RESEARCH

Central leptin regulates heart lipid content by selectively increasing PPAR β/δ expression

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Abstract

The role of central leptin in regulating the heart from lipid accumulation in lean leptin-sensitive animals has not been fully elucidated. Herein, we investigated the effects of central leptin infusion on the expression of genes involved in cardiac metabolism and its role in the control of myocardial triacylglyceride (TAG) accumulation in adult Wistar rats. Intracerebroventricular (icv) leptin infusion (0.2 µg/day) for 7 days markedly decreased TAG levels in cardiac tissue. Remarkably, the cardiac anti-steatotic effects of central leptin were associated with the selective upregulation of gene and protein expression of peroxisome proliferator-activated receptor β/δ (PPARβ/δ, encoded by *Pparb/d*) and their target genes, adipose triglyceride lipase (encoded by *Pnpla2*, herefater referred to as *Atgl*), hormone sensitive lipase (encoded by *Lipe*, herefater referred to as *Hsl*), pyruvate dehydrogenase kinase 4 (*Pdk4*) and acyl CoA oxidase 1 (*Acox1*), involved in myocardial intracellular lipolysis and mitochondrial/peroxisomal fatty acid utilization. Besides, central leptin decreased the expression of stearoyl-CoA deaturase 1 (*Scd1*) and diacylglycerol acyltransferase 1 (*Dgat1*) involved in TAG synthesis and increased the CPT-1 independent palmitate oxidation, as an index of peroxisomal β-oxidation. Finally, the pharmacological inhibition of PPARβ/δ decreased the effects on gene expression and cardiac TAG content induced by leptin. These results indicate that leptin, acting at central level, regulates selectively the cardiac expression of PPARβ/δ, contributing in this way to regulate the cardiac TAG accumulation in rats, independently of its effects on body weight.

Key Words
- leptin
- cardiac metabolism
- PPARβ/δ
- lipid metabolism

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Introduction

Leptin is a mediator of long-term regulation of energy balance that suppresses food intake and decreases body weight by affecting the expression of neuropeptides in hypothalamic nuclei and in other cell types in the central nervous system (CNS). As a consequence, this adipokine controls whole-body metabolism mainly through the CNS (Friedman & Halaas 1998, Allison & Myers 2014). Accordingly, we and others have previously reported a critical role for hypothalamic leptin action in regulating glucose and lipid metabolism in peripheral tissues in a

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tissue-specific manner (Kamohara *et al.* 1997, Minokoshi *et al.* 1999, Gallardo *et al.* 2007, Buettner *et al.* 2008, Bonzón-Kulichenko *et al.* 2009).

In isolated perfused working rat hearts, earlier work revealed that acute peripheral leptin administration decreased heart TAG content (Atkinson *et al.* 2002). A similar decrease was also observed in a transgenic mice model of myocardial steatosis upon chronic hyperleptinemia (Lee *et al.* 2004). Recent studies have shown that central and/or peripheral leptin infusion is required to reverse cardiac steatosis in obese leptin-deficient (*ob/ob*) mice (Rame *et al.* 2011, Sloan *et al.* 2011), although myocardial TAG levels were not affected by central leptin administration in low- and/or high-fat fed obese mice (Keung *et al.* 2011). Nevertheless, the role of leptin on cardiac metabolism in lean normoleptinemic animals remains incompletely understood (Karmazyn *et al.* 2008).

Regulation of cardiac metabolism occurs primarily at the transcriptional level. In particular, the members of the nuclear receptor superfamily the ligand-activated peroxisome proliferator-activated receptors (PPARs) control the expression of an array of genes involved in cardiac lipid and glucose metabolism, fuel management and mitochondrial capacity (Gilde *et al.* 2003, Burkat *et al.* 2007, Liu *et al.* 2011). Although all PPAR isoforms are involved in different aspects of lipid metabolism and their physiological role depends on tissue distribution, PPARβ/δ plays an essential role in myocardial metabolism since it controls glucose and lipid utilization (Burkat *et al.* 2007, Liu *et al.* 2011) and promotes insulin sensitivity (Finck 2007, Palomer *et al.* 2016). The activity of the PPARs and other important transcription factors in the heart is regulated by the inducible transcriptional co-activators (PGC)-1 family of proteins. PGC-1α and PGC-1β regulate mitochondrial biogenesis and genes encoding for enzymes of mitochondrial metabolism in adult heart (Rowe *et al.* 2010, Mitra *et al.* 2012, Riehle *et al.* 2012). The activity of these factors is required for the expression of genes involved in fatty acid oxidation and mice with deficient cardiac PGC-1α and PGC-1β content exhibit accelerated heart failure (Mitra *et al.* 2012). Although the function of PGC-1α is well documented, less is known about the signals that promote PGC-1β gene expression in the heart.

Knowing that PPARα repressed, whereas PPARβ/δ activated, target genes involved in heart glucose metabolism (Burkat *et al.* 2007), we hypothesized that central leptin infusion may protect the heart from lipid accumulation through the different regulation of cardiac PPARs expression. The aim of the present study was to characterize the effects of intracerebroventricular leptin administration on the expression of key enzymes and proteins involved in cardiac lipid and glucose metabolism and to test the hypothesis whether central leptin modules differently the expression of PPARs and/or PGC-1s proteins in this tissue. Our results show that central leptin decreases myocardial TAG levels, at least in part, through the selective upregulation of PPARβ/δ in rats with normal leptin sensitivity.

Methods

Animals

Male 3-months-old Wistar rats were randomly housed in individual cages under conditions of climate-controlled quarters with a 12-h light cycle and fed *ad libitum* standard laboratory diet and water. The animals were handled according to the European Union laws (2010/63/EU) and the Spanish regulations for the use of laboratory animals (RD 53/2013). The experimental protocols were approved by the Institutional Scientific Ethics Committee under Project Licence PR-2012/10-05.

Intracerebroventricular leptin administration

Rats were anesthetized with intraperitoneal ketamine/diazepam/atropine. Intracerebroventricular administration of rat leptin (0.2 µg/day) (Sigma) or its vehicle (PBS) for 7 days was performed as previously described (Gallardo *et al.* 2007). See Supplementary Materials and methods for details (see section on supplementary data given at the end of this article).

Three groups of rats were studied: (1) rats infused with leptin (0.2 µg/day); (2) rats infused with vehicle (PBS) and allowed to eat *ad libitum* and (3) rats infused with vehicle and pair-fed to the amount of food consumed by the leptin-treated rats. 7 days after minipump implantation, the animals were fasted overnight and received an intravenous (iv) injection of insulin (10 IU/kg body weight) (Eli Lilly) or saline, 30 min later, the animals were anesthetized by CO_2 inhalation and killed by decapitation. Blood was removed and centrifuged (2000 ***g***, 15 min), serum was recovered and frozen in liquid nitrogen at −70°C until use. Hearts were carefully dissected, washed in Henseleit buffer at 37°C and weighted. Hereafter, atria were removed and both ventricles were used in all analyses after frozen in liquid nitrogen for stored at −70°C until use.

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Serum metabolites, hormone analysis and myocardial TAG determination

Serum metabolites were measured and hormone analysis was performed as previously described (Gallardo *et al.* 2007) (Supplementary Materials and methods for details). Frozen rat ventricles (100 mg) were used for TAG determination as previously described (Gallardo *et al.* 2007). Homeostatic model assessment of insulin resistance (HOMA-IR), an indicator of whole-body insulin sensitivity, was calculated as (fasting insulin (μIU/mL) × fasting glucose ((mmol/L))/22.5 as described earlier (Matthews *et al.* 1985).

