes belong to complex I (*Ndufa1-2*, *Ndufa5-11*, *Ndufab1*, *Ndufb2-3*, *Ndufb5-9*, *Ndufc2*, *Ndufs1-4*, *Ndufs6-8* and *Ndufv1-2*); 4 to complex II (*Sdha*, *Sdhb*, *Sdhc* and *Sdhd*); 6 to complex III (*Uqcrb*, *Uqcrc1*, *Uqcrc2*, *Uqcrfs1*, *Uqcrh* and *Uqcrcq*); 12 to complex IV (*Cox4i1-2*, *Cox5a*, *Cox5b*, *Cox6a1-2*, *Cox6c*, *Cox7a2*, *Cox7a2l*, *Cox7b*, *Cox8a* and *Cox8c*); 18 to complex V (*Atp12a*, *Atp4a*, *Atp4b*, *Atp5a1*, *Atp5b*, *Atp5g2*, *Atp5c1*, *Atp5g2*, *Atp5h*, *Atp5j*, *Atp5l*, *Atp5o*, *Atp6ap1*, *Atp6v0a2*, *Atp6v0d2*, *Atp6v1c2*, *Atp6v1e2* and *Atp6v1g3*) and *Tbp* as housekeeping gene. Genes quantification was performed by real-timePCR on a 7900HT fast real-time PCR system (Applied Biosystems) using the pre-designed 384-well plate format. The relative expression levels of each gene, which belong to the same mitochondrial complex, were grouped and submitted to statistical analysis. On this way, we calculated the expression changes in each one the mitochondrial complexes of respiratory chain, from relative quantification of multiple genes.

2.10. Statistical analysis

Analysis of all data was carried out by one-way ANOVA followed by Bonferroni's *post-hoc* tests. Values were considered significantly different when the P value was less than 0.05. Statistical analyses were conducted with Prism software 4.00 (GraphPad, San Diego, CA, USA) with an α level of 0.05. Data are presented as mean $\pm$ SEM.

3. Results

3.1. General characteristics of the experimental groups

Daily food intake was reduced in the PF and L groups (Fig. 1A). The increase in body weight at the end of the study was lower in PF and L, with a more marked reduction in L (Fig. 1B). The weight of gastrocnemius was higher in L compared to the PF group (Fig. 1C) and basal values of serum glucose were unchanged (Fig. 1D). Serum NEFA and triglyceride levels were decreased in the L group (Fig. 1E and 1F, respectively).

Muscle total protein was increased in L and RNA content in L compared to PF group. The weight of inguinal and epididymal fat pads were reduced in PF and L, being lower in L compared to PF rats (Table 1).

3.2. PCNA-positive nuclei and area of skeletal muscle fibers are increased after intracerebroventricular leptin infusion

Immunolocalization studies showed that the number of PCNA positive nuclei were increased in the L group with respect to CF and PF groups (Fig. 2A-D). Hematoxylin staining revealed that the area of fibers was also augmented in leptin-treated rats (Fig. 2E-H).

3.3. Chronic central leptin infusion augments glucose and glycogen levels in skeletal muscle

To test the possible effects of leptin on glucose uptake, we analyzed protein levels of GLUT4. Muscle GLUT4 levels were higher in L compared to both C and PF rats (Fig. 3A). Muscle glucose content was reduced in PF and increased in L rats (Fig. 3B). Glycogen levels were higher in L compared to both C and PF rats (Fig. 3C). PEPCK protein levels were not modified (Fig. 3D).

3.4. Chronic central leptin administration changes the expression of enzymes involved in fatty acid synthesis and oxidation in skeletal muscle

Central leptin infusion modifies key enzymes involved in lipid metabolism in peripheral tissues (Gallardo et al., 2007). We have found that the relative mRNA levels of Acc β were diminished in L rats (Fig. 4A) and mRNA concentrations of Fasn were decreased in PF, with no changes in L with respect to C rats (Fig. 4B). Relative mRNA concentrations of Cpt1b were increased in L with respect to the C and PF groups (Fig. 4C).

We also analyzed NEFA and triglyceride content in the gastrocnemius. Muscle NEFA concentrations were reduced in the L group (Fig. 4D) and triglyceride content increased in PF and decreased in L rats (Fig. 4E).

3.5. Central leptin infusion increases gene expression of complexes of the mitochondrial respiratory chain in skeletal muscle

Given that central leptin infusion decreased NEFA concentration and up-regulated Cpt1b expression, we hypothesized that an increase in complexes of the mitochondrial respiratory chain may be involved in the favourable lipid profile after leptin infusion. Thus, we measured gene expression levels of complexes of the mitochondrial respiratory chain in gastrocnemius muscle from all experimental groups. The results from screening indicated that leptin increased mRNA levels of all mitochondrial complexes (I-V) in gastrocnemius compared to control and PF groups (Fig. 5A to 5E).

