ORIGINAL ARTICLE

Proanthocyanidins potentiate hypothalamic leptin/STAT3 signalling and *Pomc* gene expression in rats with diet-induced obesity

M Ibars, A Ardid-Ruiz, M Suárez, B Muguerza, C Bladé and G Aragonès

OBJECTIVE: Dietary obesity is usually linked with hypothalamic leptin resistance, in which the primary impact is an interference in the homeostatic control of body weight and appetite. Notably, proanthocyanidins (PACs), which are the most abundant phenolic compounds present in human diet, modulate adiposity and food intake. The aim of this study was to assess whether PACs could re-establish appropriate leptin signalling in both the hypothalamus and peripheral tissues.

DESIGN: Male Wistar rats were fed either a standard chow diet (STD group, $n = 7$) or a cafeteria diet (CD) for 13 weeks. The CD-fed rats were treated with either grape-seed PAC extract (GSPE) at 25 mg per kg of body weight per day (CD+GSPE group, $n = 7$) or with the vehicle (CD group, $n = 7$) for the last 21 days of the study period. Specific markers for intracellular leptin signalling, inflammation and endoplasmic reticulum stress in the hypothalamus, liver, mesenteric white adipose tissue and skeletal muscle were analysed using immunoblotting and quantitative PCR.

RESULTS: GSPE treatment significantly reduced the food intake but did not reverse the hyperleptinemia and body wt gain assessed. However, the animals treated with GSPE exhibited greater hypothalamic activation of signal transducer and activator of transcription-3, which was associated with a rise in the *Pomc* mRNA levels compared with the CD group. In addition, this restoration of leptin responsiveness was accompanied by lower local inflammation and increased *Sirt1* gene expression. The effects of the GSPE treatment in the peripheral tissues were not as evident as those in the hypothalamus, although the GSPE treatment significantly restored the mRNA levels of *Socs3* and *Ptp1b* in the skeletal muscle.

CONCLUSIONS: The use of GSPE reduces hyperphagia and improves the central and peripheral leptin resistance associated with diet-induced obesity. Our results suggest that GSPE could exert these effects partially by increasing Sirt1 expression and preventing hypothalamic inflammation.

International Journal of Obesity (2017) **41,** 129–136; doi:10.1038/ijo.2016.169

INTRODUCTION

Obesity has reached truly epidemic proportions worldwide and has become one of the most prevalent health problems that our world currently faces.[1] In mammals, energy balance is regulated by controlling food intake and energy expenditure (EE) by means of the interactions of peripheral nutrients and hormones with different neuronal subpopulations. The critical cell populations include the anorexigenic pro-opiomelanocortin (POMC)- and orexigenic Agouti-related protein (AgRP) -expressing neurons, both located in the arcuate nucleus of the hypothalamus.[2]

Leptin, a hormone secreted mainly from white adipose tissue, is the main molecule that transmits information regarding the energy stores of the periphery to the hypothalamus.[3] The interaction of leptin with its longest receptor isoform (Obrb) in the POMC- and AgRP-expressing neurons promotes the phosphorylation of the signal transducer and activator of transcription-3 (STAT3). Then, STAT3 dimerizes and translocates from the cytoplasm into the nucleus, where it binds to the POMC and AgRP promoters. This stimulates the expression of POMC and inhibits that of AgRP reducing food intake and increasing EE.[4,5]

However, leptin is completely ineffective in decreasing food intake and suppressing body weight gain in subjects with diet-induced obesity. In this condition, instead of a leptin deficiency, high circulating levels of the hormone are observed, but the high levels are associated with a loss of responsiveness.[6–8] Although the basis of leptin resistance is not completely understood, it has been generally related to several mechanisms[9–11] including reduced leptin transport across the blood–brain barrier and the enhancement of intracellular processes that attenuate Obrb signalling. These process include hypothalamic inflammation and endoplasmic reticulum (ER) stress, which, in turn, upregulates the expression of negative regulatory molecules, including suppressor of cytokine signalling 3 (SOCS3) and protein-tyrosine phosphatase 1B (PTP1B).[12] Moreover, the hypothalamic NAD+-dependent deacetylase sirtuin 1 (SIRT1) has been confirmed to be a mediator of leptin action in POMC- and AgRP-expressing neurons, in which it suppresses nuclear factor-κB (NF-κB) signalling and/or regulates ER stress reactions through the deacetylation of the active spliced form of X-box-binding protein-1 (XBP1s).[13] Therefore, hypothalamic SIRT1 activity may be an additional mechanism that is involved in the regulation of leptin signal transduction.[14]

Within this context, because pharmacologic methods to restore the leptin levels and sensitivity have not yet been found, the use of bioactive food compounds may be a useful approach that could complement the existing therapeutic strategies.[15] In this sense,

Nutrigenomics Research Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili (URV), Campus Sescelades, Tarragona, Spain. Correspondence: Professor C Bladé, Nutrigenomics Research Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili (URV), Campus Sescelades, Tarragona 43007, Spain.
E-mail: mariacinta.blade@urv.cat

natural dietary polyphenols, specifically proanthocyanidins (PACs), which are a class of flavonoids structurally complex, are bioactive food compounds present in fruits and vegetables and are significantly implicated in health promotion.[16] In particular, our group and others have reported many beneficial effects of grape-seed PACs on various obesity-associated diseases including insulin resistance, dyslipidemia, hypertension and local and systemic inflammation.[17–21] Moreover, although there have been some questionable results regarding the potential effects of these compounds on the control of body weight in diet-induced obesity,[22] latest studies have shown that grape-seed PACs have the potential to significantly modulate food intake and adiposity.[19,23,24] Thus, we hypothesized that the chronic consumption of dietary PACs could rescue the anorexigenic actions of leptin by interfering with those metabolic abnormalities that attenuate leptin signalling in diet-induced obesity. Accordingly, the aim of the present study was to evaluate the effects of a grape-seed PAC extract (GSPE), administered for 21 days to rats previously fed a cafeteria diet (CD), on the central and peripheral leptin resistance induced by the CD.

MATERIALS AND METHODS

Grape-seed PAC extract
The GSPE was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, the extract is mainly composed of phenolic compounds (total content higher that 96%) including procyanidin monomers or flavan-3-ols (21.3%), and dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5–13 units; 31.7%) of procyanidins. The phenolic composition of this extract was further analysed by Quiñones *et al.*[25]

Animals and diet
The investigation was carried out in accordance with the ethical standards and according to the Declaration of Helsinki and was approved by the Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 4249 by Generalitat de Catalunya).