Real-time RT-PCR

Total RNA was isolated from 50 to 70 mg of frozen rat ventricles and the cDNA was synthesized from 1.5 μg of DNase-treated RNA. Relative quantitation was performed using pre-developed probes (Supplementary Materials and methods and Supplementary Table 1 for details) by TaqMan real-time PCR on an ABI PRISM 7500 FAST Sequence Detection System.

Subcellular fractionation of cardiac tissue

Frozen tissue from rat ventricles (0.6–0.8 g) was thawed, minced and incubated at 4°C for 30 min before homogenization in lysis buffer supplemented with 1 M NaCl to dissociate actin filaments. After centrifugation for 2 min at 1000***g***, minced tissue was resuspended in lysis buffer (5 mL buffer/g tissue) supplemented with 20 mM NaF, 2 mM Na_3VO_4, 10 μg/mL leupeptin, 10 μg/mL aprotinin and 1 μg/mL pepstatin. After centrifugation for 5 min at 4000***g***, the supernatant was used for mitochondrial, plasma membrane (PM) and internal membrane (IM) fractionation using Optiprep (Axis-Shield, Oslo, Norway) discontinuous gradient as described (Bao *et al.* 2011) (Supplementary Materials and methods for details).

Enzyme activity measurements

Mitochondrial citrate synthase activity was performed by the method described by Srere (1969). Cytoplasmic malic enzyme activity was assayed as previously described (Andrés *et al.* 1980).

Glycogen contents

Glycogen levels were assessed in rat ventricles (10 mg) using a glycogen assay kit II (ab 169558, Abcam) following manufacturer's instruction. All samples were measured in triplicate and glycogen content was expressed as mg/g wet tissue.

Mitochondrial DNA (mtDNA) quantification

The mtDNA copy number quantification was evaluated by quantitative PCR as previously reported (Rooney *et al.* 2015) using the 16S rRNA as a mtDNA marker and resistin as a nuclear DNA (nucDNA) marker (Supplementary Materials and methods for details).

Palmitate oxidation

Palmitate oxidation was measured in fresh rat ventricle homogenates using a modified method of that described by Perdomo *et al.* (2004). Briefly, rat ventricles (200 mg) were minced in cold homogenization buffer containing, 250 mM sucrose, 1 mM EDTA and 10 mM Tris–ClH, pH 7.4, and then homogenized (10 mL/g tissue) with a Teflon pestle for 10 passes over 30 s at 1200 rpm. After centrifugation for 10 min at 420***g***, the supernatant was used as total ventricle homogenates that were incubated in assay buffer in the presence of 1.25% BSA, 0.2 mM palmitate and [9,10-^{3}H] palmitate (1 μCi/mL) final concentrations (Supplementary Materials and methods for details). Complete palmitate oxidation (as ^3H_2O) rates were measured in the absence or presence of 100 μM etomoxir, a specific and irreversible inhibitor of CPT1. The difference between complete oxidation and CPT1-independent palmitate oxidation was taken as CPT1-dependent palmitate oxidation. Palmitate oxidation was expressed as nanomoles of palmitate per milligram protein per hour.

PPARβ/δ antagonist administration

PPARβ/δ antagonist GSK0660 was dissolved first in DMSO and later in 0.9% NaCl as reported by Toral *et al.* 2015. Central saline-, pair-fed or leptin-infused rats were co-treated intraperitoneally (i.p.) with vehicle 2 mL/kg (0.062% DMSO), while another group of central leptin-infused rats were co-treated with GSK0660 at 1 mg/kg per day i.p. for 7 days.

NE determination and NE turnover rate quantification

For determination of NE turnover rate (NETO) and cardiac endogenous norepinephrine (NE) content (Supplementary Materials and methods for details).

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Table 1 Effects of leptin central infusion on the biological characteristics of the animals.

Treatment	SS	PF	Lep
ΔBody weight after i.c.v. leptin (g)	27 ± 4[a]	−15 ± 3[b]	−13 ± 2[b]
Average food intake (g/day)	20 ± 3[a]	13 ± 2[b]	13 ± 1[b]
Serum glucose (mM)	6.35 ± 0.6[a]	5.87 ± 0.5[a]	5.9 ± 0.5[a]
Blood lactate (mM)	3.5 ± 0.4[a]	3.6 ± 0.1[a]	4.7 ± 0.4[b]
Serum insulin (pmol/L)	271 ± 12[a]	243 ± 41[a]	118 ± 32[b]
Serum leptin (ng/mL)	5.9 ± 0.5[a]	5.4 ± 1[a]	5.1 ± 0.8[a]
Serum resistin (ng/mL)	21 ± 1[a]	24 ± 2[a]	18 ± 2[a]
HOMA-IR	11.1 ± 0.1[a]	9.5 ± 0.2[b]	4.8 ± 0.2[c]

Body weight and food intake were measured daily during the experiment. After 7 days of central leptin (Lep) 0.2 µg/day or vehicle (SS or PF) infusion, serum glucose, lactate, insulin, leptin and resistin were measured. HOMAR-IR score was calculated from fasting insulin and glucose concentrations using the formula HOMA-IR = (fasting glucose (mmol/L) × fasting insulin (µIU/mL))/22.5. Results are the mean ± S.E.M. of 8–10 rats per group. Different letters indicate significant differences among treatments ($P \leq 0.05$, one-way ANOVA followed by Tukey test).
Lep, leptin-infused rats; PF, vehicle-infused pair-fed rats; SS, vehicle-infused rats fed *ad libitum*.

Western blot analysis

50 µg of protein from total extracts and 15 µg of protein from PM and IM fractions were separated under reducing conditions in 7.5% SDS-PAGE, excluding GLUT4 and Na^+/K^+-ATPase, which separated under non-reducing conditions and without boiling the samples. Proteins were analyzed by Western blots (Supplementary Materials and methods for details).

Immunohistochemical analysis

The immunohistochemical detection of pY-STAT3 in hypothalamic sections from saline-infused and leptin-infused rats was performed as described earlier (Bonzón-Kulichenko *et al.* 2009).

Ex vivo glucose uptake determination

Glucose uptake was determined in fresh ventricle explants (20 mg) from saline, pair-fed and leptin-treated rats as previously described (Bonzón-Kulichenko *et al.* 2011) (Supplementary Materials and methods for details).

Statistical analysis

Data are expressed as mean ± S.E.M. Significant differences among groups were determined by one-way ANOVA followed by Tukey test. The significance of differences between two groups was determined by unpaired Student's *t*-test. Statistical analysis was performed using the GraphPad Prism, version 6.01 (GraphPad Software). Differences were considered significant at $P < 0.05$.

Results

Effects of central leptin on body weight and systemic metabolism in rats with normal leptin sensitivity

As expected, intracerebroventricular leptin infusion for 7 days reduced the daily food intake and body weight. Fasting serum glucose, leptin and resistin levels remain unchanged in the three groups of rats, whereas fasting serum lactate levels were significantly increased in leptin-treated rats (Table 1). Central leptin infusion decreased serum insulin levels compared with saline-infused pair-fed control rats. Consistent with this there was a significant decrease in HOMA-IR in leptin-treated rats (Table 1), indicating that central leptin increases the overall insulin sensitivity. Leptin reduced the serum levels of TAG (32%), increased those of NEFA (65%) and total ketone bodies (120%), whereas the levels of total cholesterol were unaffected compared with the pair-fed group (Table 2). Finally, central leptin increased the STAT3

Table 2 Effects of central leptin infusion on serum lipid profiles.

