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Resveratrol Prevents Weight Gain, Counteracts Visceral Adipose Tissue Dysfunction, and Improves Hypothalamic Leptin Sensitivity in Diet-Induced Obese Rats

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ABSTRACT

In obesity, increased adipocyte size is associated with metabolic complications, while elevated adipocyte numbers are considered a protective mechanism against metabolic disturbances. Adipose tissue dysfunction leads to decreased leptin sensitivity and disrupted energy balance regulation. Resveratrol (RSV), a bioactive compound known for potential health benefits, including obesity-related disorder prevention, has unclear modulatory effects on adipocyte dysfunction and leptin signaling in established obesity. This study investigated the impact of RSV on adiposity and hypothalamic leptin sensitivity in obesity. Rats were fed a cafeteria diet for 9 weeks and subsequently supplemented with different doses of RSV for 22 days. The 200 mg/kg RSV dose reduced leptin concentrations, body weight gain, and body fat mass in obese animals, while mitigating adipocyte hypertrophy and promoting adipocyte hyperplasia in the retroperitoneal fat depot. RSV also improved hypothalamic leptin sensitivity, shedding light on the molecular mechanisms underlying the benefits of RSV consumption for obesity-related disorders.

1 | Introduction

The prevalence of obesity and overweight, which are associated with higher all-cause mortality, continues to increase [1]. They are characterized by an imbalance between energy intake and energy expenditure, leading to an increase in body fat accumulation [2].

In this condition, expansion of white adipose tissue (WAT) occurs, resulting in an increase in adipocyte size (hypertrophy) to store more triglycerides (TAG) and/or an increase in adipocyte number (hyperplasia). When a threshold is reached in terms of TAG storage capacity, the functionality of hypertrophic adipocytes is altered. Hypertrophic adipose tissue secretes a different pattern

Abbreviations: ARC, arcuate nucleus of the hypothalamus; CAF, cafeteria diet; eWAT, epididymal white adipose tissue; iWAT, inguinal white adipose tissue; PVN, paraventricular nucleus of the hypothalamus; RER, respiratory exchange ratio; RSV, resveratrol; rWAT, retroperitoneal white adipose tissue; STD, standard chow diet.

Maria Ibars-Serra and Aïda Pascual-Serrano contributed equally to this study.

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of adipokines and inflammation markers concomitant with an impairment in the function of the adipose tissue depot [3]. Particularly, dysregulated visceral hypertrophic adipocytes are considered the most important in influencing systemic deregulations, such as insulin resistance and other obesity-related disorders [4, 5]. Conversely, increased adipocyte hyperplasia is considered a protective mechanism against metabolic complications of adipose tissue in obesity due to its higher capacity for TAG storage [6]. At molecular level, microRNAs (miRNAs) are important for adipocyte differentiation and for regulating the metabolic and endocrine functions of these cells [7]. MiRNAs constitute a class of short noncoding RNAs involved in the post-transcriptional regulation of genes. In fact, an increasing number of miRNAs has been identified to regulate key signaling pathways in this tissue [8]. Specifically, the gene expression of fatty acid synthase (*Fasn*), carnitine palmitoyltransferase 1B (*Cpt1b*), and ATP-binding cassette transporter (*Abca1*) are modulated by miR-33a [9–13], which is a master regulator of lipid metabolism and it is embedded in the *Srebp2* gene region [14].

Otherwise, the regulation of energy homeostasis and WAT metabolism is also mainly mediated by the adipokine leptin, which is primarily produced in adipocytes, and its circulating levels are proportional to body fat stores [15, 16]. However, the key organ involved in the regulation of leptin's effects on energy metabolism is the brain, specifically the hypothalamus. It receives signals from peripheral organs, such as WAT, that inform it about the body's energy status [17]. Circulating leptin reaches the arcuate nucleus in the hypothalamus and binds to the long form of the leptin receptor (ObRb). This interaction activates its signal transduction pathway, phosphorylating the signal transducer and activator of transcription 3 (STAT3) in orexigenic and anorexigenic neurons to suppress food intake and promote energy expenditure [18]. In addition, leptin's activation of acetyl-coA carboxylase (ACC), via the AMP-protein kinase (AMPK) pathway, in the arcuate (ARC) and paraventricular nuclei (PVN) is also needed to induce anorexigenic effects [19]. However, in obesity, leptin is not able to promote energy expenditure and satiety, despite highly increased levels in circulation. Several mechanisms can reduce leptin sensitivity, including altered signaling or defective transport across the blood-brain barrier [20, 21].

Plant-derived phenolic compounds that target the hypothalamic leptin system and adipose tissue have been proposed as a promising strategy to complement existing therapies against obesity [22]. Among all phenolic compounds, resveratrol (RSV) is one of the most studied non-flavonoid polyphenols. Pronounced effects of RSV have been widely reported on lipolysis, adipogenesis, and inflammation in adipose tissue in both murine animals [23–28] and humans [29]. While effects on obesity prevention have been repeatedly shown, less is known about the effects of RSV on existing obesity and information on how RSV affects the brain leptin signaling pathway is very scarce [30]. As RSV can cross the blood-brain barrier and interact with different neuronal subpopulations in the ARC [31], we hypothesize that RSV could exert part of its anti-obesity effects by modulating leptin sensitivity in the brain and/or by exerting its effects directly on the adipose tissue, since RSV metabolites have also been identified in adipose tissue [32, 33]. Thus, the aim of this study was to investigate whether RSV consumption is able to counteract

obesity by affecting central leptin signaling and adipose tissue remodeling once obesity has already been established.

2 | Materials and Methods

2.1 | Animals and Diet

Five-week old male Wistar rats with a body weight of 122.56 ± 3.11 g were purchased from Charles River Laboratories (Barcelona, Spain). Animals were housed in a 12 h light–dark cycle at 22°C and were provided with free access to tap water. During 10 days of adaptation, animals were ad libitum fed with a standard chow diet (Panlab 04, Barcelona, Spain). After the adaptation period, animals were divided into five groups composed of six animals each. One group was fed with standard chow diet (STD group) with a calorie breakdown of 20% protein, 8% fat, and 72% carbohydrate, and the other groups were fed with STD plus cafeteria diet (CAF groups) composed of 15% protein, 25% fat, and 60% carbohydrate. Animals had free access to fresh STD and CAF every day at 11:00 a.m. CAF consisted of cookies, cheese, bacon, foie-gras, sugary milk, *ensaimada* (a typical Majorcan pastry), and carrots. Weight gain and food consumption were monitored every week for 9 weeks until animals gained more than 15% body weight compared to the STD group. Then, oral treatment RSV was administered together with CAF for 22 days. The weeks before supplementation, all rats were trained to voluntarily lick the vehicle solution (low-fat condensed milk diluted in water 1:1) to avoid oral gavage. Direct measurements of fat mass were obtained in triplicate for each animal of each group ($n = 6$) using the analyzer and expressed as a percentage of total body weight. Body weight and food intake were weekly monitored until the end of the experiment. The day before the sacrifice, body composition was analyzed from rats using the magnetic resonance imaging system EchoMRI-700 (Echo Medical Systems, LLC, TX, USA).

