Supplementary materials and methods

Intracerebroventricular leptin administration
Rats were anesthetized with intraperitoneal ketamine/diazepam/atropine used at 50mg/kg, 4mg/kg and 0.2mg/kg, respectively (Parke-Davis, Roche and Braun, Spain). During 7 days, leptin or saline (PBS) was administered in the lateral ventricle through a cannula connected to an osmotic minipump (Alzet, Palo Alto, CA), with a releasing rate of 1 μl/h, and filled with 0.0082 μg/μl (0.2 μg/day) rat leptin (Sigma), or its vehicle (PBS).

Serum metabolites, hormone analysis and myocardial TAG determination
Serum glucose, TAG, nonesterified fatty acid (NEFA) and cholesterol content were measured as previously described (Gallardo et al. 2007, Bonzón-Kulichenko et al. 2011). Blood lactate levels were measured immediately using an Accutrend Glucose Analyzer (Roche Diagnostics Corp., Indianapolis, IN) and total ketone body levels were determined using an enzymatic kit from Wako Chemicals (Neuss, Germany). Serum insulin, leptin and resistin levels were assayed using specific rat ELISA kits from Spi-Bio (Montigny le Bretonneaux, France) as described (Bonzón-Kulichenko et al. 2011). 100 mg of frozen rat ventricles were used for TAG determination using enzymatic kits from Biosystems as previously described (Gallardo et al. 2007).

Mitochondrial DNA (mtDNA) quantification
Total genomic DNA was isolated from rat ventricles (25 mg) using a DNA extracting kit (Biotools, cat 21.136-4195, Madrid, Spain) following manufacturer´s instruction. To quantify the amount of mtDNA, the primer sequence located within the mitochondrial 16S rRNA region was, forward primer: 5´-AATGGTTCTTTGTTCAACGATT-3´ and
reverse primer: 5´-AGAAACCGACCTGGATTGCTC-3´. To quantify the amount of nuclear DNA (nucDNA), the primer sequence located within the nuclear resistin region was, forward primer: 5´-ACTTAACAGGATGAAGAACCTTTCA-3´ and reverse primer: 5´-GTAGGGAGCTGAAGTCTTGATTGAT-3´. Mitochondrial DNA copy number relative to nuclear DNA copy number was quantified by qPCR as reported by Rooney et al. 2015.

**Palmitate oxidation**

Fresh rat ventricles (200 mg) were minced in 400 μl of homogenization buffer containing, 250 mM sucrose, 1 mM EDTA, and 10 mM Tris-ClH, pH 7.4, and then homogenized in ice-cold homogenization buffer (10 ml/g tissue) with a Teflon pestle for 10 passes over 30 s at 1,200 rpm. After centrifugation for 10 min at 420g, the supernatant was used as total ventricle homogenates which were kept on ice until oxidation experiments were performed. Ventricle homogenates were pre-incubated at 37ºC for 30 min in assay buffer containing the following final concentrations: 100 mM sucrose, 10 mM Tris-HCl, 5 mM KH₂PO₄, 80 mM KCl, 1 mM MgCl₂, 2 mM ATP, 2 mM L-carnitine, 1 mM NAD⁺, 2 mM malate, 0.05 mM coenzyme A, 1 mM DTT, pH = 7.4. After the pre-incubation period the reaction was started by adding 1.25% BSA, 0.2 mM palmitate and [9,10-³H] palmitate (1μCi/ml) final concentrations for 3 h at 37ºC. Complete palmitate oxidation (as ³H₂O) rates were measured in the absence or presence of 100 μM etomixir, a specific and irreversible inhibitor of CPT1. At the end of the incubation period, complete palmitate oxidation was assessed by quantitative collection of the ³H₂O determined by the vapor-phase equilibration method of Hughes et al. 1993. The difference between the complete palmitate oxidation and the CPT1-independent palmitate oxidation was taken as CPT1-dependent palmitate oxidation. Palmitate oxidation was expressed as nanomole of palmitate per milligram protein per hour.
NE determination and NE turnover rate quantification