Treatment	SS	PF	Lep
VLDL-TAG (mg/dL)	81 ± 8[a]	79 ± 5[a]	54 ± 5[b]
Total cholesterol (mg/dL)	75 ± 2[a]	64 ± 4[b]	62 ± 2[b]
NEFA (mM)	0.48 ± 0.02[a]	0.28 ± 0.03[b]	0.47 ± 0.02[a]
Total ketone bodies (mg/dL)	6.9 ± 0.3[a]	2.3 ± 0.1[b]	5.2 ± 0.1[c]

After 7 days of central leptin (Lep) 0.2 µg/day or vehicle (SS or PF) infusion, VLDL-TAG, total cholesterol, NEFA and total ketone bodies were measured in serum. Results are the mean ± S.E.M. of 8–10 rats per group. Different letters indicate significant differences among treatments ($P \leq 0.05$, one-way ANOVA followed by Tukey test).
Lep, leptin-infused rats; PF, vehicle-infused pair-fed rats; SS, vehicle-infused rats fed *ad libitum*.

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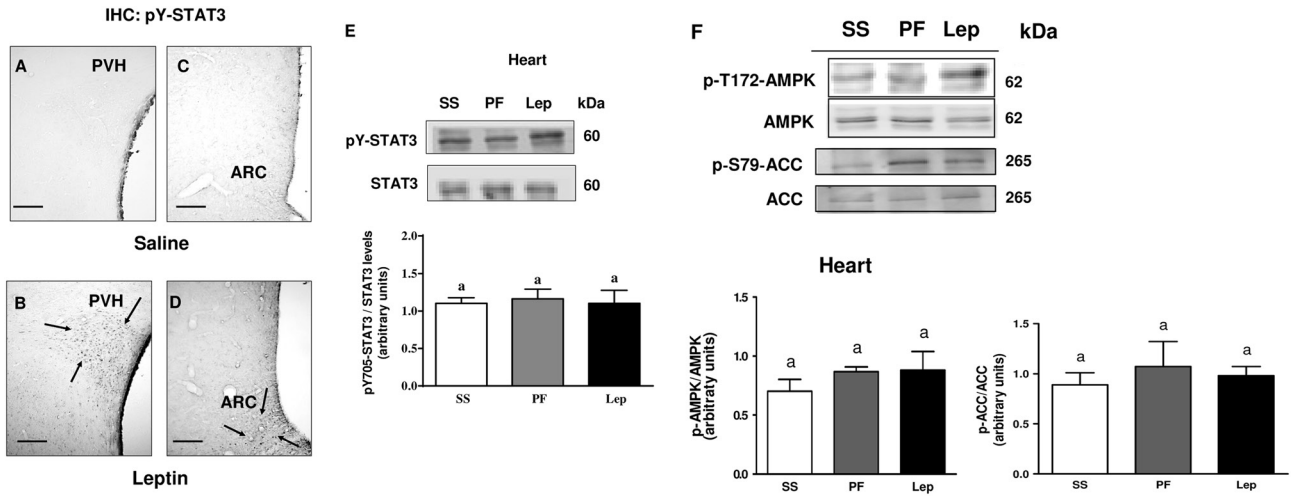

Figure 1
Central leptin effects in the phosphorylation levels of STAT3, AMPK and ACC. (A, B, C and D) Representative microphotographs of pY-STAT3 immunohistochemistry (IHC) of hypothalamic sections from saline-infused and leptin-infused rats, out of three independent experiments. After 7-day infusion of leptin (0.2 µg/day), there is an increase in the staining of tyrosine phosphorylation of STAT3 (pY-STAT3) (see arrows) in the paraventricular nucleus (PVH) (B) and arcuate nucleus (ARC) (D) with respect to saline-infused (A and C) rats. Scale bars, 150 µm. Representative Western blot out of four and quantitative densitometric analysis showing total STAT3 and pY-STAT3 (E), and total AMPK, ACC, pT-AMPK and pS-ACC (F) in 50 µg total extracts from heart after 7 days of central saline or leptin infusion. For each analyzed protein, data were expressed as a ratio of phosphorylated to protein content. Results are the mean ± S.E.M. of 8–10 rats per group. Different letters indicate significant differences among treatments ($P<0.05$, one-way ANOVA followed by Tukey test). Lep, leptin-infused rats; PF, saline-infused pair-fed rats; SS, saline-infused rats fed *ad libitum*.

tyrosine phosphorylation levels in the paraventricular and arcuate nuclei (Fig. 1B and D) with respect to saline-infused rats (Fig. 1A and C), but not in the heart (Fig. 1E). These data suggest that leptin's effects on heart were indirect and mediated via efferent pathways from the sympathetic nervous system. To assess the sympathetic nervous system activity, we determined the NE turnover rate as previously reported by Penn *et al.* (2006). Cardiac endogenous NE content was not different between treatment groups (Table 3). Nevertheless, there is a trend to increase NETO in leptin compared with pair-fed rats (Table 3), due to change in the fractional turnover rate (k) rather than in cardiac NE content at time 0 (Table 3).

Table 3 Cardiac endogenous NE content and NE turnover rate in saline-infused pair-fed and leptin-treated rats.

Group	**NE content** (ng/g of tissue) 0 h	4 h after α-MPT	k, %h	**NETO ng/g of tissue/h**
PF	9.82 ± 0.4	7.82 ± 0.8*	5.7 ± 0.4	0.56 ± 0.09
Lep	10.57 ± 0.6	8.03 ± 0.7*	6.9 ± 0.5	0.73 ± 0.13

After 7 days of central leptin (Lep) 0.2 µg/day or saline (PF) infusion, cardiac endogenous NE content was measured at time (0 h) or at time (4 h), 4 h after the administration of α-methyl-p-tyrosine (α-MPT), an inhibitor of NE synthesis. Results are the mean ± S.E.M. of 4–6 rats per group. An asterisk indicates a significant difference, ($P \leq 0.05$) vs α-MPT non-treated rats.
k, fractional turnover rate; Lep, leptin-infused rats; NE, norepinephrine; NETO, NE turnover rate; PF, saline-infused pair-fed rats.

These data suggest that leptin elicited a slight increase in sympathetic nervous activity.