On Day 22 of treatment, rats were sacrificed by decapitation. Blood was collected and serum was obtained by centrifugation ($1500 \times g$, 15 min, 4°C) and kept frozen at -80°C until assayed. Several metabolic tissues were excised, including white adipose tissue depots (inguinal, epididymal, and retroperitoneal), liver, and hypothalamus. They were weighed, immediately frozen in liquid nitrogen and stored at -80°C until further analysis. The investigation was conducted 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 University Rovira i Virgili (reference number 4249 by Generalitat de Catalunya).

2.1.1 | Dosage Information/Dosage Regimen

At 09:00 a.m. daily, animals were supplemented orally with 50, 100, or 200 mg/kg body weight of RSV (trans-resveratrol with a minimum of 98% purity, Fagron Iberica, S.A.U, Barcelona, Spain) dissolved in the vehicle for 22 days. STD and CAF groups were supplemented with the same quantity of the vehicle. The human dose equivalent to 200 mg RSV/kg for an obese adult (110 kg) should not exceed 5 g per day, for a dose of 100 mg RSV/kg 2.5 g per day, and for a dose of 50 mg 2.2 g per day of RSV based on a body

surface area (BSA) calculation [34]. The levels of RSV present in a Mediterranean diet and considering the recommendation of five servings of fruits and vegetables per day are not sufficient to meet any of the doses mentioned before [35]. Accordingly, the dose administered in this study should be covered by pharmacological supplementation.

2.2 | Quantification of Plasma Parameters

The plasma levels of insulin, leptin, and adiponectin were measured using a rat/mouse insulin ELISA kit (EZRMI-13K), rat leptin ELISA kit (EZRL-83K), and rat adiponectin ELISA kit (EZRADP-62K, Millipore Ibérica, Madrid, Spain). Plasma NEFAs were analyzed with the enzymatic colorimetric HR NEFA series kit (Wako, CA, USA). Plasma TAG, total cholesterol (TC), HDL-cholesterol (HDL-C), and glucose were measured with enzymatic colorimetric kits following the manufacturer's protocols (QCA, Barcelona, Spain).

The Homeostasis Model Assessment (HOMA) was calculated from glucose (mg/dL) and insulin (μ U/mL) levels, using the following formula:

$$\text{HOMA} = \text{fasting glucose (mg/dL)} \times \text{fasting insulin (}\mu\text{U/mL)} / 405.$$

2.3 | Respiratory Exchange Ratio and Energy Expenditure

Animals were subject to 16 h of indirect calorimetry analyses using Oxylet Pro System (Panlab, Cornellà, Spain) with free access to food and water. Measurements were taken for an 8:8 h light:dark cycle and animals had access to food and water during the entire measurement period. The oxygen consumption (VO_2) and carbon dioxide production (VCO_2) measures were used by the software Metabolism 2.1.02 (Panlab) to calculate respiratory exchange ratio (RER) as VCO_2/VO_2 and energy expenditure as $\text{VO}_2 \times 1.44 \times (3.815 + [1.232 \times \text{RQ}])$ (kcal/day/kg^{0.75}) according to Weir equation [36]. A nitrogen excretion rate (n) of 135 $\mu\text{g/kg/min}$ was assumed [37]. Total activity was measured by recording infrared beam breaks (Oxylet Pro System, Panlab).

2.4 | Adipose Tissue Morphology Analysis

Pieces of frozen rWAT (-80°C) were sent to ELDINE Patología (Tarragona, Spain), where they were thawed and fixed in 4% diluted formaldehyde. After 24 h of fixation, tissues underwent successive dehydration (alcohol/ethanol 70%, 96%, and 100%; plus xylol/dimethyl benzene) and paraffin infiltration and immersion at 52°C (Citadel 2000, HistoStar, Thermo Scientific, Madrid, Spain). The paraffin blocks were subsequently cut into successive 2- μm -thick sections (Microm HM 355S, Thermo Scientific, Madrid, Spain). The sections were deposited on slides (JP Selecta Paraffin Bath) and subjected to automated hematoxylin-eosin staining (Varistain Gemini, Shandom, Thermo) [38]. Images of the adipose sections were acquired using AxioVision Zeiss Imaging software (Carl Zeiss Iberia, S.L., Madrid, Spain). Finally, the images captured at 20 $\times$ magnification were analyzed with the Adiposoft software (CIMA, University of Navarra, Spain) to

quantify adipocyte number and areas. Five fields per sample and three samples from each group were measured. The area was calculated from the average value of the cell area in all measured fields. The total cell number in the rWAT fat depots was calculated from the fat cell volume values. Fat cell volume was obtained for each analyzed field using the formula $\frac{\pi}{6} \times [3\sigma^2 \times \bar{d} + \bar{d}^3]$ [39] (where $\bar{d}$ is the mean diameter of 100 measured cells in the field, and σ is the standard deviation of all diameters). Subsequently, fat cell density was applied to determine the fat cell weight. Finally, the total fat cell number in the whole rWAT depot of each animal was determined by applying the quotient between total fat depot weight and mean cell weight of all captured fields.

2.5 | Immunoblot Analysis

Rat hypothalamus ($n = 6$) were homogenized at 4°C using a Tissue Lyser LT (Qiagen, Barcelona, Spain) in 0.5 mL of Radio-Immunoprecipitation Assay lysis (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, 1% Tween, 0.25% Na-deoxycholate, 0.1 M PMSF) containing protease and phosphatase inhibitor cocktails (Sigma Aldrich, Madrid, Spain). The homogenates were incubated for 30 min at 4°C and centrifuged at $20\,000 \times g$ for 15 min at 4°C . The supernatant was placed in fresh tubes and was used to determine total protein and for immunoblotting analyses. The total protein content was quantified using the Pierce BCA protein assay kit (Thermo Scientific, Barcelona, Spain). Samples were denatured by mixing with loading buffer solution (Tris-HCl 0.5 M, pH 6.8, glycerol, SDS, β -mercaptoethanol, and bromophenol blue) and then heated at 99°C during 5 min using a thermocycler (Multigen Labnet, Barcelona, Spain). Acrylamide gels were prepared using TGX Fast Cast Acrylamide Kit, 10% (Bio-Rad, Barcelona, Spain) and 30 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using electrophoresis buffer (glycine 192 mM, Tris base 25 mM and 1% SDS). Proteins were electrotransferred onto supported polyvinylidene difluoride membranes (Trans-Blot Turbo Mini PVDF Transfer Packs, Bio-Rad). After blocking with 5% of non-fat dried milk, membranes were incubated with gentle agitation overnight at 4°C with specific antibodies for Tyr705 phosphorylated STAT3 (pSTAT3) (Abcam), diluted 1:2500 and total STAT3 (Cell Signaling); diluted 1:2000, Ser79 phosphorylated ACC and total ACC (Cell Signaling); diluted 1:1000, ObRb (Abcam, Cambridge, UK); diluted 1:1000, SOCS3 (Cell Signaling); diluted 1:1000 and SIRT1 (Cell Signaling) at a 1:1000 dilution. β -actin was used as loading control, membranes were incubated with a rabbit anti- β -actin primary antibody (Sigma), diluted 1:1000. Finally, membranes were incubated with anti-rabbit horseradish peroxidase secondary antibody (GE Healthcare, Barcelona, Spain), diluted 1:10 000. 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). Lastly, protein bands were quantitated by densitometry using ImageJ software (NIH, Bethesda, MD, USA), each band was normalized by the corresponding β -actin band, and finally the treatment groups were normalized by the control group (STD).