The sympathetic nervous system activity was determined by measuring the NE turnover rate (NETO) based on the rate of decline in tissue NE content after inhibition of tyrosine hydroxylase with α-methyl-p-tyrosine (α-MPT) injection, a competitive inhibitor of the rate-limiting enzyme in NE biosynthesis. α-MPT was prepared in glacial acetic acid (0.5 μl/mg) and then diluted with sterile PBS to 300 mg/ml final concentration and injected intraperitoneally at 300 mg/kg. Briefly, on the 7th day of central infusion and after an overnight fasting, saline-infused pair-fed or leptin-infused rats were injected intraperitoneally either with 300 mg α-MPT/kg (time 0h), and 2 h later they were given a second injection with 150 mg α-MPT/kg. 4 h after the first injections, the animals were anesthetized by CO₂ inhalation and sacrificed by decapitation (time 4h). Blood and cardiac tissue were obtained and processed as before. Cardiac endogenous NE content from non-injected (time 0h) [NE]₀ and α-MPT injected (time 4h) [NE]₄ was measured using a NE research ELISA kit (Demeditec Diagnostics GmbH, Kiel, Germany), following the manufacturer’s protocol. Only two time points were used for the measurement of turnover as previously reported (Penn et al. 2006), since previous publications have shown linearity of the logarithmic decline in NE content. NETO was calculated as the product of fractional turnover rate (k) and the cardiac endogenous NE content at time 0h [NE]₀. The fractional turnover rate (k) was calculated as described by Penn et al. 2006: (k) = \( \frac{\log(\text{mean} \ [NE]_0) - \log(\text{mean} \ [NE]_4)}{(0.434 \times 4)} \).

Real-time RT-PCR

Total RNA was isolated from 50 to 70 mg of frozen rat ventricles previously pulverized under liquid N₂, resuspended in Trizol reagent (Invitrogen) and homogenized with Polytron homogenizer (PT 3000 Kinematic AG) for three 15s burst at 12000 rpm, on ice. The cDNA was synthesized from 1.5 μg of DNase-treated RNA by using the reverse-transcriptase activity from Moloney murine leukaemia virus (Gibco-BRL), and p[dn]₅ (Boehringer Mannheim, Germany) as random primer. Relative quantitation was performed using pre-developed probes (Supplementary Table S1) for fatty acid transporter (FAT/CD36), adipose triglyceride lipase (ATGL), hormone sensitive lipase.
(HSL), diacylglycerol acyltransferase 1 (DGAT-1), ATP citrate lyase (ACL), stearoyl-CoA desaturase 1 (SCD-1), cytoplasmic malic enzyme (ME1), peroxisome proliferator-activated receptor alpha, beta/delta and gamma (PPARα, PPARβ/δ and PPARγ); PPARγ coactivator-1 alpha and beta (PGC-1α and PGC-1β), mitofusin 2 (Mfn2); carnitine palmitoyltransferase Ib (CPT-1b); acyl CoA oxidase 1 (Acox1), uncoupling protein 3 (UCP3), pyruvate dehydrogenase kinase 4 (PDK4) mRNA levels, by TaqMan real-time PCR according to the manufacturer's protocol on an ABI PRISM 7500 FAST Sequence Detection System instrument and software (PE Applied Biosystem, Foster City, CA). To standardize the amount of sample cDNA added to the reaction, amplification of endogenous control 18S rRNA was included in separate wells using VIC (TaqMan Assay) as real-time reporter. The ΔΔCT method was used to calculate the relative differences between experimental conditions and control groups as fold change in gene expression.

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 100 mM Tris-HCL lysis buffer, pH 7.4, containing 100 mM sucrose, 10 mM EDTA, 46 mM KCl, supplemented with 1 M NaCl to dissociate actin filaments. After centrifugation for 2 min at 1000g, minced tissue was resuspended in lysis buffer (5 ml buffer/g tissue) supplemented with 20 mM NaF, 2 mM Na3VO4, 10 μg/mL leupeptin, 10 μg/mL aprotinin and 1 μg/mL pepstatin. Samples were homogenized with 30 passes of a loose fitting A pestle and 10 passes of a tight fitting B pestle. After centrifugation for 5 min at 4000g, the supernatant was saved and the pellet fraction was resuspended, homogenized and centrifuged as above. The two 4000g supernatants were combined and used for mitochondrial, plasma membrane (PM) and internal membrane (IM) fractionation using Optiprep (Axis-Shield, Norway) discontinuous gradient as described (Bao et al. 2001). Briefly, discontinuous gradients were setup in 4.4 ml tubes; 3 ml of the 4000g supernatants adjusted to 5% Optiprep final concentration were placed above 0.5 ml of 0.5 ml 15% Optiprep and overlaid with 0.5ml homogenization buffer. After centrifugation at 200,000g for 150 min, PM and IM were harvested from the 0%/5% and 5%/15% Optiprep interface, while the pellet constitutes the mitochondrial enriched fraction. All fractions were diluted with 10mM Tris-HCl buffer, pH 7.4, 1 mM EDTA, and recovered by centrifugation. The mitochondrial fraction, PM and IM were resuspended in homogenization buffer.
supplemented with protease and phosphates inhibitors as above and stored at −70°C until use. Protein concentration was determined by Bradford (Protein Assay, Bio-Rad, Spain) using BSA as standard.