Central leptin decreases heart TAG content and regulates the expression of enzymes involved in lipid metabolism

Central leptin infusion significantly reduced (~50%) the cardiac TAG content when compared with saline-infused pair-fed animals (Fig. 2A). Moreover, consistent with previous observations (Atkinson *et al.* 2002, Keung *et al.* 2011), central leptin infusion did not alter the phosphorylation levels of cardiac AMPK and ACC (Fig. 1F) suggesting that the anti-steatotic effects of leptin in the heart are independent of changes in the AMPK-ACC axis. Next, we analyzed the mRNA levels of enzymes and proteins involved in lipid metabolism. Leptin administration enhanced the gene expression of the lipolytic enzymes *Atgl* and *Hsl* (Fig. 2B) and decreased those involved in the TAG synthesis *Scd1* and *Dgat1* when compared with the pair-fed animals (Fig. 2D). In addition, the expression of the lipogenic enzymes ACC and *Acly* were not significantly changed by leptin (Figs 1F and 2D). Interestingly, leptin administration significantly induced the expression of genes associated with mitochondrial and peroxisomal fatty acid catabolism *Pdk4*, *Ucp3* and *Acox1*, independently of its anorectic

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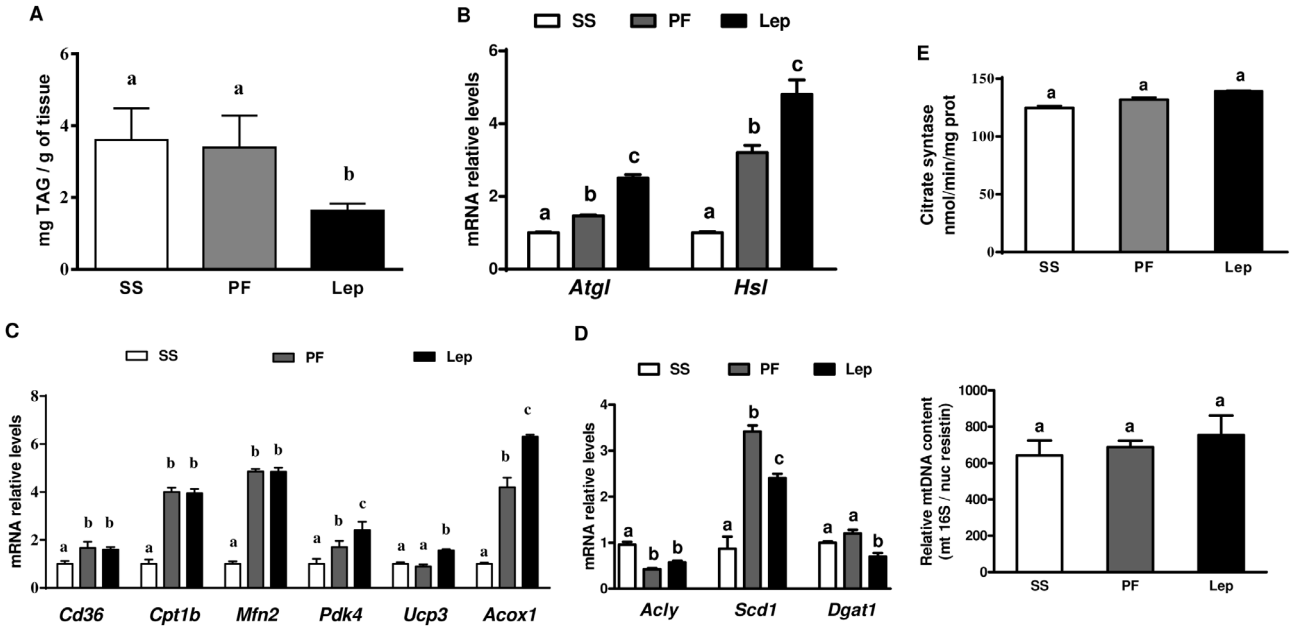

Figure 2

Intracerebroventricular leptin decreases cardiac triacylglyceride content and regulates the expression of genes involved in cardiac metabolism. (A) TAG content in heart after 7 days of central infusion of leptin (0.2 μg/day) (Lep), saline-infused rats fed *ad libitum* or saline-infused pair-fed rats. (B) mRNA levels of *Atgl, Hsl* in leptin or saline-infused rats. (C) mRNA levels of *Cd36, Cpt1b, Mnf2, Pdk4, Ucp3, Acox1* in leptin or saline-infused rats. (D) Effect of leptin or saline infusion on mRNA levels of *Acly, Scd1, Dgat1*. (E) Effect of leptin or saline infusion on citrate synthase activity and relative mtDNA content. Results are the mean ± S.E.M. of 8–10 rats per group. Different letters indicate significant differences among treatments, ($P \leq 0.05$, one-way ANOVA followed by Tukey test). Lep, leptin-infused rats; PF, saline-infused pair-fed rats; SS, saline-infused rats fed *ad libitum*.

effects (Fig. 2C). All these data are consistent with the decrease in cardiac TAG levels elicited by central leptin infusion reported herein. On the other hand, the mRNA levels of *Cd36*, *Cpt1b* and *Mfn2*, were increased in both, the leptin and the pair-fed groups of rats (Fig. 2C). Next, we determined the mtDNA content and the activity of the enzyme citrate synthase, as index of mitochondrial biogenesis and oxidative capacity. The results indicate that there were no relevant changes either in heart mtDNA content, as assessed by the ratio of mtDNA to nuclearDNA, or the citrate synthase activity in leptin-treated and pair-fed rats (Fig. 2D).

Central leptin increases the mRNA and protein levels of PPARβ/δ and PGC-1β in the heart

The results presented herein indicate that the cardiac mRNA (Fig. 3A) and protein levels of PGC-1α (encoded by *Pgc1a*) were increased in saline-infused pair-fed and leptin-treated rats compared with *ad libitum*-fed rats (Fig. 3B and C). On the other hand, despite the fact that cardiac mRNA levels of PPARα (endcoded by *Ppara*) were clearly decreased in the pair-fed and leptin-treated rats (Fig. 3A), the protein levels of PPARα were also upregulated in both, the pair-fed and leptin-treated rats compared with saline-infused *ad libitum*-fed rats (Fig. 3B and C). Together, these results suggest that the cardiac expression of PPARα and PGC-1α appears to depend, at least in part, of the leptin-mediated reduction in food intake.

Next, we further analyzed the effect of central leptin on the cardiac expression of PPARβ/δ, PPARγ and PGC-1β. Interestingly, our results indicated that leptin markedly increased the cardiac mRNA and protein levels of PPARβ/δ and PGC-1β (Fig. 3D, E and F). These results are consistent with the upregulation of *Atgl, Hsl, Pdk4, Ucp3* and *Acox1* (Fig. 2B and C). Finally, central leptin decreased the mRNA, but not the protein levels, of PPARγ (encoded by *Pparg*) when compared with the pair-fed animals (Fig. 3D, E and F).

Effect of central leptin on palmitate oxidation

Consistent with our previous data (Fig. 2C and E), complete cardiac palmitate oxidation rates were not significantly different between pair-fed and leptin-treated rats (Fig. 4A). Interestingly, the CPT1-independent palmitate oxidation,

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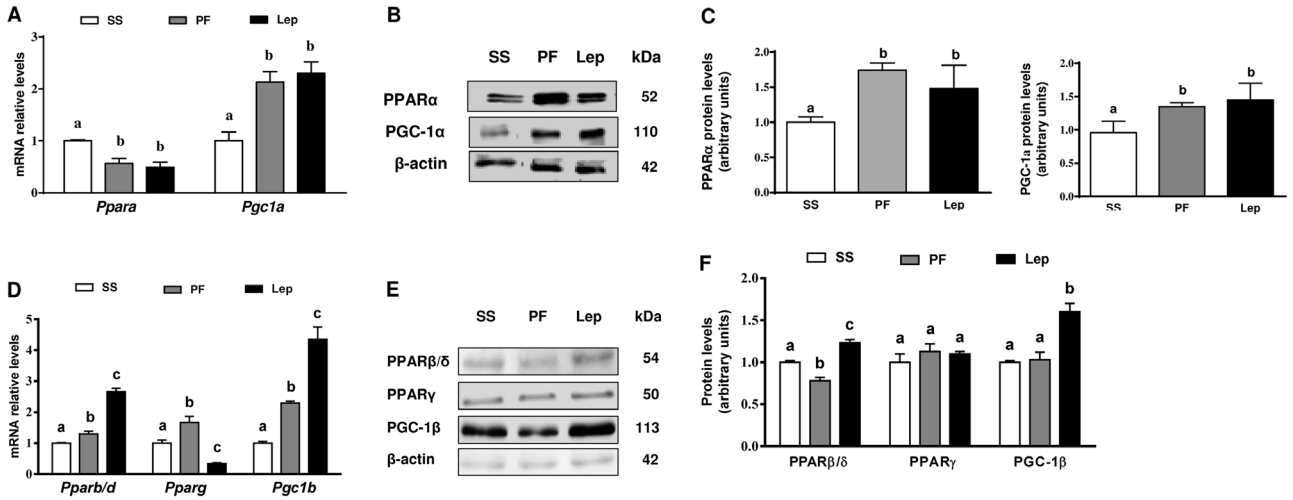