Male Wistar rats of 200 ± 50 g body wt were purchased from Charles River Laboratories (Barcelona, Spain). The animals were singly housed in a 12 h light–dark cycle at 22 ºC, fed a standard chow diet (STD, Panlab 04, Barcelona, Spain) *ad libitum* and were provided access to tap water during the adaptation week. After the adaptation week, animals were distributed into three equivalent groups of seven animals and housed individually in cages to permit control of their food intake. One group was fed with STD with a calorie breakdown of 14% protein, 8% fat and 73% carbohydrates, and the other groups were fed with STD plus CD composed by 14% protein, 35% fat and 51% carbohydrates. The animals had access during the dark phase to STD and water and to CD *ad libitum*. CD consisted of bacon, carrots, cookies, foie-gras, cupcakes, cheese and sugary milk. Ten weeks later, an oral treatment was administered in combination with the CD diet for 21 days. The treated group (CD+GSPE) received 25 mg of GSPE per kg body weight dissolved in 5% gum arabic (G9752, Sigma-Aldrich, Madrid, Spain), and the CD group received 1 ml of gum arabic. The rats received the treatment in the afternoon and then were allowed *ad libitum* access to STD and CD at night. On day 21 of treatment, the rats were fasted for 3 h before anaesthesia with 50 mg per kg of body weight of sodium pentobarbital (Fagron Iberica, Barcelona, Spain) and killed by abdominal aorta exsanguination. Blood was collected using heparin (Deltalab, Barcelona, Spain) as the anticoagulant. The plasma was obtained by centrifugation (1500 g, 4 °C, 15 min) and stored at -80 °C. The hypothalamus, liver, muscle and mesenteric white adipose tissue (mWAT) were excised, weighed, immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

Adiposity index
The adiposity index was determined by the sum of the mWAT, perirenal white adipose tissue (pWAT) and epididymal white adipose tissue (eWAT) depot weights. Results were expressed as percentage of the total body wt.

Plasma leptin levels
The plasma leptin levels were determined using an enzyme immunoassay kit according to the manufacturer's instructions (Biosource International, Inc., San Diego, CA, USA).

Indirect calorimetry
Indirect calorimetry analyses were performed on day 20 of the treatment using a Oxylet Pro System (PANLAB). Food was removed at 0900 hours, and the animals were fasted for 7 h (from 0900 to 1600 hours). After an initial acclimatization period of 1 h, oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured for 6 h. The respiratory quotient (RQ), EE and substrate oxidation were calculated as previously described.[26] Briefly, the RQ was calculated as the VCO2/VO2 ratio and the EE in kcal day^{-1} kg^{-1} 0.75 as $VO2 \times 1.44 \times [3.815+(1.232 \times RQ)]$. The rate of carbohydrate and fat oxidation were calculated in g min^{-1} as $4.55 \times VCO2 - 3.21 \times VO2 - 2.87 n$ and as $1.67 \times VO2 - 1.67 \times VCO2 - 1.92 n$, respectively. A nitrogen excretion rate (n) of 135 µg/kg/min was assumed. Finally, to obtain the values of fat and carbohydrate oxidation in kJ min^{-1}, the fat and carbohydrate rates were multiplied by 37 and 16, respectively, using the Atwater general conversion factor.

Total RNA isolation
Total RNA from the hypothalamus, liver, muscle and mWAT was extracted using the TRIzol LS Reagent (Life Technologies, Uppsala, Sweden) and RNeasy Mini Kit (Qiagen, Barcelona, Spain) according to the manufacturers' protocols. The quantity and purity of RNA was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Madrid, Spain). RNA integrity was evaluated on denaturing electrophoretic gels stained with SYBR Green dye (Bio-Rad, Barcelona, Spain), and only samples with an adequate RNA concentration (A260/A280 $\geqslant$ 1.8) and purity (A230/A260 $\geqslant$ 2.0) were selected for reverse transcription.

Gene expression analysis
The cDNA was generated using the High-Capacity complementary DNA Reverse Transcription Kit from Life Technologies and was subjected to quantitative PCR using the CFX96 real-time system-C1000 Touch Thermal Cycler (Bio-Rad) with SYBR Green PCR Master Mix (Bio-Rad). The forward and reverse primers for the various genes used in this study are shown in Supplementary Table 1 and were obtained from Biomers.net (Ulm, Germany). A cycle threshold (Ct) value was defined by setting the threshold during the geometric phase of the cDNA sample amplification. The fold change in expression of each mRNA was calculated with respect to the STD group using the $\Delta\Delta$Ct method corrected for the primer efficiency and converted to relative expression ratio with *Ppia* as the reference gene.[27]

Western blot analysis
Activated STAT3 in the hypothalamus, liver, mWAT and skeletal muscle was visualized using a phospho-specific antibody that recognized Tyr705-phosphorylated STAT3 (p-STAT3). In addition, the protein levels of the ObRb leptin receptor isoform as well as total and phosphorylated eIF2α in the hypothalamus were also determined by western blot analysis. Tissues were homogenized at 4 °C in 0.5 ml of Radio-Immunoprecipitation Assay lysis buffer containing protease and phosphatase inhibitor cocktails using a TissueLyser LT (Qiagen). The homogenate was incubated for 30 min at 4 °C and then centrifuged at 20 000 g for 15 min at 4 °C. The supernatant was used for total protein and western blot analyses. The total protein content was measured using the Pierce BCA protein assay kit (Thermo Scientific).

A total of 100 µg of protein was solubilized and boiled for 10 min in a loading buffer solution containing Tris HCl 0.5 M, pH 6.8; glycerol, SDS, β-mercaptoethanol and Bromophenol Blue. The total protein extracts were separated using SDS–polyacrylamide gel electrophoresis (10% polyacrylamide) and electrotransferred onto supported polyvinylidene difluoride membranes (Trans-Blot Turbo Mini polyvinylidene difluoride Transfer Packs from Bio-Rad). After blocking, the membranes were incubated with agitation overnight at 4 °C with antibodies specific for ObRb (Abcam, Cambridge, UK), p-STAT3 (Abcam) or total or phosphorylated eIF2α (Cell Signalling, Izasa SA, Barcelona, Spain), diluted 1:1000 and then with the goat anti-rabbit secondary antibodies (Sigma-Aldrich), diluted 1:10 000. For β-actin analysis, the membranes were incubated with a rabbit anti-actin primary antibody (Abcam) and then with a goat anti-rabbit secondary antibody (GE Healthcare, Barcelona, Spain), using the same dilutions

specified above. The protein levels were detected with the chemiluminescent detection reagent ECL Select (GE Healthcare) and using GeneSys image acquisition software (G:Box series, Syngene, Barcelona, Spain). Finally, protein band quantification was performed using ImageJ (NIH, MD, USA).