2.6 | Leptin Sensitivity Assessment

Consistent with hypothalamic pSTAT3 levels are typically attributable to leptin action, leptin sensitivity was estimated as

the ratio of hypothalamic pSTAT3 levels to leptin concentration in plasma.

2.7 | Gene Expression Analysis

Total RNA was isolated from the frozen rWAT using Trizol reagent (Ambion, MA, USA) according to the manufacturer's protocol including a step of incubation with isopropanol at -20°C overnight instead of the 10 min at room temperature in order to obtain the RNA pellet also small RNAs for a subsequent miRNA quantification. The quantity, quality, and integrity were checked with Agilent 2100 Bioanalyzer system (Agilent Technologies, Madrid, Spain).

2.7.1 | mRNA Quantification by Real-Time qRT-PCR

Relative mRNA levels of ATP binding cassette subfamily A member 1 (*Abca1*), carnitine palmitoyltransferase 1B (*Cpt1b*), fatty acid synthase (*Fasn*), fatty acid binding protein 4 (*Fabp4*), insulin receptor substrate 2 (*Irs2*), leptin (*Lep*), lipoprotein lipase (*Lpl*), peroxisome proliferator-activated receptor gamma (*Pparg*) were analyzed by real-time PCR in rWAT using cyclophilin A (*Ppia*) as the endogenous control. Total RNA was reverse transcribed using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, MA, USA) according to the manufacturer's protocol. Gene expression was evaluated with the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, Barcelona, Spain) using the SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Barcelona, Spain) and gene-specific SYBR primers designed for each gene using the FastPCR software (Table S1). The results were normalized to *Ppia*. Amplification was performed following the temperature steps of 95°C for 30 s followed by 40 cycles at 95°C for 5 s and 60°C for 5 s. The fold-change in the mRNA level was calculated by the $2^{-\Delta\Delta\text{Ct}}$ method, where $\Delta\text{Ct} = \text{Ct mRNA} - \text{Ct Ppia}$ and $\Delta\Delta\text{Ct} = \Delta\text{Ct treated samples} - \Delta\text{Ct untreated controls}$.

2.7.2 | miRNA Quantification by Real-Time qRT-PCR

Single-stranded cDNAs were synthesized by using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) and the miRNA specific reverse transcription primers (Applied Biosystems, Madrid, Spain). The reaction was performed in a final volume of $7.5\ \mu\text{L}$ at the following temperature cycles: 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. The cDNA products of the reaction were mixed with the appropriate volume of TaqMan Universal PCR master mix (Applied Biosystems, Madrid, Spain) following the manufacturer's recommendations and with the associated specific PCR primers provided in the Taqman MicroRNA kit (Applied Biosystems, Madrid, Spain). Then the mixture was used for a quantitative polymerase chain reaction (qPCR) amplification. A specific Taqman probe was used for the microRNA analysis as follows: 5'-UGGAGUGUGACAAUGGUGUUUG-3' for microRNA-122 (002245, Applied Biosystems, Madrid, Spain); 5'-GUGCAUUGUAGUUGCAUUGCA-3' for microRNA-33a (002135, Applied Biosystems, Madrid, Spain); and U87 small nuclear RNA (001712, Applied Biosystems, Madrid, Spain) were used as an endogenous control and the results were normalized

to its expression. The amplification reaction was run on an ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems, Madrid, Spain) at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Fold change in the miRNA level was calculated according to the equation: $2^{-\Delta\Delta\text{Ct}}$, where $\Delta\text{Ct} = \text{Ct miRNA} - \text{Ct U87}$ and $\Delta\Delta\text{Ct} = \Delta\text{Ct treated samples} - \Delta\text{Ct untreated controls}$.

2.8 | Statistical Analyses

The results are expressed as mean $\pm$ standard error of the mean (S.E.M). Multiple independent groups were compared with one-way ANOVA followed by Tukey or Dunnett's T3 post-hoc analysis according to Levene's test. Repeated-measures ANOVA was used in indirect calorimetry analyses. GraphPad Prism 6 was used for statistical analyses and graphs (GraphPad Software, La Jolla, CA, USA). A p value ≤ 0.05 was considered statistically significant.

3 | Results

3.1 | High Doses of RSV Improved Obesity-Related Parameters

The group CAF+RSV200, which consumed the highest dose of RSV, tended to decrease the final body weight at the end of the study and prevented the body weight gain (Table 1). Moreover, at this dose, the percentage of fat mass was decreased compared to CAF animals. The percentages of fat mass in inguinal WAT (iWAT) and epididymal WAT (eWAT) depots but not in rWAT were significantly lower in the CAF+RSV200 group compared to the CAF group and not different from the STD group. RSV consumption did not modify the levels of food intake in the CAF groups (Table 1), showing that the effects on body weight and adiposity observed in these animals were not the result of differences in energy intake.

Circulating leptin concentrations showed a significant increase in CAF group compared to the STD group (Table 2), indicating that our experimental model developed hyperleptinemia after 22 weeks of CAF. In agreement with the body weight and fat mass results, consumption of 200 mg/kg RSV showed a reduction in leptin levels, comparable to the level of the STD group. No effects on leptin levels were seen in the CAF+RSV50 and CAF+RSV100 groups, compared to CAF. Glucose and insulin levels tended to be decreased in the CAF+RSV200 group, resulting in a significantly improved HOMA-IR index, compared to the CAF group. Of note, TAG plasma levels were significantly decreased in the CAF+RSV200 group compared to the CAF group. RSV did not have any effects on cholesterol levels.

3.2 | High Doses of RSV Increased Fat Oxidation

We reasoned that if the consumption of RSV did not have significant effects on food intake, it could enhance energy expenditure and lead to the utilization of fat as the principal energy source. As expected, animals fed with the standard chow diet used carbohydrates as a main energetic substrate and, therefore, presented RER values very close to 1 (Figure 1A,B). In

TABLE 1 | Effects of different doses of resveratrol on body weight, body composition, fat deposition, and food intake in diet-induced obese rats.

	STD	CAF	CAF+RSV50	CAF+RSV100	CAF+RSV200
Initial body weight (g)	223.3 ± 8.8 ^a	247.5 ± 6.6 ^a	255.2 ± 6.6 ^a	245.5 ± 11.1 ^a	240.9 ± 8.9 ^a
Final body weight (g)	437.7 ± 17.1 ^a	513.8 ± 9.3 ^b	634.8 ± 30.8 ^c	549.7 ± 24.2 ^b	499.7 ± 16.6 ^{ab}
Body weight gain treatment period (g)	29.2 ± 2.7 ^a	54.6 ± 4.1 ^{bc}	70.4 ± 5.2 ^c	46.1 ± 8.7 ^{ab}	30.8 ± 3.2 ^a
Lean mass (%)	80.7 ± 1.3 ^a	65.9 ± 1.6 ^b	64.5 ± 3.6 ^{bc}	70.3 ± 1.1 ^{bc}	74.7 ± 0.8 ^c
Fat mass (%)	12.6 ± 1.2 ^a	26.1 ± 1.4 ^b	29.5 ± 3.4 ^{bc}	26.7 ± 2.8 ^{bc}	18.9 ± 0.8 ^c
Liver weight (%)	2.91 ± 0.12 ^a	2.82 ± 0.12 ^a	3.13 ± 0.02 ^a	2.99 ± 0.16 ^a	3.12 ± 0.08 ^a
iWAT weight (%)	0.22 ± 0.02 ^a	0.96 ± 0.24 ^b	0.43 ± 0.04 ^{ab}	0.8 ± 0.2 ^{bc}	0.34 ± 0.04 ^{ac}
rWAT weight (%)	0.89 ± 0.8 ^a	1.77 ± 0.22 ^b	1.41 ± 0.1 ^{ab}	1.83 ± 0.39 ^b	1.21 ± 0.02 ^{ab}
eWAT weight (%)	2.03 ± 0.15 ^a	4.01 ± 0.4 ^b	4.05 ± 0.36 ^b	3.37 ± 0.22 ^{bc}	2.86 ± 0.27 ^{ac}
Energy intake (kJ/24 h)	235.2 ± 20.4 ^a	848.7 ± 66.5 ^b	699.9 ± 146.7 ^b	794.7 ± 48.93 ^b	878.3 ± 63.5 ^b