Figure 3
Effects of central leptin on cardiac expression levels of PPARs and PGC-1s. (A) mRNA levels of *Ppara* and *Pgc1a* in leptin or saline-infused rats. (B) Representative Western blot out of four and (C) quantitative densitometric analysis of PPARα and PGC-1α protein levels in 50 µg of total extracts from cardiac ventricles after central saline or leptin infusion. (D) mRNA levels of *Pparb/d, Pparg* and *Pgc1b*. (E) Representative Western blot out of four and (F) quantitative densitometric analysis of PPARβ/δ, PPARγ and PGC-1β protein levels in 50 µg of total extracts from cardiac ventricles after central saline or leptin infusion. β-Actin was used as control for protein loading and for densitometric normalization. Results are the mean ± S.E.M. of 8–10 rats per group. Different letters indicate significant differences among treatments, ($P \leq 0.05$, one-way ANOVA folowed by Tukey test). Lep, leptin-infused rats; PF, saline-infused pair-fed rats; SS, saline-infused rats fed *ad libitum*.

as a measure of peroxisomal activity supplying chain-shortened fatty acids to the mitochondria for complete oxidation, was significantly higher in leptin compared with pair-fed rats (Fig. 4B). As a result, the contribution of peroxisomes to the complete plamitate oxidation was increased from 39% in pair-fed to 63% in leptin-infused

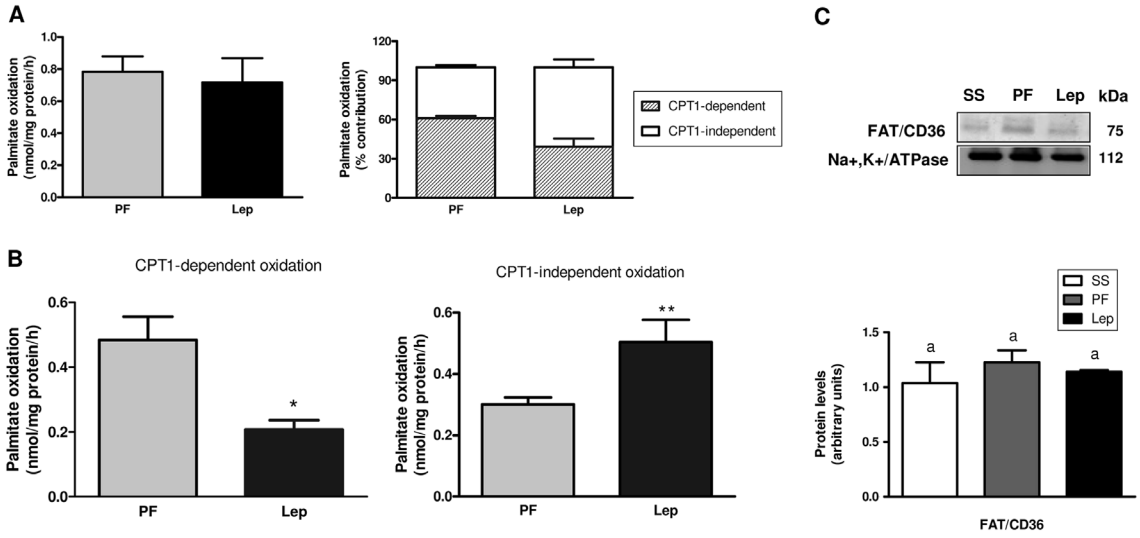

Figure 4
Effects of central leptin on cardiac palmitate oxidation in cardiac homogenates. (A) Complete palmitate oxidation and percent contribution of etomoxir-dependent inhibition of palmitate oxidation (CPT1-dependent) or etomoxir-independent (CPT1-independent) palmitate oxidation in saline-infused pair-fed and leptin-infused rats. (B) Effect of leptin infusion on etomoxir-dependent inhibition (CPT1-dependent) or etomoxir-independent (CPT1-independent) palmitate oxidation in saline-infused pair-fed and leptin-infused rats. (C) Representative Western blot out of four and quantitative densitometric analysis of FAT/CD36 protein levels in 15 µg of PM fraction from cardiac ventricles after 7 days of central saline or leptin infusion. Na^+/K^+-ATPase was used as control for PM protein loading and for densitometric normalization. Results are the mean ± S.E.M. of 8–10 rats per group. Different letters indicate significant differences among treatments, ($P \leq 0.05$, one-way ANOVA followed by Tukey test). Lep, leptin-infused rats; PF, saline-infused pair-fed rats; SS, saline-infused rats fed *ad libitum*.

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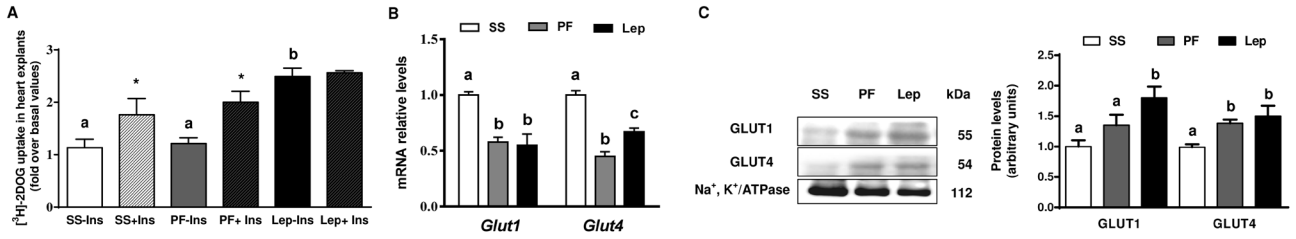

Figure 5
Central leptin stimulates cardiac basal glucose uptake. (A) Cardiac ventricular explants were used for [3H]-2-deoxyglucose uptake measurements in the absence or presence of 80 nM insulin stimulation for 10 min after 7 days of central infusion of leptin or saline. Data shown are from four independent experiments per group. (B) *Glut1* and *Glut4* glucose transporters mRNA levels in leptin or saline-infused rats. (C) Representative Western blot out of four and quantitative densitometric analysis of GLUT1 and GLUT4 levels in 15 µg of PM fraction from cardiac ventricles after 7 days of central saline or leptin infusion. Na^+/K^+-ATPase was used as control for PM protein loading and for densitometric normalization. Results are the mean ± S.E.M. of 8–10 rats per group. Different letters indicate significant differences among treatments, ($P \leq 0.05$, one-way ANOVA followed by Tukey test). Student's *t*-test, $^*P \leq 0.05$ vs basal, without insulin. Lep, leptin-infused rats; PF, saline-infused pair-fed rats; SS, saline-infused rats fed *ad libitum*.

rats (Fig. 4A). Finally, central leptin did not change PM levels of cardiac FAT/CD36 (Fig. 4C).