**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 transferred to nitrocellulose sheets (0.2 µm; Bio-Rad, Spain) and incubated overnight at 4ºC with the corresponding primary antibody followed by incubation at room temperature for 30 min with secondary antibody conjugated with horseradish peroxidase. Primary rabbit polyclonal antibodies were: anti-pT102-AMPK (1:1000, ab23875), anti-AMPK (1:500, ab39644), anti-pS79-ACC (1:2000, ab31931), anti-ACC (1:2000, ab45174), anti-PPARα (1:1000, ab8934), anti-PPARγ (1µg/mL, ab27649), anti-PPARβ/δ (2µg/mL, ab23673), anti-PDK4 (1:500, ab89295), anti-FAT/CD36 (1:500, ab36977) from Abcam, Cambridge, UK, anti-pS473-AKT2 (1:1000, 9271), anti-AKT2 (1:1000, 9272), anti-p-Y705-STAT3 (1:1000, 9131), anti-STAT3 (1:1000, 9132) from Cell Signaling, anti-GLUT1 (1:200, Sc7903) from Santa Cruz Biotechnology, and secondary antibody goat anti-rabbit conjugated with horseradish peroxidase (1:5000, 172-1019, Bio-Rad, Spain). Primary mouse monoclonal antibody were anti-Na+/K+-ATPase (1:500, ab7671), anti-PGC-1β (1:1000, ab176328), anti-β-actin (1:1000, ab8226) from Abcam, Cambridge, UK, anti-GLUT4 (IF8) (1µg/mL, 2213) from Cell Signaling, anti-EEA1 (1:2500, Nº 610457) from BD Transduction laboratories and secondary antibody goat anti-mouse conjugated with horseradish peroxidase (1:5000, 170-6516, Bio-Rad, Spain).

Blots were repeated 3 times to assure the reproducibility of the results. The immunocomplexes formed were visualized using the ECL Western-blotting detection kit (Amersham Biosciences, Inc., Piscataway, NJ) with a G-Box Densitometer, and bands were quantified by scanning densitometry with the exposure in the linear range using Gene Tools software (Syngene, Cambridge, UK). β-actin, Na⁺/K⁺-ATPase and early endosome antigen 1 (EEA1) were used as controls for protein loading of the total tissue extract, PM, and IM fractions, respectively. Samples from rats infused with vehicle or leptin in all experimental conditions were run on the same gel to allow a direct comparison. The densitometric values of pY705-STAT3, pT172-AMPK, pS79-
ACC and pS473-AKT2 were normalized to the densitometric values of the corresponding amount of protein mass in the same sample. Data were expressed as a ratio of pY705-STAT3/STAT3, pT172-AMPK/AMPK, pS79-ACC/ACC and pS473-AKT2/AKT2. Finally, 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. Explants were placed immediately after dissection in 0.5 ml Krebs-Ringer-HEPES (KRH) (pH 7.4) supplemented with 1.2 mM CaCl₂ and 2% BSA (KRH/Ca²⁺-BSA), and were further incubated in the absence or presence of 80 nM insulin for 10 min at 37°C. 2-Deoxyglucose (0.2 mM) and [³H]-2-deoxyglucose (2 µCi/ml) (Amersham Pharmacia Biotech) were used to analyze the basal glucose uptake and insulin-mediated glucose uptake, as previously described (Bonzón-Kulichenko et al. 2011).

**REFERENCES**