Statistical analysis

The data are expressed as the means ± s.e.m. A two-tailed Student *t*-test was used to evaluate the differences between two groups. Multiple independent groups were compared with a one-way analysis of variance followed by Tukey or Dunnett's T3 *post hoc* test when necessary. Outliers were determined by Grubbs' test. The statistical analyses were performed using SPSS (SPSS, Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

GSPE treatment ameliorated food intake but did not reverse the obesity and hyperleptinemia induced by the CD

As shown in Table 1, CD for 13 weeks consistently resulted in obesity and loss of leptin sensitivity in our experimental model as indicated by significant increases in the body wt and circulating leptin levels. Moreover, CD increased the adiposity index and the wt of all of the WAT depots studied including the mWAT, pWAT and eWAT depots, which confirmed the robust metabolic correlation between leptin and fat mass (rho = 0.901, $P < 0.001$). However, the administration of GSPE to the CD-fed rats for 21 days did not significantly exert anti-obesity effects, indicating that GSPE consumption during 21 days did not prevent the total body wt gain measured at the end of the experimental period and did not significantly reduce the adiposity index and the weights of all the WAT depots studied (Table 1). A very similar pattern was also observed for the leptin levels, which exhibited a slight decrease (9.4% lower) in CD+GSPE group, but the difference from that of the CD group was not statistically significant ($P = 0.41$).

Notably, the energy intake in the GSPE-treated group was significantly lower than that of the CD rats (Table 2). In addition, because leptin has been reported to maintain high-EE even though it reduces energy intake, we reasoned that the GSPE treatment, despite its effects on energy intake, could also enhance EE and lead to the utilization of fat as the main energy source. Thus, we measured the EE and the RQ in both CD- and GSPE-treated rats. However, no significant differences were observed between the groups in the RQ values, EE or substrate oxidation (Table 2).

GSPE treatment activated the hypothalamic leptin receptor-STAT3 pathway in the CD-fed rats.

To determine whether the observed decrease in energy intake indicated that the GSPE treatment affected the functions of the POMC- and AgRP-expressing neurons, we initially assessed the leptin signalling pathway by measuring the STAT3 activation in the hypothalamus using a phospho-specific antibody that recognized Tyr705-phosphorylated STAT3 (p-STAT3). Indeed, CD did lead to a significant decrease in the basal levels of p-STAT3 in the hypothalamus, and when GSPE was administered to the CD group, the levels of p-STAT3 increased significantly to basal levels. This showed that the GSPE treatment reversed the phenomenon observed in CD rats (Figure 1a) and indicated that treatment of the CD rats with GSPE for 21 days was sufficient to rescue the leptin signalling in this tissue.

Next, we determined whether the modulation of hypothalamic p-STAT3 was directly mediated by enhanced cell surface content of the long leptin receptor isoform Obrb. However, the CD group showed only a slight decrease in the mRNA levels of Obrb compared with the STD group, and no statistically significant difference between these groups were detected. In addition, the mRNA levels of Obrb were not enhanced after the administration of GSPE to the CD rats (Figure 1b). This result indicated that if GSPE is, indeed, a true leptin sensitizer, its effects are not mediated by a rise in the Obrb content of the hypothalamic cells. Accordingly, the Obrb protein levels as assessed by western blotting were also not affected by the GSPE treatment. Finally, the gene expression of both the short leptin receptor isoform Obra and low-density lipoprotein-related protein 2 (Lrp2), also called megalin, was assessed to determine whether the modulation of hypothalamic p-STAT3 was a result of an enhanced transcellular transport of leptin into hypothalamus. However, the mRNA levels of both Obra and Lrp2 were similar in all three groups of animals, and the differences among the groups did not reach statistical significance (Figure 1c).

To further investigate the effects of GSPE treatment on the regulation of the leptin signalling pathway, the gene expression of negative feedback regulatory molecules, namely SOCS3 (Socs3) and PTP1B (Ptp1b), was also assessed by quantitative PCR. Notably, the Socs3 mRNA levels were decreased in the CD-fed rats compared with the STD group, and the GSPE treatment significantly increased its transcript levels relative to the CD-fed rats. In addition, the mRNA expression of Ptp1b was reduced in the

Table 1. Body and adipose tissue weights, adiposity index and plasma concentrations of leptin in rats fed the STD or the CD and treated with GSPE or the vehicle

	STD	CD	CD+GSPE
Initial body wt (g)	288 ± 9	289 ± 14	292 ± 17
Final body wt (g)	443 ± 11[a]	531 ± 15	511 ± 17
Body wt gain (g)	155 ± 23[a]	242 ± 31	219 ± 43
Mesenteric WAT (g)	6.26 ± 0.6[a]	14.00 ± 1.5	13.33 ± 0.9
Perirenal WAT (g)	8.96 ± 0.4[a]	20.17 ± 2.0	19.51 ± 0.6
Epididymal WAT (g)	10.02 ± 0.6[a]	21.61 ± 2.5	18.10 ± 0.8
Adiposity index (%)	5.80 ± 0.3[a]	10.42 ± 0.8	9.89 ± 0.2
Leptin (ng ml^{-1})	10.09 ± 1.4[a]	27.85 ± 2.5	25.24 ± 2.4

Abbreviations: ANOVA, analysis of variance; CD, cafeteria diet; GSPE, grape-seed proanthocyanidin extract; STD, standard chow diet; WAT, white adipose tissue; wt, weight. The rats were fed a STD (STD group) or a CD for 10 weeks. After 10 weeks, the rats fed the cafeteria diet were treated orally with GSPE (25 mg per kg of body wt) (CD-GSPE group) or the vehicle (CD group) for 21 days. The adiposity index was computed for each animal as the sum of the different WAT weights, expressed as a percentage of body weight. The values are the means ± s.e.m. of seven samples from each group. [a]Denotes significant differences ($P < 0.05$) with respect to both CD and CD+GSPE groups assessed using one-way ANOVA and Tukey's *post hoc* test.

Table 2. Food intake, substrate oxidation and energy expenditure in in rats fed with a CD and supplemented with GSPE or vehicle

	CD	CD+GSPE
Energy intake (kJ day^{-1} per animal)	1197.32 ± 31.1	1105.83 ± 45.4[a]
Respiratory quotient	0.89 ± 0.1	0.88 ± 0.1
Energy expenditure (kcal day^{-1} kg^{-1} 0.75)	68.28 ± 2.2	66.73 ± 5.3
Carbohydrate oxidation (kJ min^{-1} kg^{-1} 0.75)	127.39 ± 17.9	152.59 ± 34.9
Fat oxidation (kJ min^{-1} kg^{-1} 0.75)	51.76 ± 11.4	61.33 ± 9.5

Abbreviations: CD, cafeteria diet; GSPE, grape-seed proanthocyanidins extract; STD, standard chow diet. After 10 weeks rats fed a CD received oral treatment of GSPE (25 mg per kg of body wt) (CD-GSPE group) or vehicle (CD group) for 21 days. Values are mean ± s.e.m. of seven samples from each group. [a]Denotes significant differences ($P < 0.05$) with respect to STD group assessed by Student's *t*-test.

CD-fed rats compared with STD group, but the GSPE treatment did not induce any significant change after the treatment period compared with the CD-fed rats (Figure 1d).