3.6. Chronic central leptin promotes activation of muscle insulin signaling

We studied the levels and/or activation of several key targets of insulin/PI3K signaling pathways. We first analyzed whether levels of IR were affected by leptin infusion. There was an increase in muscle IR levels in L group (Fig. 6A). Phosphorylation of Akt at Thr308 and Ser473 was increased in PF and L, being higher in L compared to PF rats (Fig. 6B and 6C, respectively), while PTEN phosphorylation at Ser380 residue was reduced in L (Fig. 6D).

We also analyzed whether the increase in glycogen levels were due to changes in GSK3 β activation. As glycogen synthase is activated by inactivation of GSK3 β , mainly mediated through phosphorylation of GSK3 β at Ser9 (Mottet et al., 2003), we analyzed if leptin changed the degree of phosphorylation in this residue. Our results showed an increase in GSK3 β phosphorylation in leptin-treated rats, with no changes in the PF group (Fig. 6E).

3.7. Increase in insulin signaling is associated with the activation of protein-related synthesis

Activation of PI3K pathways increases activation of mTOR (Tan et al., 2016). We found that phosphorylation of mTOR at Ser2448 was augmented in the L group (Fig. 7A). Myogenin mRNA levels were increased in gastrocnemius of PF and L rats (Fig. 7B). CREB phosphorylation was augmented in PF and L rats (Fig. 7C), whereas mRNA levels of UCP2 were reduced in L rats with respect to the C and PF groups (Fig. 7D). Finally, phosphorylation of p70S6K1 at Thr389 residue was increased in L with respect to C and PF groups (Fig. 7E).

3.8. Central leptin infusion modifies the activation or expression of factors involved in the regulation of translational capacity

We analyzed several factors implicated in the possible modulation of ribosome biogenesis and translational capacity. Phosphorylation of AMPK was reduced in PF and L groups (Fig. 8A). We also studied the activation of ERK1/2 that was increased in L rats with respect to C and PF groups (Fig. 8B). *Rps18* mRNA levels were increased in gastrocnemius of L rats (Fig. 8C) and relative mRNA concentrations of *Rpl13a* were augmented in L with respect to C and PF groups (Fig. 8D).

4. Discussion

The anabolic effects of insulin in the regulation of muscle protein metabolism have been extensively reported (Meek et al., 1998, Gordon et al., 2013). Moreover, the effects of insulin on muscle mass, as well as the role of insulin favoring glucose uptake through an increase in GLUT4 are known (Hall et al., 2013). The study presented here demonstrates that chronic central leptin infusion regulates key factors that participate in the intracellular mechanisms mediating insulin signaling in the gastrocnemius muscle. Our major finding is that the increased insulin-related signaling in leptin-treated rats activates factors involved in protein synthesis and this may contribute to muscle mass accretion. We also show that rats treated with central leptin present a reduction in enzymes involved in fatty acid synthesis, as well as an increase in those implicated in fatty acid oxidation. Thus, these favorable changes in lipid metabolism may contribute to potentiate insulin sensitivity in the gastrocnemius muscle. The experimental model of icv infusion of leptin used here changes peripheral insulin sensitivity (Lin et al., 2002, Keung et al., 2011). Here we found that chronic central leptin infusion increased protein levels of IR and reduced PTEN phosphorylation that explain the activation of Akt. Indeed, insulin and leptin activate PI3K (Sohn et al., 2016).

Disorders in skeletal muscle fatty acid metabolism play a role in the development of insulin resistance (van der Kolk et al., 2016). We demonstrated that in leptin-treated rats not only NEFA and triglycerides levels were reduced in serum, but also in muscle. This effect was not seen in pair-fair and may be attributed to a direct