Effects of central leptin on cardiac glucose transport and glucose metabolism

Next, we investigated the effects of central leptin on glucose uptake in heart explants. In the absence of insulin, basal rates of glucose transport were significantly increased in leptin-infused compared with the pair-fed group of rats (Fig. 5A). Accordingly, GLUT1 protein levels in PM increased in hearts from leptin compared with saline-infused pair-fed animals, whereas the protein levels of GLUT4 increased to the same extent than the pair-fed rats (Fig. 5C). However, *in vivo* insulin stimulation did not further increase the already high levels of glucose uptake manifested by heart explants of leptin-treated rats (Fig. 5A). Additionally, central leptin upregulates the cardiac mRNA levels of GLUT4 (encoded by *Slc2a4*, hereafter referred to as *Glut4*), but not the mRNA levels of GLUT1 (encoded by

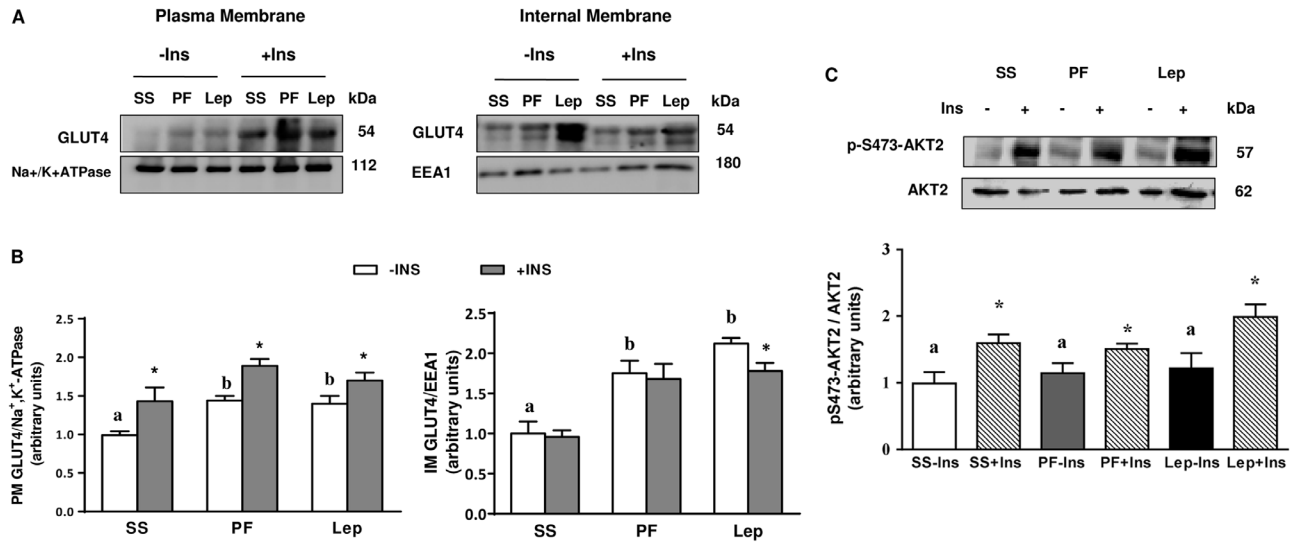

Figure 6
Central leptin infusion did not alter the subcellular distribution of GLUT4 in response to insulin in heart. (A) Representative Western blot out of three and (B) quantitative densitometric analysis of GLUT4 glucose transporter in 15 µg of protein from plasma membrane (PM) and internal membrane (IM) of heart from central leptin or saline-infused rats after 7 days of treatment and 30 min of *in vivo* insulin (10 IU/kg body weight) stimulation. Na^+/K^+-ATPase and EEA1 were used as control for protein loading for PM and IM, respectively, and for densitometric normalization. (C) Representative Western blot out of three and quantitative densitometric analysis of p-S473-AKT2 and AKT2 in 50 µg of total extracts from cardiac ventricles after 7 days of central saline or leptin infusion, and 30 min of *in vivo* insulin (10 IU/kg body weight) stimulation. Data are expressed as ratios of phosphoserine content in AKT2 normalize to the corresponding amount of AKT2 protein. Results are the mean ± S.E.M. of 6–8 rats per group. Different letters indicate significant differences among treatments, ($P \leq 0.05$, one-way ANOVA followed by Tukey test). Student's *t*-test, $^*P \leq 0.05$ vs basal, without insulin. Lep, leptin-infused rats; PF, vehicle-infused pair-fed rats; SS, vehicle-infused rats fed *ad libitum*.

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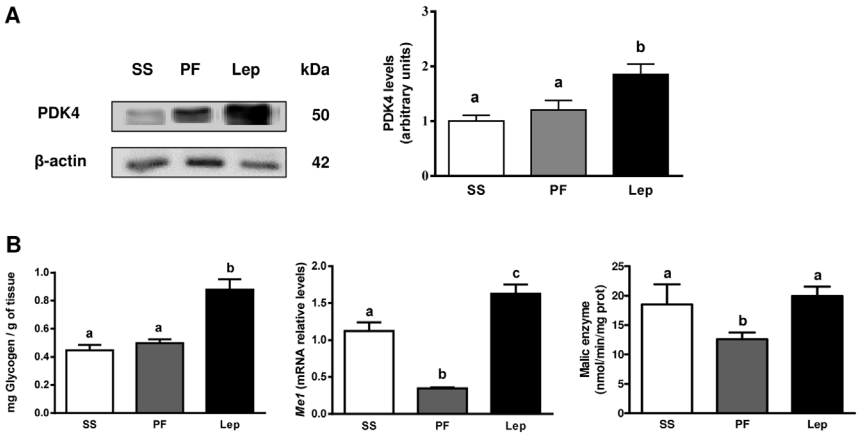

Figure 7
Central leptin increases cardiac protein levels of PDK4, glycogen content and activity of malic enzyme. (A) Effect of leptin infusion on protein levels of PDK4. Representative Western blot out of four and quantitative densitometric analysis of PDK4 protein levels in 50 µg of total extract from cardiac ventricles of leptin or saline-infused rats after 7 days of treatment. β-Actin was used as control for protein loading and for densitometric normalization. (B) Effect of leptin infusion on glycogen levels, mRNA levels and activity of malic enzyme. Results are the mean ± S.E.M. of 6–8 rats per group. Different letters indicate significant differences among treatments, ($P \leq 0.05$, one-way ANOVA followed by Tukey test). Lep, leptin-infused rats; PF, saline-infused pair-fed rats; SS, saline-infused rats fed *ad libitum*.

Slc2a1, hereafter referred to as *Glut1*) (Fig. 5B). Finally, the subcellular distribution of cardiac GLUT4 did not significantly change upon central leptin infusion neither under basal or insulin-stimulated conditions (Fig. 6A and B), supporting that the enhanced glucose transport in heart from central leptin-infused rats was independent of insulin signaling. In fact, central leptin treatment did not affect the cardiac basal or insulin-stimulated phosphorylation of AKT2 (Fig. 6C).