GSPE treatment selectively regulated the expression of hypothalamic peptides involved in appetite regulation

Then we evaluated the hypothalamic mRNA levels of POMC (*Pomc*), agouti-related peptide (*Agrp*) and neuropeptide Y (*Npy*). Interestingly, the *Pomc* mRNA levels were significantly increased in the GSPE-treated rats compared with both the STD group and CD group (Figure 2a). Furthermore, and in contrast to our expectations, we found that the *Agrp* gene expression levels were also significantly increased although to a much lower degree than the *Pomc* levels (Figure 2b). The *Npy* mRNA levels were not statistically altered by either the CD or GSPE treatment (Figure 2c).

GSPE treatment significantly potentiated the gene expression of hypothalamic *Sirt1* in a manner consistent with an attenuation of local inflammation

To elucidate the intracellular effects by which GSPE treatment potentially rescues leptin signalling in the hypothalamus, we assessed the impact of these compounds on the molecular processes associated with leptin signalling disruption, including local inflammation, ER stress and loss of SIRT1 activity in this tissue.

The contributions of these processes to leptin resistance were initially investigated using the gene expression of inducible nitric oxide synthase (*inos*), which is an important marker of neuroinflammation. Indeed, although the CD did not induce local inflammation in this tissue, as indicated by the similar mRNA levels in the STD and CD groups, the GSPE treatment significantly downregulated the *inos* gene expression (Figure 3a), which confirmed the ability of these compounds to prevent local
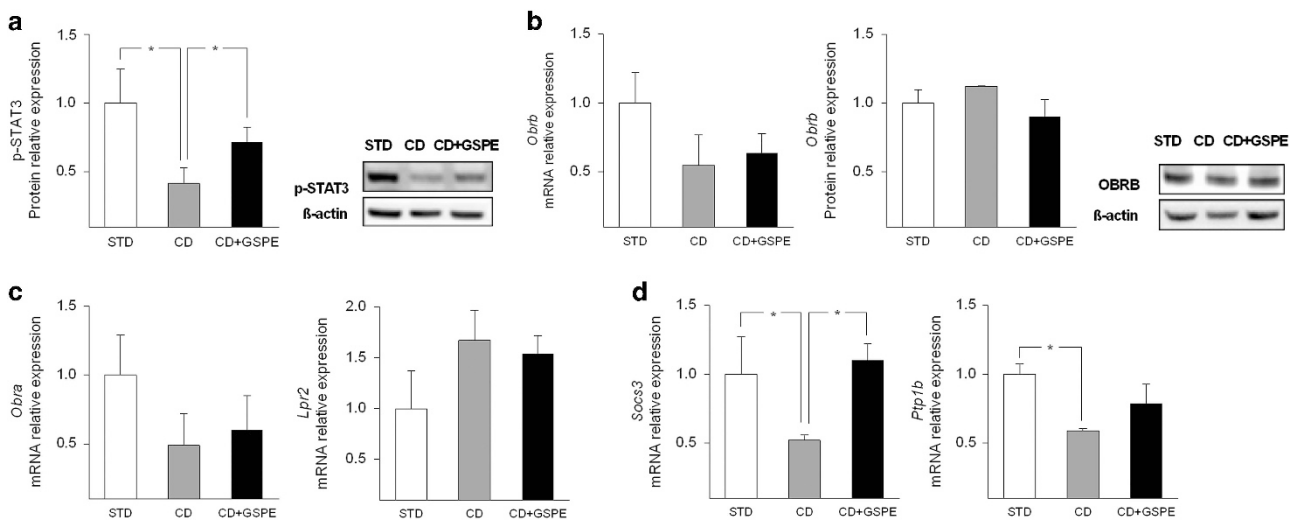

Figure 1. Effect of GSPE treatment on the hypothalamic leptin signalling. The leptin signalling pathway was primarily assessed by evaluating the STAT3 activation in the hypothalamus using a phospho-specific antibody that recognized Tyr705-phosphorylated STAT3 (p-STAT3) (**a**) and by the determination of the cell surface content of the long leptin receptor isoform b (Obrb) (**b**). In addition, quantitative PCR was used to investigate the gene expression of both the short leptin receptor isoform a (Obra) and low-density lipoprotein-related protein 2 (Lrp2) (**c**). Finally, the gene expression levels of the negative feedback regulatory molecules Socs3 and Ptp1b were also determined in this tissue (**d**). The rats were fed either the STD (*n* = 7) or CD for 13 weeks. The CD-fed rats were treated with either GSPE at 25 mg per kg of body wt per day (CD+GSPE group, *n* = 7) or with the vehicle (CD group, *n* = 7) during the last 21 days of the study. The values shown are the means ± s.e.m. *indicates significant differences between the groups at $P \leqslant 0.05$, as assessed using one-way analysis of variance.
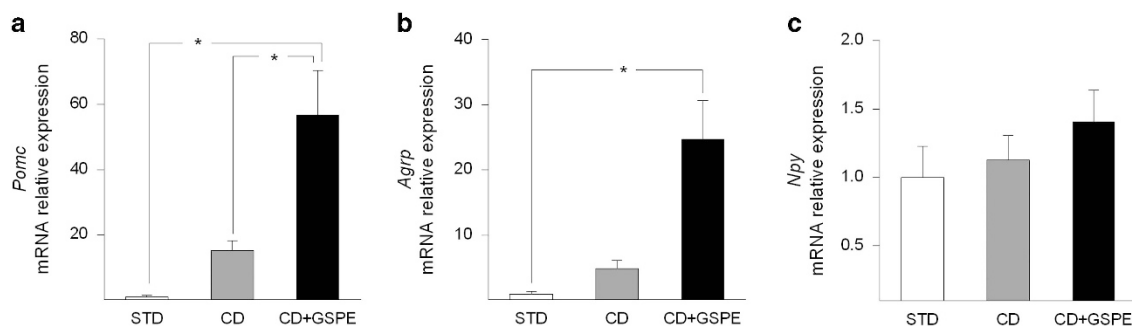