effect of leptin on fatty acid metabolism. This is in agreement with studies reporting that a reduction in food intake alone is insufficient to improve lipid metabolism (Ebihara et al., 2001) and that leptin administration diminishes skeletal muscle triacylglycerol content in patients with lipodystrophy (Kusakabe et al., 2009). Activation of insulin signaling may also explain the favorable lipid profile in gastrocnemius here reported, given that improvement of insulin sensitivity is associated with reduced Acc and up-regulated Cpt1 expression in the liver of high-fat diet-induced obese mice (Naowaboot et al., 2016), similar to that seen in the gastrocnemius of leptin-infused rats. Moreover, anti-diabetic agents increase leptin receptor levels, decrease triglyceride levels and lipogenic gene expression, including Fasn and Acc (Tang et al., 2016). There was an increase in mitochondrial respiratory chain complexes and this effect may be parallel to up-regulation of Cpt1, as previously reported (Bruce et al., 2009). Muscle is the main organ for the utilization of NEFA and leptin activates mitochondrial function (Holmström et al., 2013). In addition, the increase in the expression of several components of the mitochondrial respiratory chain is related to insulin-mediated myogenesis (Litwiniuk et al., 2016), whereas inhibitors of the respiratory chain reduced muscle differentiation (Pawlikowska et al., 2006). Taken together, our data suggest that increased NEFA oxidation may also be a positive factor for the improvement in insulin action, not only in lipid metabolism but also in myogenesis. In addition, the reduction in fat pad weight in leptin-treated rats suggest that fatty acid oxidation could be also enhanced in this localization.

We have previously reported that this experimental model provokes an increase in serum leptin levels (Burgos-Ramos et al., 2015), as it has been previously demonstrated (Morrison et al., 2001). In fact, icv injected leptin goes to the periphery and contributes to hyperleptinemia (Maness et al., 1998; Al-Barazanji et al., 2001). Evidence indicates that leptin enhances insulin sensitivity and glucose uptake in skeletal muscle through central and peripheral relays (Denroche et al., 2012; Yau et al., 2014). The present study also showed that leptin successfully enhanced GLUT4

protein levels in the gastrocnemius. *In vitro* studies in muscle tissue demonstrated that leptin increases glucose uptake (Ceddia et al., 1998), which is in accordance with increase in muscle glucose content observed here. These effects could be to a direct action of leptin or mediated by activation of insulin signaling, as Akt activation increases GLUT4-mediated glucose uptake (Ramachandran and Saravanan, 2015). In addition to increased glucose content in the gastrocnemius muscle, we found an augment in glycogen levels. Rodents on a high fat diet present a decrease in skeletal muscle glucose uptake and glycogen synthesis in the gastrocnemius, whereas leptin normalized rates of glycogen synthesis (Yaspelkis et al., 2004). Our results may indicate that increased glycogen content is also related to activation of the insulin cascade. In fact, leptin treatment potentiates insulin signaling, activating Akt to phosphorylate GSK3 β at Ser9, producing an inhibitory effect (Cross et al., 1995) and so rising glycogen levels. However, there was no reduction in PEPCK protein content in leptin-treated rats. Although activation of the insulin cascade is related to inhibition of glucose synthesis, we should take into consideration that gluconeogenic transcription is repressed by insulin under feeding conditions (Oh et al., 2013) and leptin-treated rats presented a reduction in food intake that could explain, at least in part, the absence of changes in muscle PEPCK levels.

The growth of an organ may be mediated by increase in both cell size and cell number, with the activation of insulin targets playing a crucial role in the control of skeletal muscle growth (Bodine, 2006). We found an increase in PCNA in the gastrocnemius and its mass accrual after chronic central leptin infusion related to activation of PI3K/Akt signaling. A role for leptin as a possible mitogen was suggested by data showing that peripheral leptin treatment of leptin-deficient *ob/ob* mice stimulates expression of cyclin D1 in muscle (Sáinz et al., 2009).

The increase in mTOR phosphorylation after activation of Akt at Thr308 and Ser473 residues has been extensively reported (Sekulić et al., 2000, Perry et al., 2016). Activation of mTOR stimulates protein translation and cell growth by up-

regulating cell cycle genes, whereas rapamycin inhibition provokes cell cycle arrest (Tan et al., 2016). Additionally, Akt-mediated inhibition of GSK3 β may play a role in protein synthesis and cell growth; thus, inhibition of this kinase in response to insulin treatment promotes eukaryotic initiation factor 2B activity (Gordon et al., 2013). Another possible explanation is that the increase in mTOR activation and subsequent protein synthesis could be associated with the CREB activation here reported, as its phosphorylation modulates the activity of many eukaryotic transcription factors (Altarejos and Montminy, 2011), as well as mTOR phosphorylation (Tseng et al., 2016). Finally, our results suggest a role of mTORC1, since we found an increase in p70S6K1 phosphorylation, which is related to augmented protein synthesis (Gordon et al., 2015). In this line, we have found not only an increase in muscle fiber size but only in total protein content in leptin-treated rats. In spite of this, we cannot exclude a role of mTORC2 because it activates Akt through phosphorylation at Ser473 (Sarbasov et al., 2005).