Next, we measured the protein levels of PDK4, an inhibitor of the glucose oxidation and a direct PPARβ/δ target gene (Degenhardt *et al.* 2007). In agreement with gene expression data, central leptin also upregulated the protein levels of PDK4 in the heart (Fig. 7A), suggesting a decrease in glucose oxidation in the hearts of central leptin-infused rats. To analyze the fate of the enhanced glucose uptake mediated by central leptin in heart, we measured glycogen levels. As can be seen, cardiac glycogen levels were significantly increased in central leptin-infused rats (Fig. 7B). Additionally, we also analyzed the effects of central leptin infusion on gene expression and activity of the cytoplasmic malic enzyme, an anaplerotic enzyme that generates malate for transfer into mitochondria as an alternate route for glucose oxidation (Pound *et al.* 2009). Central leptin infusion enhanced significantly both, the gene expression and the activity of the cytoplasmic malic enzyme compared with pair-fed rats (Fig. 7B).

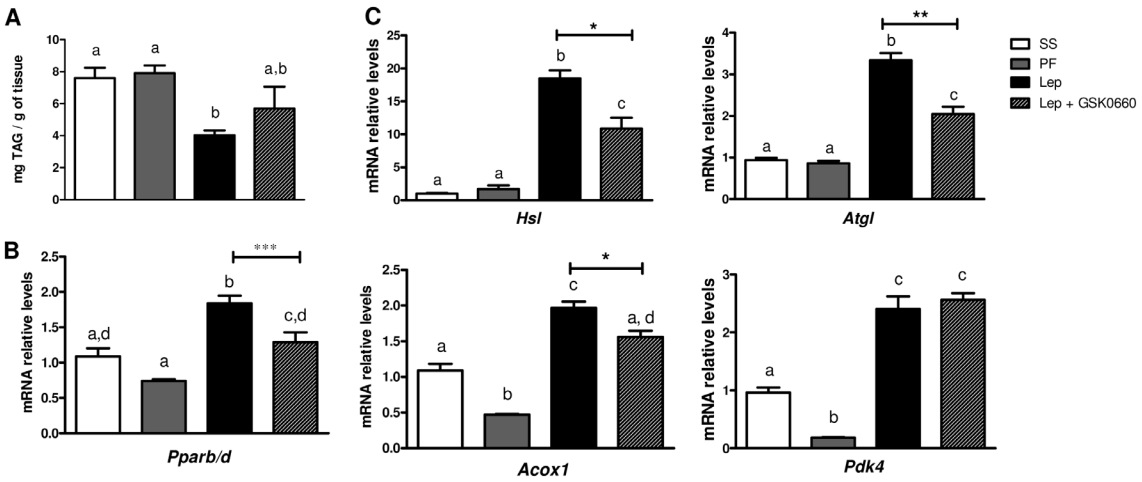

Figure 8
The PPARβ/δ antagonist GSK0660 reduces the effects of central leptin on both, cardiac triacylglyceride content and the expression of PPARβ/δ target genes. Central saline- or leptin-infused rats were co-treated with either vehicle (0.062% DMSO) or GSK0660 (1 mg/kg per day i.p.) for 7 days. (A) TAG content in heart after 7 days of central infusion in rats, as described in Fig. 2, co-treated with either vehicle or GSK0660. (B) Effect of GSK0660 on mRNA levels of *Pparb/d*. (C) Effects of GSK0660 on mRNA levels of target genes of PPARβ/δ. Results are the mean ± S.E.M. of 4–6 rats per group. Different letters indicate significant differences among treatments, ($P \leq 0.05$, one-way ANOVA followed by Tukey test). Student's *t*-test, $^*P \leq 0.05$ vs leptin. Lep, leptin-infused rats; PF, saline-infused pair-fed rats; SS, saline-infused rats fed *ad libitum*.

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The pharmacological inhibition of PPARβ/δ reduces the effects mediated by central leptin in heart

To confirm that the cardiac anti-steatotic effects of central leptin are associated to the selective upregulation of PPARβ/δ expression and their target genes, we investigated whether the pharmacological inhibition of PPARβ/δ decreases the effects mediated by central leptin in the heart. In central leptin-infused rats, PPARβ/δ antagonist treatment *in vivo* substantially increased cardiac TAG content (Fig. 8A), while markedly decreased the expression of *Pparb/d* (Fig. 8B) and their target genes involved in lipolysis (*Atgl* and *Hsl*) and in peroxisomal fatty acid oxidation (*Acox1*) (Fig. 8C).

Discussion

Earlier and recent works have established that acute or chronic peripheral leptin administration decreased cardiac TAG content (Atkinson *et al.* 2002, Lee *et al.* 2004, Rame *et al.* 2011). However, there are conflicting data about the role of central leptin infusion in regulating cardiac TAG accumulation (Keung *et al.* 2011, Sloan *et al.* 2011). Here, we demonstrated that central leptin upregulates the expression of genes involved in myocardial intracellular lipolysis (ATGL and HSL) and mitochondrial/peroxisomal fatty acid utilization (PDK4, UCP3, Acox1), whereas decreases those involved in TAG synthesis (SCD-1 and DGAT1). Besides, the peroxisomal palmitate oxidation is increased in central leptin-infused rats. As a result, we found a marked reduction in cardiac TAG content. These changes occur in parallel with selective upregulation of PPARβ/δ. Interestingly, the pharmacological inhibition of PPARβ/δ decreased the effects on gene expression and TAG content induced by central leptin. Moreover, our data also suggest that central leptin's effects on heart may be, at least in part, mediated by the sympathetic nervous system, as indicated by the slight increase in the cardiac NE turnover rate.

Previously, we showed that central leptin reduces TAG content in liver (~36%) and adipose tissue (~33%) by regulating the expression of PPARα and PGC-1α in a tissue-specific manner (Gallardo *et al.* 2007). The data presented herein demonstrate that the anti-steatotic effects of central leptin in heart were higher than those in liver and/or adipose tissue, decreasing ~50% the cardiac TAG content. Interestingly, the decrease in cardiac TAG content is paralleled with increased expression of PPARβ/δ and PGC-1β, whereas the protein levels of PPARα and PGC-1α were unaffected upon central leptin infusion. The cardiac anti-steatotic effects induced by central leptin were reduced by GSK0660, a selective inhibitor of PPARβ/δ, sustaining a central role of PPARβ/δ as mediator on central leptin's effects on cardiac metabolism. In contrast to our study, a recent report found that peripheral leptin administration in obese leptin-deficient (*ob/ob*) mice reduced cardiac steatosis in parallel with increased mRNA levels of *Ppara* and *Pgc1a*, and no significant change in *Pparb/d* and *Pgc1b* (Rame *et al.* 2011). Taken together, these data suggest remarkable differences between indirect or direct leptin actions in heart with respect to the regulation of protein expression of PPARs–PGC-1s in cardiac tissue.

There is abundant evidence about the role of the PPARα/PGC-1α pathway stimulating fatty acid catabolism in the heart especially in response to fasting (Leone *et al.* 1999). Besides, increased fatty acid oxidation has been previously observed in cardiomyocytes and/or heart overexpressing PPARα/PGC-1α pathway (Leone *et al.* 1999, Finck *et al.* 2002, Gilde *et al.* 2003, Benton *et al.* 2008). In this work, we observed that the restriction in food intake elicited by leptin and pair-feeding upregulates the protein levels of PPARα and PGC-1α in the heart in both groups of rats. In line with this observation, we shown that both treatments significantly induced the expression of their target genes *Cd36* and *Cpt1b*, associated with fatty acid availability/transport and oxidation, respectively, as well as Mfn2 involved in mitochondrial fusion/metabolism. Besides, both groups of rats presented similar cardiac mtDNA content and citrate synthase activity suggesting a comparable capacity for mitochondrial β-oxidation. Consistent with these results, we found that the complete palmitate oxidation rates in heart were unchanged in pair-fed and leptin-infused rats. Our results agree with previous data obtained in central leptin-infused mice (Keung *et al.* 2011, Sloan *et al.* 2011) and in cardiac-specific PPARβ/δ-overexpressing mice (Burkat *et al.* 2007).