Figure 2. Effect of GSPE treatment on the regulation of hypothalamic neuropeptide gene expression. The mRNA levels of hypothalamic pro-opiomelanocortin (Pomc) (**a**), agouti-related peptide (Agrp) (**b**) and neuropeptide Y (Npy) (**c**) were assessed using quantitative PCR. The mRNA levels of the selected neuropeptides were normalized to those of Ppia. The rats were fed either the STD (*n* = 7) or CD for 13 weeks. The CD-fed rats were treated with either GSPE at 25 mg per kg of body wt per day (CD+GSPE group, *n* = 7) or with the vehicle (CD group, *n* = 7) during the last 21 days of the study. The values shown are the means ± s.e.m. *indicates significant differences between the groups at $P \leqslant 0.05$, as assessed using one-way analysis of variance.
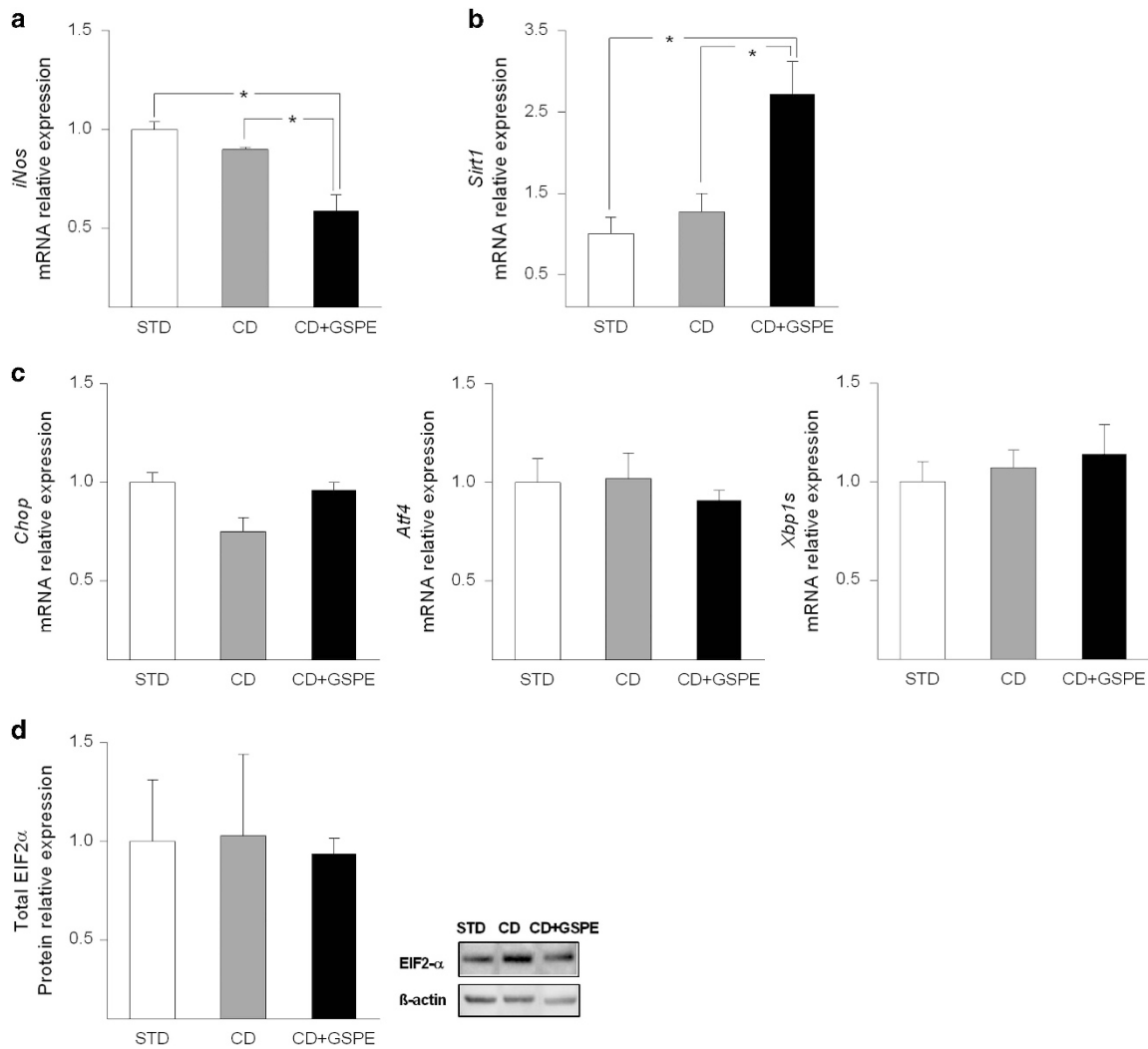
Figure 3. Effect of GSPE treatment on hypothalamic inflammation, sirtuin expression and ER stress. To evaluate the possible mechanisms responsible for the GSPE effects on hypothalamic leptin signalling, the gene expression of inducible nitric oxide synthase (inos) (**a**) and sirtuin 1 (Sirt1) (**b**) was investigated in this tissue. In addition, the hypothalamic mRNA levels of the ER stress markers X-box-binding protein-1 (XBP1) spliced form, and ATF4 and CHOP were determined using quantitative PCR (**c**). The mRNA levels of these selected genes were normalized to those of Ppia. In addition, the protein levels of total and phosphorylated eIF2α were also assessed by immunoblotting (**d**). The rats were fed either the STD ($n = 7$) or CD for 13 weeks. The CD-fed rats were treated with either GSPE at 25 mg per kg of body wt per day (CD+GSPE group, $n = 7$) or with the vehicle (CD group, $n = 7$) during the last 21 days of the study. The values shown are the means ± s.e.m. *indicates significant differences between the groups at $P \leqslant 0.05$, as assessed using one-way analysis of variance.

inflammation. Notably, GSPE treatment resulted in a significant upregulation (threefold higher) of the Sirt1 mRNA levels in a manner consistent with reduced hypothalamic inflammation (Figure 3b).

Finally, to address the molecular implications of ER stress in leptin resistance, hypothalamic ER stress markers, including the spliced form of X-box-binding protein-1 (XBP1s) and the levels of ATF4 and CHOP mRNA, were also determined by quantitative PCR. However, our results indicated that GSPE treatment did not modify the gene expression levels of any of these indicators (Figure 3c). In addition, other ER stress markers were assessed by western blotting. However, the total eIF2α protein levels did not display significant differences among the groups (Figure 3d), and the phosphorylated form of eIF2α was not detected in any group of animals. These results indicate not only that the CD did not induce ER stress in this tissue but that these markers of ER stress were also not significantly affected by the GSPE treatment.

GSPE treatment distinctively modulated leptin signalling in the liver, mWAT and skeletal muscle of the CD-fed rats
Alternatively, to assess the contribution of the metabolic signals derived from peripheral tissues to the regulation of energy intake and energy homeostasis, we next investigated the leptin signal transduction in the liver, mWAT and skeletal muscle. Indeed, a decrease in the level of p-STAT3 was observed in the livers of the CD and GSPE groups compared with STD group but the differences did not reach statistical significance (Figure 4a). Furthermore, no significant differences were observed in the mRNA levels of Obrb, Socs3 and Ptp1b among the three groups of animals indicating that GSPE had no effect on restoring leptin sensitivity in this tissue.