Note: Rats were fed a standard chow diet (STD) or a cafeteria diet (CAF) for 9 weeks. From Week 10 animals fed a CAF diet were supplemented orally with either a vehicle (CAF) or resveratrol (CAF+RSV groups) at the doses of 50, 100, or 200 mg/kg body weight/day for a period of 22 days. Lean and fat mass are presented as parameters of body composition and expressed as a percentage of total body weight. Data are the mean ± s.e.m. of $n = 6$ per group. Different letters denote statistical significance ($p \leq 0.05$) between groups assessed by one-way ANOVA and post-hoc multiple comparisons tests Tukey or Dunnett's T3 according to Levene's test.

Abbreviations: eWAT, epididymal white adipose tissue; iWAT, inguinal white adipose tissue; rWAT, retroperitoneal white adipose tissue.

contrast, the CAF group presented lower RER values compared to the STD group, indicating that they are not fully using carbohydrates since they have a diet rich in fat. Notably, RSV consumption significantly modulated RER in a dose-dependent manner. Specifically, 100 and 200 mg/kg RSV significantly lowered RER in comparison to CAF vehicle animals, indicating that RSV consumption at these doses significantly potentiates the utilization of lipids as the principal energetic substrate. However, energy expenditure measures could not fully explain these effects (Figure 1C,D). In this case, the dose of 100 mg/kg showed decreased energy expenditure compared to STD and CAF groups.

3.3 | RSV Induced a Healthier Expansion of Visceral rWAT

As visceral adipose tissue accumulation is more related to metabolic disorders than subcutaneous depots, we examined how rWAT morphology was modified by the CAF diet and RSV (Figure 2A). CAF animals showed a significant increase in both adipocyte area and volume (Figure 2B,C) compared to STD. Interestingly, all the doses of RSV reduced adipocyte size and volume after 22 days of supplementation, showing a reduced hypertrophy. Hypertrophy was completely counteracted with the highest dose of RSV.

In addition, supplementation with 200 mg/kg of RSV showed a size distribution of adipocytes more comparable to that of the STD-fed rats, while 50 and 100 mg/kg doses did not show any significant changes (Figure 2D). The total adipocyte number remained unchanged when the CAF diet was given to rats, but after 22 days of RSV administration, a tendency to increase adipocyte numbers was observed for RSV doses of 50 and 100 mg/kg, while at a dose of 200 mg/kg the adipocyte number was significantly increased by 150% (Figure 2E).

3.4 | RSV Downregulated *Lpl* and Upregulated *Irs2* mRNA Expression in rWAT

mRNA expression of some genes involved in lipid handling and adipogenesis was analyzed in rWAT to determine adipocyte functionality. Gene expression of Peroxisome Proliferator-Activated Receptor gamma (*Pparg*), the key regulator of adipogenesis, and adipocyte biology in general [40, 41] did not show any statistical differences produced by the dietary interventions. The fatty-acid binding protein 4 (*Fabp4*), involved in fatty acid uptake and known to interact with PPARγ [42], was also not affected significantly (Figure 3A,B). Gene expression of lipoprotein lipase (*Lpl*), which is crucial for triglyceride breakdown [43], was not affected by CAF (Figure 3C), but a dose-dependent decrease in gene-expression was observed in response to RSV supplementation. When Leptin (*Lep*) mRNA levels were analyzed, no significant changes were observed either by the CAF diet or by the RSV supplementation, although a dose of 200 mg/kg of RSV resulted in the lowest levels of leptin gene expression (Figure 3D).

Gene expression of genes involved in lipid metabolism was also analyzed in rWAT to study the effect of CAF diet and RSV. Specifically, *Fasn*, involved in fatty acid synthesis [44]; *Cpt1b*, which is essential in metabolizing fat in order to obtain energy [45]; and *Abca1*, relevant in the reverse cholesterol transport [46, 47]. *Fasn* and *Cpt1b* gene expressions were not significantly affected in any of the conditions (Figure 4A,B). Cholesterol transporter (*Abca1*) was downregulated by the CAF diet, with a tendency for alleviation by RSV at the highest doses, but a significant increase at the dose of 50 mg/kg of RSV (Figure 4C). Gene expression of insulin receptor substrate 2 (*Irs2*), which mediates insulin effects in adipose tissue [48], was reduced by 30% with the CAF diet, while RSV 200 mg/kg fully reversed that effect (Figure 4D).

Srebp2 and miR-33a expression levels were also quantified in these animals but were not affected, neither by the CAF diet nor

TABLE 2 | Effects of different doses of resveratrol on hormones, plasma metabolites, insulin resistance, and atherogenic indexes in diet-induced obese rats.

	STD	CAF	CAF+RSV50	CAF+RSV100	CAF+RSV200
Leptin (ng/mL)	10.8 ± 0.4 ^a	42.4 ± 3.2 ^{bc}	58.2 ± 9.3 ^b	47.6 ± 6.4 ^{bc}	25.3 ± 0.9 ^{ac}
Adiponectin (µg/mL)	28.1 ± 2.2 ^a	57.7 ± 5.5 ^b	49.1 ± 4.0 ^{bc}	41.3 ± 2.9 ^{bc}	34.7 ± 3.4 ^{ac}
Insulin (ng/mL)	2.7 ± 0.2 ^a	7.5 ± 1.1 ^{ab}	8.7 ± 1.4 ^b	5.4 ± 0.9 ^{ab}	4.4 ± 0.4 ^{ab}
Glucose (mg/dL)	126.1 ± 4.0 ^a	186.5 ± 14.8 ^b	153.0 ± 10.2 ^{ab}	152.2 ± 10.5 ^{ab}	139.5 ± 4.0 ^{ab}
HOMA-IR	21.3 ± 2.7 ^a	66.9 ± 5.8 ^b	83.1 ± 15.6 ^{ab}	48.5 ± 9.6 ^{ab}	36.8 ± 4.8 ^a
QUICKI	0.25 ± 0.004 ^a	0.22 ± 0.004 ^b	0.22 ± 0.005 ^b	0.23 ± 0.006 ^b	0.24 ± 0.004 ^{ab}
R-QUICKI	0.27 ± 0.008 ^a	0.23 ± 0.007 ^b	0.23 ± 0.005 ^b	0.25 ± 0.009 ^{ab}	0.25 ± 0.006 ^{ab}
TAG (mg/dL)	69.3 ± 12.7 ^a	152.0 ± 7.2 ^b	109.1 ± 17.2 ^{ab}	58.6 ± 6.2 ^a	67.6 ± 6.8 ^a
NEFAs (mmol/L)	0.55 ± 0.05	0.78 ± 0.13	0.73 ± 0.09	0.56 ± 0.05	0.66 ± 0.05
TC (mg/dL)	79.6 ± 4.52	99.8 ± 7.17	100.9 ± 2.7	81.1 ± 6.2	81.1 ± 0.6
HDL-c (mg/dL)	41.0 ± 4.1 ^a	24.8 ± 1.4 ^b	23.3 ± 3.2 ^b	22.9 ± 2.1 ^b	22.5 ± 2.5 ^b
Non-HDL-c (mg/dL)	34.9 ± 1.7 ^a	71.0 ± 8.8 ^b	67.2 ± 4.8 ^b	53.9 ± 3.7 ^{ab}	59.0 ± 2.5 ^b
HDL-c / non-HDL-c	1.2 ± 0.11 ^a	0.42 ± 0.01 ^b	0.35 ± 0.05 ^b	0.42 ± 0.03 ^b	0.39 ± 0.05 ^b
TC / HDL-c	2.0 ± 0.2 ^a	4.1 ± 0.7 ^b	4.2 ± 0.6 ^b	3.6 ± 0.2 ^{ab}	4.4 ± 0.7 ^b