Our results demonstrated that central leptin administration favored muscle accretion, by increasing muscle fiber size in parallel with an upregulation of PCNA-positive nuclei and myogenin mRNA levels. These factors are up-regulated during tissue regeneration (Song et al., 2015), with a parallel modulation of these hyperplasia markers with muscle fiber area (Alami-Durante et al., 2014), as reported here. The increase in PCNA positive nuclei may be associated with the higher Akt activation in leptin-treated rats. Accordingly, Akt activation is directly associated with the number of PCNA positive cells (Fang et al., 2016); whereas the frequency of PCNA positive cells is lower after reduction of insulin signaling (Costa et al., 2012). We also observed a reduction in Ucp2 gene expression that may be inversely associated with proliferative markers, as Ucp2 might serve as a brake for myogenic differentiation (Chen et al., 2009) and deficiency of this uncoupling protein improves insulin sensitivity (Zhou et al., 2016).

Changes in the activation of cell cycle markers may be linked to ribosome biogenesis and translational capacity after central leptin infusion. In this regard, activation of mTORC1-related signaling after leptin infusion may play a role in the regulation of ribosome biogenesis (Hannan et al., 2003; Chaillou et al., 2014). Our results showed that leptin increases ERK phosphorylation, which reportedly activated proteins implicated in ribosomal assembly and biogenesis (Kim et al., 2005), while inhibition of ERK decreases protein synthesis (Mariappan et al., 2011). In addition, it has been stated that phosphorylation of AMPK suppresses ribosome biogenesis (Cao et al., 2017), whereas our data revealed a reduction in AMPK phosphorylation after leptin infusion that may favor ribosomal biogenesis. We have also found an increase in the gene expression of ribosomal proteins *Rpl13a* and *Rps18*. Interestingly, insulin and insulin/IGF-I related signaling up-regulates 18S RNA (Fain and Bahouth, 1998; McMahon et al., 2010) and *Rpl13a* play a key role as a rRNA methylation factor (Chaudhuri et al., 2007) and is also a protein that may be involved in ribosome biogenesis (Das et al., 2013).

5. Conclusions

Our results suggest that leptin is implicated in the accretion of gastrocnemius skeletal muscle through activation of insulin signaling cascade. Additionally, our findings indicate that leptin increases the complexes of the mitochondrial respiratory chain and show a favorable lipid profile in the gastrocnemius muscle that could increase insulin sensitivity in this tissue. Finally, our data seem to denote that the augment in muscle fiber size may be related to a possible increase in translational capacity. The current data may help to understand the role of leptin in skeletal muscle accrual and its possible relation with insulin-signaling targets and highlight the need of future research concerning the interaction between leptin and insulin in this tissue, which will provide noteworthy advances into the muscle physiology as well as pathophysiology related to states of leptin and insulin resistance.

Conflict of interest

All authors declare no conflict of interest.

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Figure legends

Fig. 1. General characteristics of experimental groups. (A) Daily food intake in control rats (C), pair-fed rats (PF) and rats treated with chronic icv leptin infusion (L). (B) Increase in body weight at the end of study. (C) Gastrocnemius weight. (D) Serum glucose concentrations. (E) Serum non-esterified fatty acid (NEFA) concentrations (F) Serum triglyceride levels. NS, non-significant; * $p<0.05$, ** $p<0.01$ by one-way ANOVA.

Fig. 2. Central leptin increases PCNA-positive nuclei and area of muscle fibers. (A-C) Immunohistochemistry for proliferating cell nuclear antigen (PCNA)-positive nuclei in the gastrocnemius muscle of control rats (C), pair-fed rats (PF) and rats treated with chronic icv leptin infusion (L), respectively. (D) PCNA-positive nuclei per 100 fibers. (E-G) Hematoxylin-eosin staining in the gastrocnemius of C, PF and L groups, respectively. H. Area of muscle fibers. Magnification 200X; * $p<0.05$, ** $p<0.01$ by one-way ANOVA.

Fig. 3. Muscle glucose content is augmented after central leptin infusion. (A) Relative protein levels of glucose transporter 4 (GLUT4) in the gastrocnemius muscle of control rats (C), pair-fed rats (PF) and rats treated with chronic icv leptin infusion (L). (B) Free glucose levels. (C) Glycogen concentrations. (D) Relative protein levels of phosphoenolpyruvate carboxykinase (PEPCK). DU, densitometry units; NS, non-significant; * $p<0.05$, ** $p<0.01$ by one-way ANOVA.