In the mWAT (Figure 4b), the CD-fed rats showed a significant increase in p-STAT3 compared with the STD-fed rats, whereas the GSPE treatment significantly restored the p-STAT3 values to those of the STD-fed rats. Furthermore, the mRNA levels of Obrb, Socs3
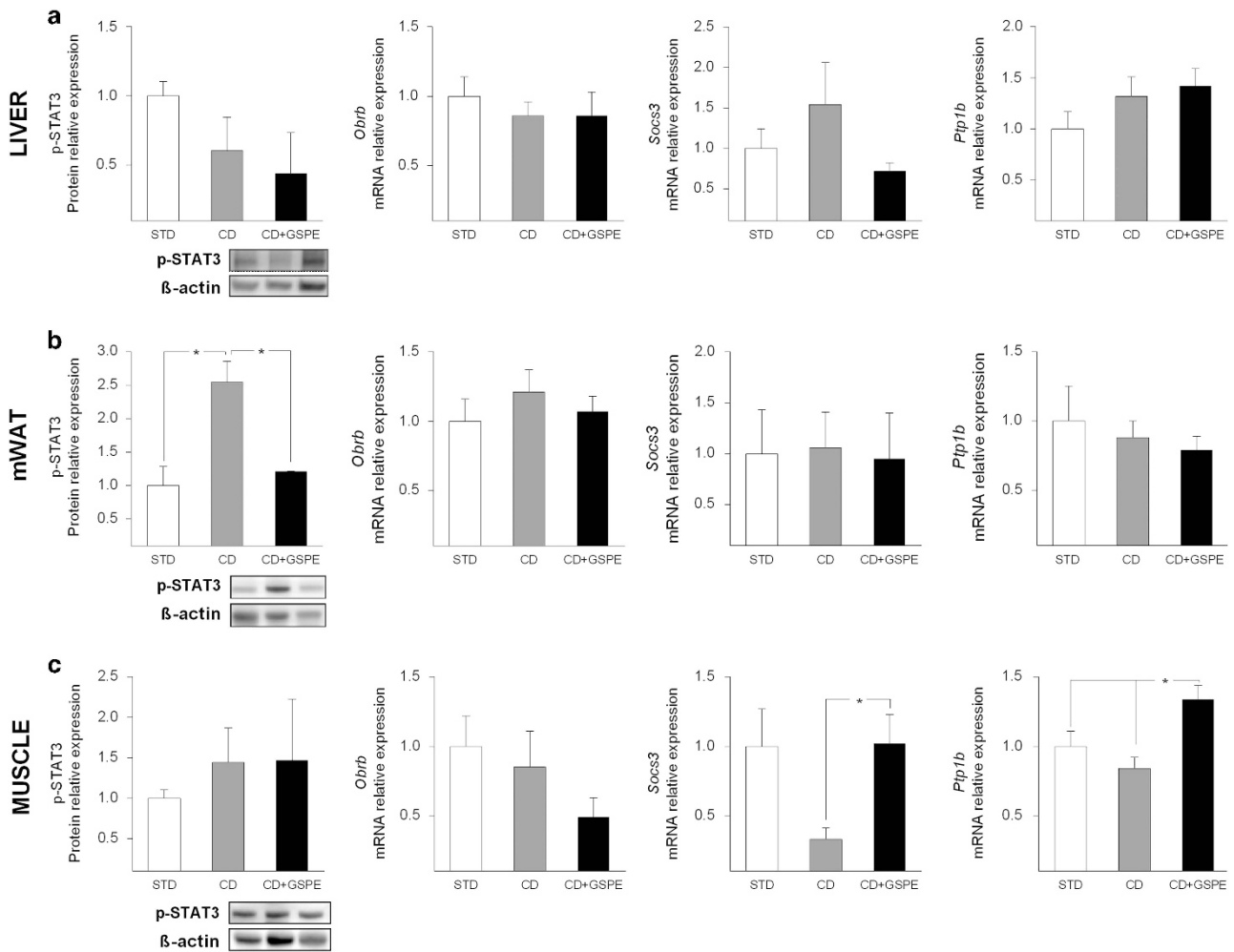
Figure 4. Effect of GSPE treatment on the peripheral leptin signalling. The leptin signalling pathway was investigated in the liver (**a**), mWAT (**b**) and skeletal muscle (**c**). STAT3 phosphorylation (p-STAT3) was assessed using western blotting, and the mRNA levels of the long leptin receptor isoform b (Obrb), Socs3 and Ptp1b were determined using quantitative PCR. The mRNA levels of these selected genes were normalized to those of Ppia. The rats were fed either the standard chow diet (STD group, $n = 7$) or cafeteria diet (CD) for 13 weeks. The CD-fed rats were treated with either GSPE at 25 mg per kg of body wt per day (CD+GSPE group, $n = 7$) or with the vehicle (CD group, $n = 7$) during the last 21 days of the study. The values shown are the means ± s.e.m. *indicates significant differences between the groups at $P \leqslant 0.05$, as assessed using one-way analysis of variance.

and *Ptp1b* in mWAT were also similar in all three groups of animals.

Finally, a slight increase of p-STAT3 was observed in the skeletal muscles of the GSPE-treated rats compared with STD and CD groups (Figure 4c). Importantly, in contrast to the liver and mWAT, the *Socs3* and *Ptp1b* gene expression levels were significantly lower in the CD-fed rats than in the STD group; furthermore, the GSPE treatment induced a robust increase of their expression levels compared with the CD-fed rats. These results indicated that the skeletal muscle is the tissue most sensitive to the GSPE treatment with respect to leptin signalling. In addition, as observed in the hypothalamus, the *Obrb* mRNA levels were not altered by the GSPE treatment.

DISCUSSION

Previous results from our group have indicated that chronic consumption of grape-seed PACs are unable to counteract the body weight gain and the hyperleptinemia induced by a CD in rats, but consumption of these compounds significantly reduces the food intake.[23] The hyperphagia observed in animals fed a

highly palatable diet has been related to a dysfunctional melanocortin system.[28] We therefore determined whether chronic ingestion of GSPE was able to reverse this dysfunction and normalize the leptin signalling in the hypothalamus of rats fed a CD.

Importantly, our CD model exhibited central leptin resistance as indicated by the decrease in the hypothalamic p-STAT3 levels in rats fed the CD for 13 weeks. Notably, the impairment of leptin-induced STAT3 phosphorylation in the hypothalamus has been considered to be one of the leading markers for cellular leptin signal attenuation in hyperleptinemic rats with diet-induced obesity.[29] Conversely, the CD-fed rats in this study did not display altered gene expression of *Obrb*, *Pomc*, *Agrp* or *Npy* in the hypothalamus. Contradictory results have been published regarding the effect of a CD on *Pomc* expression in the hypothalamus, with both repression[30] and overexpression[31] having been reported. Thus, the duration of the CD and the grade of obesity achieved can affect the severity of the melanocortin system dysfunction in rats.

Remarkably, 21 days of GSPE treatment normalized the level of p-STAT3 and the gene expression of *Socs3* and *Ptp1b* in the

hypothalamus, all of which had been repressed by the CD. Interestingly, this normalization was associated with a high overexpression of *Pomc*, suggesting that the upregulation of *Pomc* could be mediated by the increase in p-STAT3 induced by the GSPE treatment. Together, these results indicate that chronic consumption of GSPE clearly improved the central leptin signalling in the CD-fed rats. Notably, POMC is an anorexigenic neuropeptide.[2] Thus, the overexpression of POMC induced by GSPE treatment could mediate the significant reduction of food intake and adiposity observed in the CD+GSPE group. Other polyphenols and polyphenol extracts modulate the neuropeptides involved in food intake and EE (reviewed in Panickar[32]). For instance, resveratrol reduces *Npy* and *Agrp* expression,[33] and apigenin increases *Pomc* expression[34] in neuronal cell lines. These results reinforce the idea that specific polyphenols could improve central leptin signalling.