Note: Rats were fed a standard chow diet (STD) or a cafeteria diet (CAF) for 9 weeks. From Week 10 animals fed a CAF diet were supplemented orally with either a vehicle (CAF) or resveratrol (CAF+RSV groups) at the doses of 50, 100, or 200 mg/kg body weight/day for a period of 22 days. Lean and fat mass are presented as parameters of body composition and expressed as percentage of total body weight. Data are the mean ± s.e.m. of $n = 6$ per group. Different letters denote statistical significance ($p \leq 0.05$) between groups assessed by one-way ANOVA and post-hoc multiple comparisons tests Tukey or Dunnett's T3 according to Levene's test. Abbreviations: HDL-c / non-HDL-c, the ratio between HDL-c and non-HDL-c levels; HDL-c, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment-estimated insulin resistance; NEFAs, non-esterified fatty acids; non-HDL-c, non-high-density lipoprotein cholesterol; QUICKI, quantitative insulin sensitivity check index; R-QUICKI, revised quantitative insulin sensitivity check index; TAG, triglycerides; TC/HDL-c, ratio between TC and HDL-c levels; TC, total cholesterol.

by the RSV treatments (Figure 4E,F). Together this suggests that lipid handling in rWAT is not modulated by RSV through changes in gene expression, with the possible exception of lipolysis.

The expression levels of miR-33a and of miR-122 were also analyzed in the liver as this tissue is responsible for the lipidic metabolism [49] (Figure S1). miR-33a was increased by the CAF diet and normalized in CAF+RSV100 and CAF+RSV200. In addition, liver *Srebp2* gene expression levels displayed the same trend as miR-33a, without reaching significance. Finally, the hepatic expression levels of miR-122, *Cpt1a* (liver isoform of *Cpt1*), and *Abca1* were not affected by any RSV dose.

3.5 | High Doses of RSV Improved Hypothalamic Leptin Sensitivity in Obese Rats via Two Distinct Pathways

The determination of pSTAT3 levels is the gold standard experimental marker for cellular leptin signaling [50]. We analyzed pSTAT levels in the hypothalamus to determine if different doses of RSV could modulate hypothalamic leptin signaling in obesogenic conditions. The activation of STAT3 was assessed by immunoblotting and band density was analyzed (Figure 5A,B). Contrary to our expectations, CAF did not lead to a significant decrease in the basal levels of pSTAT3 in the hypothalamus, but when RSV was administered to obese rats, the levels of pSTAT3 increased with respect to CAF, although only the dose of 100 mg/kg of RSV reached statistical significance. Considering

that hypothalamic pSTAT3 levels are mainly attributable to leptin action, the next we performed a ratio between hypothalamic pSTAT3 and plasma leptin levels to estimate the degree of sensitivity to leptin of this tissue. In particular, the leptin sensitivity of CAF animals was significantly reduced when compared to the STD group (Figure 5C). However, we observed that hypothalamic leptin sensitivity was improved in a dose-dependent manner by RSV, reaching significance at a dose of 200 mg/kg RSV. This may suggest that the consumption of high doses of this phenolic compound could potentially rescue leptin action in this tissue. Leptin signaling leads to the activation of the PI3K pathway, which mediates the acute effects of leptin in the hypothalamus [51–53]. A regulatory point of this pathway is the conversion of acetyl-CoA to malonyl-CoA by ACC. ACC is inactivated by AMPK via phosphorylation. Leptin inhibits AMPK which in turn will activate ACC, increasing malonyl-CoA which induces an anorexigenic response [54, 55]. Interestingly, the dose of 200 mg/kg of RSV significantly reduced the levels of phosphorylated ACC (pACC) (Figure 5D,E), indicating increased ACC activation in the hypothalamus of these animals.

3.6 | RSV Modulated the Hypothalamic Leptin Receptor and Leptin-Signaling Factors in Obese Animals

The leptin receptor *Obrb* was only increased in the hypothalamus by RSV at a dose of 100 mg/kg (Figure 6A,B). Additionally, CAF resulted in a significant decrease in protein levels of SOCS3