A major finding of the present work is the marked upregulation of genes involved in myocardial intracellular lipolysis, *Atgl* and *Hsl*. To our knowledge, this is the first report showing the upregulation of cardiac expression of ATGL mediated by central leptin. Several lines of evidence suggest that ATGL-dependent lipolysis may have a crucial role in the regulation of cardiac TAG content and PPARs activation. In hearts of *Atgl*-deficient (*Atgl*-KO) mice and/or in mice with cardiac-specific ATGL deletion, the TAG content is increased (Haemmerle *et al.* 2011), whereas the

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TAG content is significantly reduced in hearts from cardiomyocyte-specific *Atgl*-overexpressing mice (Kienesberger *et al.* 2012). Second, the expression of *Acox1* and *Pdk4* is markedly reduced in heart of ATGL-KO mice, which is reverted to normal values upon cardiomyocyte-specific overexpression of ATGL in *Atgl*-KO mice (Haemmerle *et al.* 2011). Thus, the gene expression data reported herein are consistent with these observations and suggest that central leptin-induced lipolysis, through the upregulation of cardiac *Atgl* expression, might provide ligands or ligand precursors for the selective upregulation of *Pparb/d* and its target genes *Pdk4*, *Ucp3* and *Acox1*. In addition, it has been recently reported that *Scd1* deficiency decreased cardiac lipid content independently of PPARα by reducing lipogenesis and activating lipolysis (Bednarski *et al.* 2016). Thus, an enhanced lipolysis and low lipogenesis, as supported by the decreased expression of SCD-1 and DGAT1 mediated by leptin at central level, may provide higher fatty acid availability for mitochondrial and peroxisomal oxidation, which can explain the reduction in cardiac TAG content reported herein. In fact, although no changes were observed in complete palmitate oxidation, central leptin-infused rats present higher peroxisomal palmitate oxidation rates compared with pair-fed rats. It is known that peroxisomal fatty acid oxidation is incomplete and yields acetyl-CoA that may be used as a precursor to synthesize other complex molecules (Wanders & Waterham 2006) and that in isolated perfused rat hearts not all acetyl-CoA generated in the peroxisomes is transferred to the mitochondria for oxidation (Bian *et al.* 2005). Thus, it can be hypothesized that, for the maintenance of a normal cardiac contractile function, central leptin-infused rats would require an enhanced peroxisomal fatty acid oxidation rates in order to obtain the same amount of energy generated via mitochondrial fatty acid oxidation. This suggestion might explain the decrease in cardiac TAG content reported here in leptin compared with pair-fed rats despite similar food intake. Nevertheless, further work has to be done before a final conclusion may be drawn.

The contribution of PPARβ/δ in regulating the expression of genes involved in cardiac lipid metabolism is basically similar to PPARα (Finck 2007, Palomer *et al.* 2016). In fact, the expression of genes involved in mitochondrial fatty acid oxidation is decreased to similar levels in hearts of *Ppara*-null mice and/or in mice with cardiac-specific deletion of *Pparb/d* (Watanabe *et al.* 2000, Cheng *et al.* 2004). However, the expression of genes involved in peroxisomal fatty acid oxidation, such as *Acox1*, may be differently regulated. In fact, the gene expression of *Acox1* is unaffected in hearts of *Ppara*-null mice (Watanabe *et al.* 2000), while is significantly reduced in hearts from mice with cardiac-specific deletion of *Pparb/d* (Cheng *et al.* 2004). Conversely, an enhanced expression of *Acox1* has been reported in hearts of mice expressing a constitutively active form of *Pparb/d* (Liu *et al.* 2011). Consistent with these data, we show that upon central leptin infusion, the cardiac mRNA levels of *Acox1* are upregulated in association with increased mRNA and protein levels of PPARβ/δ, while *Acox1* mRNA levels are decreased after the pharmacological inhibition of PPARβ/δ. Overall, these observations suggest that PPARα and PPARβ/δ have complementary roles regulating the expression of shared target genes involved in cardiac lipid metabolism (Finck 2007, Palomer *et al.* 2016).

Earlier studies have reported that central and/or peripheral leptin administration enhances glucose uptake in heart, among other peripheral tissues (Kamohara *et al.* 1997, Minokoshi *et al.* 1999). Herein, we also found that central leptin enhanced basal rates of glucose transport in heart without altering GLUT4 protein levels. These results agree with previous data in skeletal muscle in which GLUT4 protein levels were unchanged upon leptin administration despite a significant increase in glucose uptake (Wang *et al.* 1999). Nevertheless, our data indicate for the first time that, the enhanced basal glucose uptake induced by central leptin in cardiac tissue may be attributed to the increased GLUT1 protein levels upon central leptin infusion. Besides, these changes occurred in parallel with the marked upregulation of cardiac PPARβ/δ expression and without significant changes in PPARα protein levels. In this sense, it has been reported that basal myocardial glucose uptake and expression of both GLUT1 and GLUT4 were enhanced in adult mice constitutively expressing an active form of *Pparb/d* in the heart (Burkat *et al.* 2007, Liu *et al.* 2011). Conversely, basal myocardial glucose transport and *Glut1* mRNA and protein levels were decreased in transgenic mice with cardiac-specific deletion of *Pparb/d*, despite increased GLUT4 total protein levels (Wang *et al.* 2010). On the other hand, although we did not directly measure cardiac glucose oxidation rates, the upregulation of PDK4 expression might suggest a decrease in glucose oxidation through pyruvate dehydrogenase. Nevertheless, we studied an alternate route for glucose oxidation, the anaplerotic cytoplasmic malic enzyme, as a compensatory pathway that may contribute to maintain the tricarboxylic acid cycle flux (Pound *et al.* 2009). In fact, we found that central leptin increase both, cardiac

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gene expression and activity of the cytoplasmic malic enzyme that generates malate for transfer and oxidation into mitochondria and consumes NADPH required for TAG formation, preventing the accumulation of TAG in the heart as we reported herein. Finally, consistent with previous data in adult mice constitutively expressing an active form of *Pparb/d* in the heart (Burkat *et al*. 2007), cardiac glycogen levels were significantly increased in central leptin-infused rats.

In conclusion, we demonstrate that leptin, acting at central level, regulates cardiac TAG content in rats with normal leptin sensitivity through the specific upregulation of PPARβ/δ and their target genes involved in cardiac metabolism. The cardiac gene expression pattern reported herein suggests that, central leptin probably increases lipolysis and reduces lipogenesis through the ATGL/HSL and SCD-1/DGAT1 pathways, respectively, and promotes higher peroxisomal fatty acid oxidation rates via PPARβ/δ/Acox1 in the heart, as a compensatory adaptation for lipid oxidation to control cardiac TAG content and lipotoxicity. Therefore, progress in strategies for preventing hypothalamic leptin resistance and/or improving leptin action in situations associated with central leptin resistance, as seen in human obesity, may represent a therapeutic approach to ameliorate cardiac steatosis and prevent cardiac dysfunction.

Supplementary data

This is linked to the online version of the paper at https://doi.org/10.1530/JOE-17-0554.

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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