The induction of central leptin resistance in diet-obesity models has been mainly attributed to hypothalamic inflammation[35,36] as a result of the induction of the pro-inflammatory signalling molecules JNK and NF-κB and ER stress resulting from over-nutrition. Remarkably, our results showed that GSPE treatment reduced the hypothalamic inflammation, as indicated by the *inos* gene expression levels, which suggested that the local anti-inflammatory activity of PACs in this tissue could be one of the mechanisms by which GSPE treatment re-established normal central leptin sensitivity. In addition, SIRT1 activity has been highlighted as a mediator of central leptin action.[13,14] Thus, the hypothalamic overexpression of *Sirt1* induced by GSPE treatment could be another part of the mechanism by which GSPE treatment reduced the central leptin resistance.

The improvement of central leptin signalling could be secondary to the peripheral actions of GSPE on metabolism and hormones or the communication by the afferent nervous system to the brain. However, the capacity of GSPE to modulate *Sirt1* gene expression levels and inflammation in the hypothalamus itself, together with the facts that GSPE compounds can cross the brain-blood barrier[37] and that their metabolites have been found in the rat brain,[38] suggest a direct action of the PACs at hypothalamic level.

In addition to the hypothalamus, peripheral tissues such as the liver, skeletal muscle and adipose tissue are targets of leptin, and peripheral leptin resistance has been associated with obesity.[39] Notably, the GSPE treatment normalized the leptin cascade disruptions caused by the CD in the mWAT and skeletal muscle. Remarkably, leptin resistance in WAT has been associated with the excessive fat mass accumulation that characterizes obesity.[39] Accordingly, the normalization of leptin signalling observed in the mWAT of rats supplemented with GSPE was associated with a decline in the body adiposity. Moreover, we have demonstrated in a previous study that CD-fed rats supplemented with GSPE at the same dose and time period used in this study show an activation of muscle β-oxidation and an improvement in mitochondrial function.[24] These effects of GSPE in muscle are also consistent with the upregulation of fatty acid oxidation, which is an insulin-sensitizing effect of leptin in this tissue.[40] Therefore, these results clearly indicate that GSPE treatment was also effective in improving peripheral leptin sensitivity in CD-fed rats.

Despite this improvement of leptin sensitivity, rats treated with GSPE did not displayed a significant body weight reduction indicating that GSPE, at the dose and time used in this experiment, was not sufficient to totally reverse leptin dysfunction induced by a high-fat diet. However, body weight gain and epididymal fat mass of rats treated with GSPE were 10 and 16% lesser that those of rats not treated, respectively. Therefore, the improvement of leptin sensitivity induced by GSPE could behind a body mass rearrangement that, in turn, can improve obesity outcomes.

Intriguingly, GSPE did not reduce significantly the body weight, despite GSPE decreasing energy intake and not affecting EE nor substrate utilization. However, measurements of EE and substrate utilization were performed in the fasted state rather than the fed state. Thus, the lack of effects of GSPE on body weight combined with a decrease in energy intake suggest that EE in the fed state may be reduced by GSPE.

Importantly, translation of the daily dose of PACs (25 mg per kg of body wt per day) used in this study to the human doses[41] estimated for a 70 kg human indicates that the equivalent intake would be between 250 and 280 mg of GSPE per day. Humans consuming a polyphenol-rich diet can easily achieve or even exceed this PAC intake.[42] Therefore, the inclusion of PAC-rich foods in diets of obese people could be a good strategy to reduce the appetite and improve central and peripheral leptin sensitivity, thus complementing dietary therapies intended to promote weight loss in obese subjects. However, the effect of an obesogenic diet on leptin resistance have been described to be sex-dependent.[43] Therefore, as this study has been performed in males, further studies are warranted in order to determine the potential sex differences of PACs in relation to energy intake and body weight.

In conclusion, 21 days of GSPE treatment normalized the CD-induced leptin signalling disorders observed mainly in the hypothalamus and in the skeletal muscle of rats with diet-induced obesity. This improvement in leptin signalling resulted in part from neuroprotection against diet-induced inflammation and from the increase in hypothalamic sirtuin expression. Together, these results strongly suggest that PACs could reduce energy intake and adiposity by re-establishing central and peripheral leptin sensitivity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1 WHO (World health Organization). Obesity and overweight. Fact sheet N°311. 2015. Available at: http://www.who.int/mediacentre/factsheets/fs311/en/# (accessed on 15 March 2016).

2 Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. *Nature* 2006; **443**: 289–295.

3 Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature* 1998; **395**: 763–770.

4 Bates SH, Stearns WH, Dundon TA, Schubert M, Tso AWK, Wang Y et al. STAT3 signalling is required for leptin regulation of energy balance but not reproduction. *Nature* 2003; **421**: 856–859.

5 Iskandar K, Cao Y, Hayashi Y, Nakata M, Takano E, Yada T et al. PDK-1/FoxO1 pathway in POMC neurons regulates Pomc expression and food intake. *Am J Physiol Endocrinol Metab* 2010; **298**: E787–E798.

6 Frederich RC, Hamann A, Anderson S, Löllmann B, Lowell BB, Flier JS. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nat Med* 1995; **1**: 1311–1314.

7 Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* 1996; **334**: 292–295.

8 Halaas JL, Boozer C, Blair-West J, Fidahusein N, Denton DA, Friedman JM. Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proc Natl Acad Sci USA* 1997; **94**: 8878–8883.

9 Myers MG, Heymsfield SB, Haft C, Kahn BB, Laughlin M, Leibel RL et al. Challenges and opportunities of defining clinical leptin resistance. *Cell Metab* 2012; **15**: 150–156.

10 Jung CH, Kim M-S. Molecular mechanisms of central leptin resistance in obesity. *Arch Pharm Res* 2013; **36**: 201–207.

11 Thaler JP, Yi C-X, Schur EA, Guyenet SJ, Hwang BH, Dietrich MO et al. Obesity is associated with hypothalamic injury in rodents and humans. *J Clin Invest* 2012; **122**: 153–162.

136

12 Zhang X, Zhang G, Zhang H, Karin M, Bai H, Cai D. Hypothalamic IKKβ/NF-κB and ER stress link overnutrition to energy imbalance and obesity. *Cell* 2008; **135**: 61–73.

13 Sasaki T, Kikuchi O, Shimpuku M, Susanti VY, Yokota-Hashimoto H, Taguchi R *et al.* Hypothalamic SIRT1 prevents age-associated weight gain by improving leptin sensitivity in mice. *Diabetologia* 2014; **57**: 819–831.