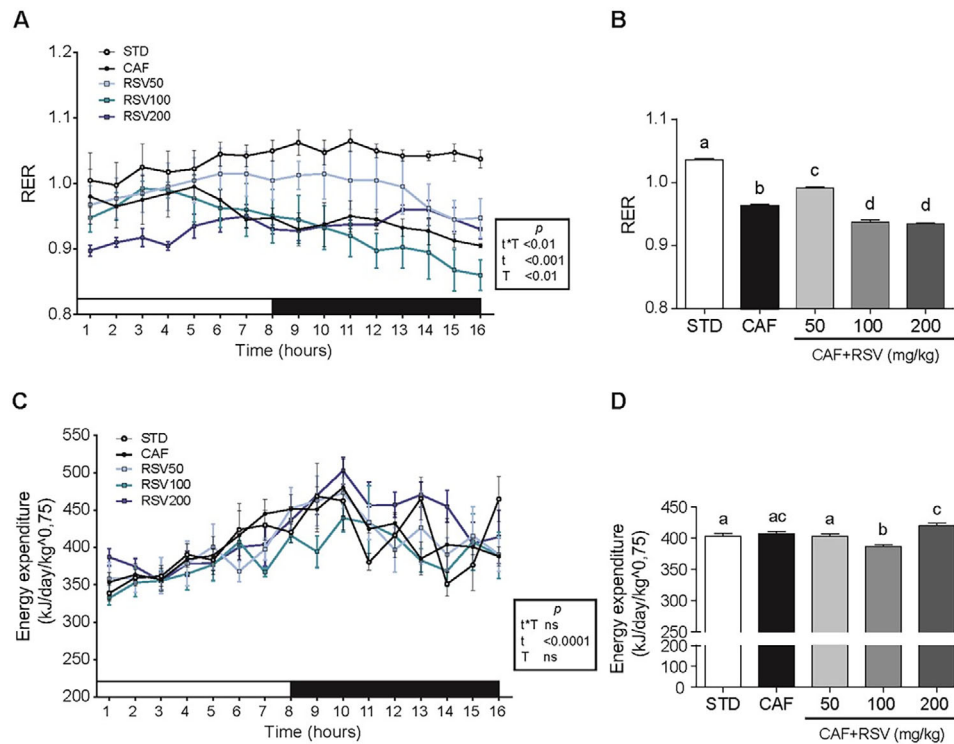


FIGURE 1 | Effect of resveratrol treatment on RER and energy expenditure of obese rats. 16-h indirect calorimetry, displayed as the respiratory exchange ratio (RER) hourly measurements (A), average 16-h RER (B), energy expenditure hourly measurements (C), and average 16-h energy expenditure (D). Animals were fed a STD or a CAF diet for 9 weeks. After this, STD group was supplemented with vehicle ($n = 6$) and CAF group ($n = 18$) was divided in three groups. Animals received either a daily low dose of 50 mg/kg (CAF+50), a medium dose of 100 mg/kg (CAF+100), or a high dose of 200 mg/kg (CAF+200) of resveratrol for a period of 22 days. Values are mean $\pm$ SEM of six animals per group. Significant differences ($p \leq 0.05$) in graphs A and C were assessed by repeated-measures ANOVA; t , time effect; T , resveratrol treatment effect, and $t \times T$ interaction of time and resveratrol treatment. Significant differences ($p \leq 0.05$) in graphs B and D were assessed by one-way ANOVA followed by post-hoc multiple comparisons tests Tukey or Dunnett's T3 according to Levene's test. Different letters denote significant difference between groups.

compared to STD group, and RSV supplementation tended to normalize SOCS3 levels (Figure 6C). Neuronal SIRT1 is required to protect against diet-induced obesity, and RSV is a known activator of this enzyme [56–58]. Rats fed CAF showed a strongly reduced levels of SIRT1, which was normalized by RSV at the doses of 50 and 100 mg/kg (Figure 6C).

4 | Discussion

In obesity there is an excessive energy surplus, and the additional lipid accumulation can lead to dysfunctional hypertrophic adipocytes and to an impaired expandability of the adipose tissue. This process inside visceral fat depots has been correlated to a high risk of developing obesity-related diseases [59]. RSV has been demonstrated to ameliorate health state in obesity without affecting body weight [60], and thus, we considered as seen with other polyphenols, that RSV could exert its beneficial effects on obesity through adipose tissue remodeling by counteracting hypertrophy and increasing adipocyte number once obesity is established. The administration of RSV for 22 days together with the CAF diet in established obesity reduced the percentage of fat mass only in iWAT and eWAT. These heterogeneous effects observed by RSV were previously reported with a mix containing polyphenols in CAF rats also showing a reduction of iWAT and eWAT but not in rWAT [61]. These results could be

explained by the intrinsic differences among fat deposits. In this sense, variations within fat depots had been reported regarding glucose and lipid metabolism, cytokine profile, proliferative and thermogenic capacities, and vascularization [62].

Although RSV especially reduced the percentage of iWAT and eWAT mass, we examined rWAT as a visceral fat depot, because especially visceral fat depots have been correlated to a high risk of developing obesity-related diseases [63]. Furthermore, rWAT was shown to be more sensitive to modulation of adipocyte size and number by phenolic compounds than subcutaneous fat [64]. Other studies in different models, including humans, have also observed reduced visceral WAT hypertrophy by RSV [65, 66], without reporting increased hyperplasia. Furthermore, previous animal studies were done with RSV administered at the start of the high-fat diet feeding, suggesting hypertrophic-preventive effects [67]. Here, RSV was administered during 22 days after obesity had been developed, showing a notable RSV anti-hypertrophic and pro-hyperplastic properties. Visceral WAT dysfunction is related to hypertrophic adipocytes, is unable to further expand, and is strongly associated with metabolic syndrome. Therefore, RSV induced remodeling of rWAT, increasing its expandability and potentially its lipid storage capacity that could have implications in ameliorating metabolic complications. Indeed, our results showed normalization of TAG serum levels and an improved HOMA-IR index, a surrogate marker of insulin