Fig. 4. Changes in lipid metabolism of skeletal muscle after central leptin administration. Relative mRNA levels of acetyl-CoA carboxylase beta (Accb) (A), fatty acid synthase (Fasn) (B) and carnitine palmitoyltransferase 1b (Cpt1b) (C) in the gastrocnemius muscle of control rats (C), pair-fed rats (PF) and rats treated with chronic icv leptin infusion (L). Non-esterified fatty acid (NEFA) (D) and triglyceride content (E) in the gastrocnemius muscle. * $p<0.05$, ** $p<0.01$ by one-way ANOVA.

Fig. 5. Gene expression levels of factors involved in mitochondrial respiratory chain are increased after central leptin infusion. Relative mRNA levels of Complex I (A), II (B), III (C), IV (D) and V (E) in the gastrocnemius muscle of control rats (C), pair-fed rats (PF) and rats treated with chronic icv leptin infusion (L). ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA.

Fig. 6. Chronic central leptin administration induces the activation of insulin signaling in skeletal muscle. Relative protein levels of the insulin receptor beta chain (IR β) (A), phosphorylated (p) Akt at Thr308 (pThr308Akt) (B), phosphorylated Akt at Ser473 (pSer473Akt) (C), phosphorylated phosphatase and tensin homolog on chromosome 10 (PTEN) at Ser380 (pSer380PTEN) (D) and phosphorylated glycogen synthase kinase (GSK)3 β at serine 9 (pSer9GSK3 β) (E) in the gastrocnemius muscle of control rats (C), pair-fed rats (PF) and rats treated with chronic icv leptin infusion (L). DU, densitometry units; MFI, median fluorescent intensity; * $p < 0.05$, ** $p < 0.01$ by one-way ANOVA.

Fig. 7. Central leptin increases markers of protein synthesis. (A) Relative phosphorylated (p) mechanistic target of rapamycin (mTOR) at Ser2448 (pSer2448mTOR) protein levels in the gastrocnemius muscle of control rats (C), pair-fed rats (PF) and rats treated with chronic icv leptin infusion (L). (B) Relative mRNA levels of myogenin (Myog). (C) Relative phosphorylated cyclic AMP response element-binding protein (CREB) at Ser133 (pSer133CREB) protein levels. (D) Relative mRNA levels of uncoupling protein 2 (Ucp2). (E) Relative phosphorylated p70S6 kinase 1 at Thr389 (pThr389p70S6K1) protein levels. MFI, median fluorescent intensity; * $p < 0.05$, *** $p < 0.001$ by one-way ANOVA.

Fig. 8. Changes in molecules involved in the regulation of ribosomal biogenesis after leptin administration. (A) Relative phosphorylated (p) AMP-activated protein kinase (AMPK) protein levels in the gastrocnemius muscle of control rats (C), pair-fed

rats (PF) and rats treated with chronic icv leptin infusion (L). (B) Relative phosphorylated extracellular signal-regulated kinase (ERK)1/2 protein levels. (C) Relative mRNA levels of ribosomal protein S18 (Rps18). (D) Relative mRNA levels of ribosomal protein L13a (Rpl13a). DU, densitometry units; MFI, median fluorescent intensity; * $p<0.05$, ** $p<0.01$ by one-way ANOVA.

TABLES

Table 1. Protein and RNA content in gastrocnemius muscle and weight of fad pads.

Group	Control	Pair-fed	Leptin
Protein ($\mu\text{g}/\text{mg}$ tissue)	190.9 ± 2.1	184.7 ± 6.4	212.5 ± 3.3 * #
RNA ($\mu\text{g}/\text{mg}$ tissue)	1.92 ± 0.11	1.71 ± 0.09	2.07 ± 0.32 #
Inguinal fat (g)	2.60 ± 0.18	2.25 ± 0.24 *	1.69 ± 0.32 * #
Epididymal fat (g)	3.81 ± 0.26	2.45 ± 0.30 **	1.70 ± 0.16 ** ##

* $p < 0.05$, ** $p < 0.01$ vs. control; # $p < 0.05$, ## $p < 0.01$ vs. pair-fed group by one-way ANOVA

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Highlights of the manuscript: The increase in fiber size in male rat gastrocnemius after chronic central leptin infusion is related to activation of insulin signaling

Bullet points:

1. Central leptin infusion increases muscle size
2. Higher muscle size is related to insulin signaling activation
3. Improved insulin signaling may increase protein synthesis markers
4. Central leptin up-regulates mitochondrial respiratory chain complexes
5. Insulin sensitivity is associated with a favorable lipid profile