14 Sasaki T. Age-associated weight gain, leptin, and SIRT1: a possible role for hypothalamic SIRT1 in the prevention of weight gain and aging through modulation of leptin sensitivity. *Front Endocrinol* 2015; **6**: 109.

15 Aragonès G, Ardid-Ruiz A, Ibars M, Suárez M, Bladé C. Modulation of leptin resistance by food compounds. *Mol Nutr Food Res* 2016; **60**: 1789–1803.

16 Bladé C, Aragonès G, Arola-Arnal A, Muguerza B, Bravo FI, Salvadó MJ *et al.* Proanthocyanidins in health and disease. *Biofactors* 2016; **42**: 5–12.

17 Terra X, Pallarés V, Ardèvol A, Bladé C, Fernández-Larrea J, Pujadas G *et al.* Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. *J Nutr Biochem* 2011; **22**: 380–387.

18 Pinent M, Bladé C, Salvadó MJ, Blay M, Pujadas G, Fernández-Larrea J *et al.* Procyanidin effects on adipocyte-related pathologies. *Crit Rev Food Sci Nutr* 2006; **46**: 543–550.

19 Caimari A, del Bas JM, Crescenti A, Arola L. Low doses of grape seed procyanidins reduce adiposity and improve the plasma lipid profile in hamsters. *Int J Obes* 2012; **37**: 576–583.

20 Pons Z, Guerrero L, Margalef M, Arola L, Arola-Arnal A, Muguerza B. Effect of low molecular grape seed proanthocyanidins on blood pressure and lipid homeostasis in cafeteria diet-fed rats. *J Physiol Biochem* 2014; **70**: 629–637.

21 Quesada H, del Bas JM, Pajuelo D, Díaz S, Fernandez-Larrea J, Pinent M *et al.* Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver. *Int J Obes (Lond)* 2009; **33**: 1007–1012.

22 Salvadó MJ, Casanova E, Fernández-Iglesias A, Arola L, Bladé C. Roles of proanthocyanidin rich extracts in obesity. *Food Funct* 2015; **6**: 1053–1071.

23 Serrano J, Casanova-Martí À, Gil-Cardoso K, Blay MT, Terra X, Pinent M *et al.* Acutely administered grape-seed proanthocyanidin extract acts as a satiating agent. *Food Funct* 2016; **7**: 483–490.

24 Casanova E, Baselga-Escudero L, Ribas-Latre A, Cedó L, Arola-Arnal A, Pinent M *et al.* Chronic intake of proanthocyanidins and docosahexaenoic acid improves skeletal muscle oxidative capacity in diet-obese rats. *J Nutr Biochem* 2014; **25**: 1003–1010.

25 Quiñones M, Guerrero L, Suarez M, Pons Z, Aleixandre A, Arola L *et al.* Low-molecular procyanidin rich grape seed extract exerts antihypertensive effect in males spontaneously hypertensive rats. *Food Res Int* 2013; **51**: 587–595.

26 Crescenti A, del Bas JM, Arola-Arnal A, Oms-Oliu G, Arola L, Caimari A. Grape seed procyanidins administered at physiological doses to rats during pregnancy and lactation promote lipid oxidation and up-regulate AMPK in the muscle of male offspring in adulthood. *J Nutr Biochem* 2015; **26**: 912–920.

27 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**: 402–408.

28 Hansen MJ, Ball MJ, Morris MJ. Enhanced inhibitory feeding response to alpha-melanocyte stimulating hormone in the diet-induced obese rat. *Brain Res* 2001; **892**: 130–137.

29 Levin BE. Obesity-prone rats have normal blood-brain barrier transport but defective central leptin signaling before obesity onset. *AJP Regul Integr Comp Physiol* 2003; **286**: 143 R–14150.

30 Plut C. Hypothalamic leptin receptor and signaling molecule expressions in cafeteria diet-fed rats. *J Pharmacol Exp Ther* 2003; **307**: 544–549.

31 Torri C, Pedrazzi P, Leo G, Müller EE, Cocchi D, Agnati LF *et al.* Diet-induced changes in hypothalamic pro-opio-melanocortin mRNA in the rat hypothalamus. *Peptides* 2002; **23**: 1063–1068.

32 Panickar KS. Effects of dietary polyphenols on neuroregulatory factors and pathways that mediate food intake and energy regulation in obesity. *Mol Nutr Food Res* 2013; **57**: 34–47.

33 Kim S-J, Lee YH, Han M-D, Mar W, Kim W-K, Nam K-W. Resveratrol, purified from the stem of *Vitis coignetiae* Pulliat, inhibits food intake in C57BL/6 J Mice. *Arch Pharm Res* 2010; **33**: 775–780.

34 Myoung H-J, Kim G, Nam K-W. Apigenin isolated from the seeds of *Perilla frutescens* britton var crispa (Benth.) inhibits food intake in C57BL/6 J mice. *Arch Pharm Res* 2010; **33**: 1741–1746.

35 Benzler J, Ganjam GK, Pretz D, Oelkrug R, Koch CE, Legler K *et al.* Central inhibition of IKKβ/NF-κB signaling attenuates high-fat diet-induced obesity and glucose intolerance. *Diabetes* 2015; **64**: 2015–2027.

36 de Git KCG, Adan RAH. Leptin resistance in diet-induced obesity: the role of hypothalamic inflammation. *Obes Rev* 2015; **16**: 207–224.

37 Janle EM, Lila MA, Grannan M, Wood L, Higgins A, Yousef GG *et al.* Pharmacokinetics and tissue distribution of 14C-labeled grape polyphenols in the periphery and the central nervous system following oral administration. *J Med Food* 2010; **13**: 926–933.

38 Margalef M, Pons Z, Bravo FI, Muguerza B, Arola-Arnal A. Tissue distribution of rat flavanol metabolites at different doses. *J Nutr Biochem* 2015; **26**: 987–995.

39 Sáinz N, Barrenetxe J, Moreno-Aliaga MJ, Martínez JA. Leptin resistance and diet-induced obesity: central and peripheral actions of leptin. *Metabolism* 2015; **64**: 35–46.

40 Dyck DJ. Adipokines as regulators of muscle metabolism and insulin sensitivity. *Appl Physiol Nutr Metab* 2009; **34**: 396–402.

41 Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB J* 2008; **22**: 659–661.

42 Knaze V, Zamora-Ros R, Luján-Barroso L, Romieu I, Scalbert A, Slimani N *et al.* Intake estimation of total and individual flavan-3-ols, proanthocyanidins and theaflavins, their food sources and determinants in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. *Br J Nutr* 2012; **108**: 1095–1108.

43 Priego T, Sánchez J, Palou A, Pico C. Effect of high-fat diet feeding on leptin receptor expression in white adipose tissue in rats: depot- and sex-related differential response. *Genes Nutr* 2009; **4**: 151–156.

Supplementary Information accompanies this paper on International Journal of Obesity website (http://www.nature.com/ijo)