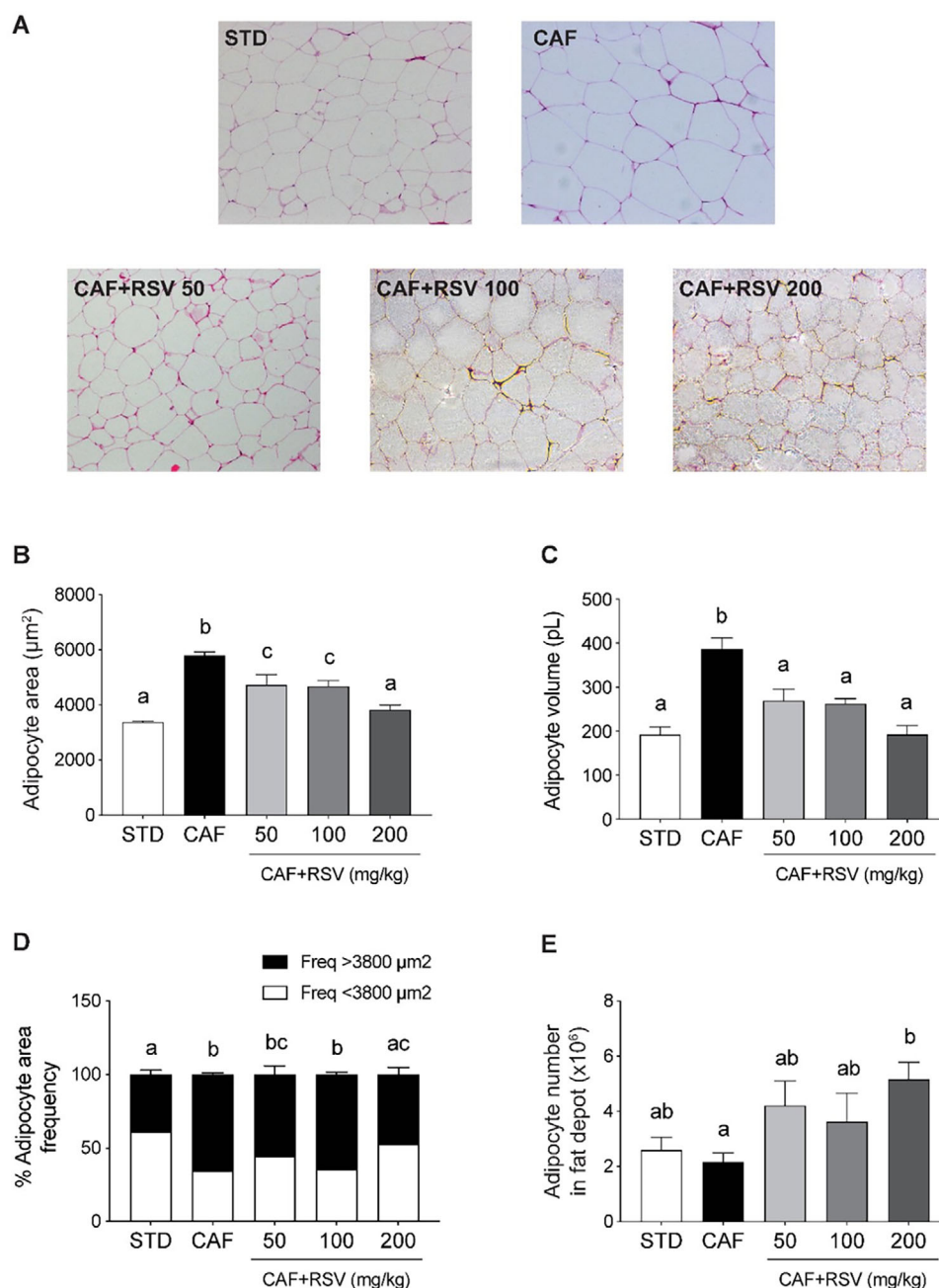


FIGURE 2 | Effect of three different doses of resveratrol administration on adipocyte size and number in retroperitoneal white adipose tissue of obese rats. Samples of retroperitoneal white adipose tissue (rWAT) were stained with hematoxylin and eosin. Representative light microscopy images captured at 20 $\times$ magnification from each group (A) were used to measure adipocyte area (B), adipocyte volume (C), and the frequency of adipocyte size (D). Total adipocyte number (E) was extrapolated from the size of adipocytes and the weight of rWAT. Animals were fed a STD or a CAF diet for 9 weeks. After this, STD group was supplemented with vehicle ($n = 6$) and CAF group ($n = 18$) was divided in three groups. Animals received either a daily low dose of 50 mg/kg (CAF+50), a medium dose of 100 mg/kg (CAF+100), or a high dose of 200 mg/kg (CAF+200) of resveratrol for a period of 22 days. The values are the mean $\pm$ SEM of five fields per animal from three animals per group. Different letters indicate significant differences ($p \leq 0.05$) assessed by one-way ANOVA statistical analysis followed by multiple comparisons tests Tukey or Dunnett's T3 according to Levene's test.

resistance, in response to 200 mg/kg of RSV. Furthermore, at this dose, RSV also normalized circulating leptin concentrations, in agreement with a reduced adiposity, iWAT mass, and body weight gain.

In view of the rWAT improved expandability and the general metabolic state of animals after RSV administration, we studied RSV mechanisms by gene expression analysis of rWAT. These

analyses showed hardly any effects for most of the genes examined, except for *Lpl*. This downregulations of *Lpl* gene expression, a key enzyme in the hydrolysis of TAG [68], could explain the normalization of TAG with the dose of 200 mg/kg. Consistent with our results, *Lpl* expression was also shown to be reduced by RSV administration exerting anti-obesogenic effects in other animal studies, although in those studies RSV was administered from the start of the experimental period, concomitant with

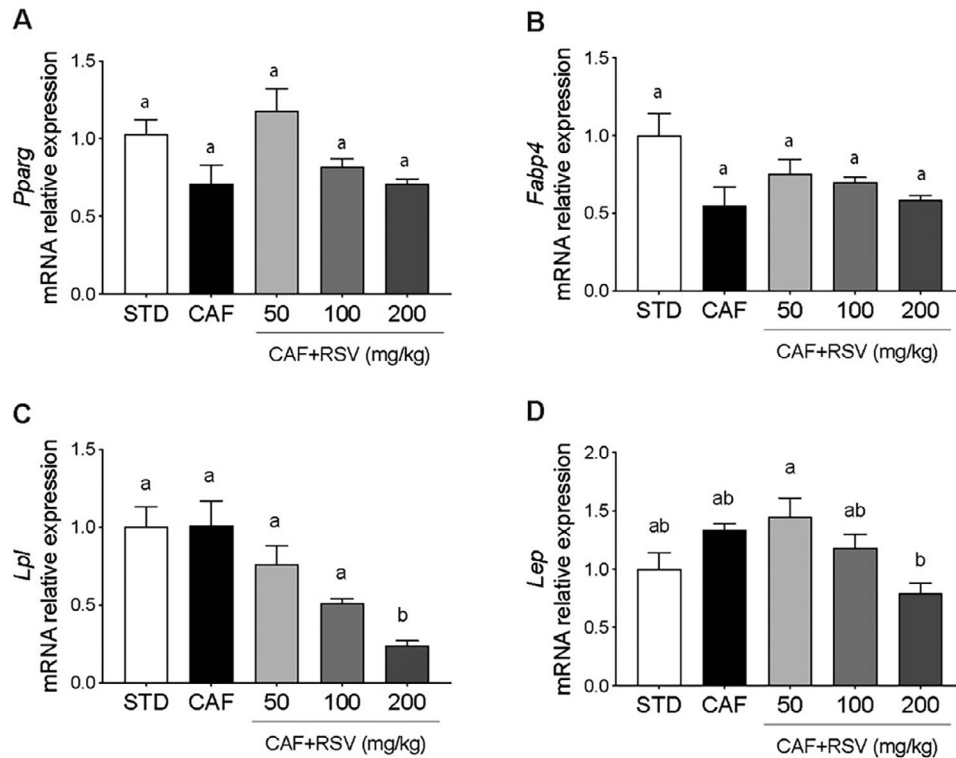


FIGURE 3 | Effect of resveratrol administration on mRNA levels of genes related to adipogenesis in retroperitoneal white adipose tissue of obese rats. Peroxisome proliferator-activated receptor gamma (*Pparg*) (A); fatty acid binding protein 4 (*Fabp4*) (B); Lipoprotein Lipase (*Lpl*) (C); Leptin (*Lep*) (D) mRNA levels. Animals were fed a STD or a CAF diet for 9 weeks. After this, STD group was supplemented with vehicle ($n = 6$) and CAF group ($n = 18$) was divided in three groups. Animals received either a daily low dose of 50 mg/kg (CAF+50), a medium dose of 100 mg/kg (CAF+100), or a high dose of 200 mg/kg (CAF+200) of resveratrol for a period of 22 days. The values are the mean $\pm$ SEM of six animals per group. Different letters indicate significant differences ($p \leq 0.05$) assessed by one-way ANOVA statistical analysis followed by multiple comparisons tests Tukey or Dunnett's T3 according to Levene's test.

obesity induction [69]. Somewhat unexpected, in view of the observed alterations in adipocyte size and number, was not to observe significant changes in the expression of the genes examined, including no changes in the expression of *Pparg*, the master regulator of adipogenesis. The discrepancy may be due to these studies being in vitro or preventive in vivo studies with RSV [70].

Phenolic compounds have been described for modulating the expression of miRNAs [71, 72]. However, we did not observe modulation of miR-33a neither its host gene *Srebp2* nor its targets *Abca1* and *Fasn* in rWAT. This indicates an absence of miR-33a regulation in rWAT. In contrast, we observed a normalization of miR-33a by RSV in a dose-dependent manner in the liver, with a similar pattern for *Srebp2*. This result is in agreement with an in vitro study in the HepG2 liver cell line, which showed RSV could modulate miR-33 by direct binding [73]. Our differential results for miR-33 between rWAT and liver suggest that RSV targets multiple tissues, which are together responsible for the metabolic improvements. This makes it advisable to use a multi-tissue analysis approach for future studies.

Alterations in leptin levels in adipose tissue are related with adipose tissue deregulation and whole energy homeostasis imbalance. Therefore, it is crucial to study the connection between adipose tissue and hypothalamus since this brain part is where

leptin will ultimately control food intake and energy expenditure [74]. Our results show that RSV supplementation to diet-induced obese rats normalizes plasma leptin levels and enhances hypothalamic leptin action. Accordingly, in vitro [75] and in vivo [76] studies showed a reduction of leptin levels by RSV. In agreement, it was previously reported that the treatment of offspring of high-fat diet fed mice with 30 mg RSV/kg rescued hyperleptinemia and increased pSTAT3 levels in ARC [77]. However, contradictory results have been widely published regarding the effect of RSV compound on circulating leptin levels and a meta-analysis of randomized controlled trials did not find changes in plasma leptin of obese and non-obese subjects supplemented with different doses of RSV [78]. Possible explanations for these contrasting data could be different study setups and different experimental conditions, for example, the different range of doses used and the treatment length. For clarity, the focus of future studies should be on systematic elucidation of mechanisms of action of RSV and other polyphenols, which is the only way to clarify conflicting data.

The observed concomitant decrease in plasmatic leptin concentration with increasing hypothalamic pSTAT3 levels is consistent with higher leptin sensitivity in this tissue. If hypothalamic STAT3 phosphorylation is mainly the consequence of leptin-induced activation of ObRb signaling, the levels of hypothalamic pSTAT3 relative to the levels of leptin in plasma could, according

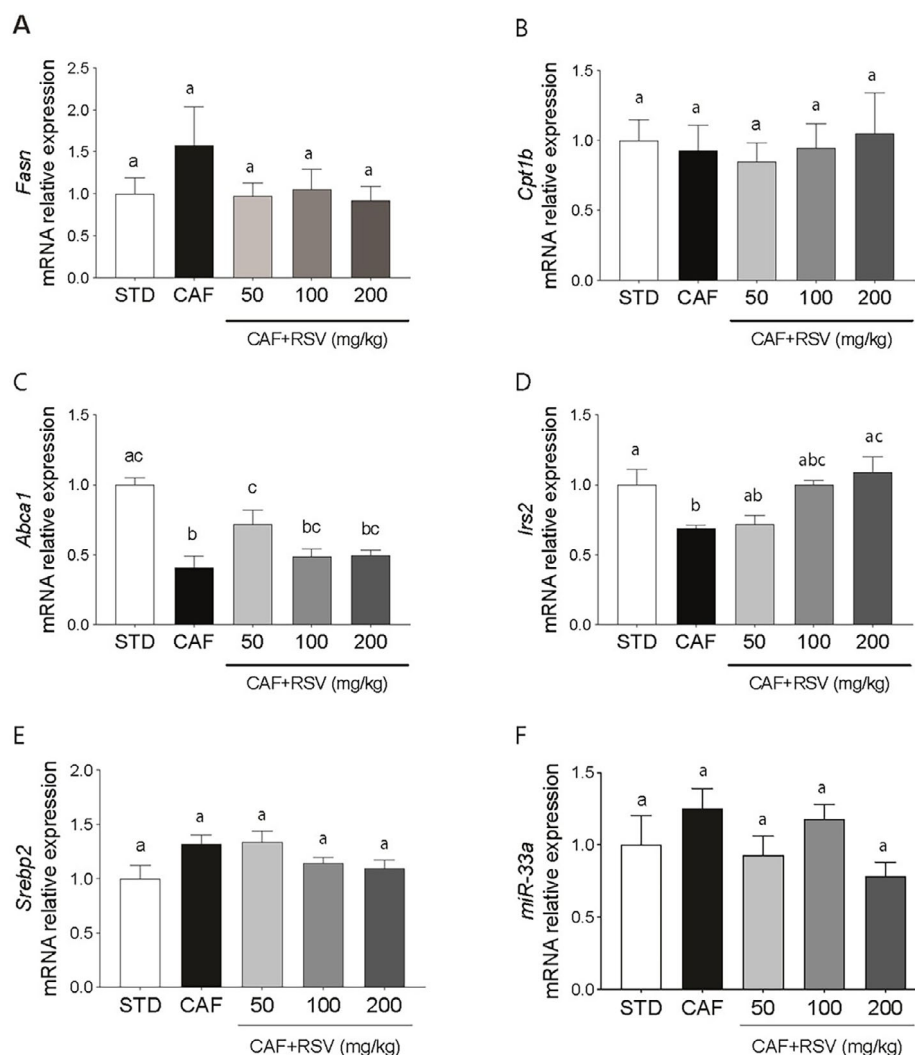


FIGURE 4 | Effect of resveratrol administered on mRNA levels of genes related to lipid metabolism in retroperitoneal white adipose (rWAT) of diet-induced obese rats. Fatty acid synthase (*Fasn*) (A), carnitine palmitoyltransferase 1B (*Cpt1b*) (B), ATP binding cassette subfamily A member 1 (*Abca1*) (C), insulin receptor substrate 2 (*Irs2*) (D), sterol regulatory element binding protein 2 (*Srebp2*) (E), and miR-33a levels (F). Animals were fed a STD or a CAF for 9 weeks. After this, STD group was supplemented with vehicle ($n = 6$) and CAF group ($n = 18$) was divided in three groups. Animals received either a daily low dose of 50 mg/kg (CAF+50), a medium dose of 100 mg/kg (CAF+100), or a high dose of 200 mg/kg (CAF+200) of resveratrol for a period of 22 days. The values are the mean $\pm$ SEM of six animals per group. Different letters indicate significant differences ($p \leq 0.05$) assessed by one-way ANOVA statistical analysis followed by multiple comparisons tests Tukey or Dunnett's T3 according to Levene's test.

to our reasoning, be reliable estimation of leptin sensitivity in the hypothalamus. Using this estimation, our results showed for the first time that RSV, at dose of 200 mg/kg, restored the hypothalamic sensitivity of leptin in obese rats. Besides, the 200 mg/kg RSV was also able to reduce pACC levels indicating activation of leptin's signaling pathway. Several authors have indicated that the metabolic disturbances observed in animals fed a highly palatable diet are firmly related to a dysfunctional leptin system in the hypothalamus [79]. Accordingly, our results showed that this restoration of leptin action in obese animals was concomitant to reduced body weight gain that, in turn, was associated with increased lipid oxidation but not with a reduction in energy intake. Although, it has been reported that RSV is able to decrease food intake in rodents fed chow diet via modulation of melanocortinergic system, we did not find an effect of food intake despite increased leptin sensitivity, which may possibly be explained by interactive effects of the various

pathways that play a role in food intake regulation, such as the ghrelin, cholecystokinin, glucagon-like-peptide-1, and peptide YY (PYY) signaling pathways. In a similar way, we observed a tendency toward SOCS3 level restoration by RSV. The functional interpretation is complicated, because on the one hand SOCS3 negatively regulates leptin signaling, but on the other hand its transcription is activated by higher levels of pSTAT3, working as a negative feedback mechanism, and in that case high levels of SOCS3 would indicate enhanced leptin signaling [80]. We also observed a significant increase in hypothalamic SIRT1 expression. This is in agreement with Ramadori et al. [81], who showed that RSV was able to mediate anti-diabetic effects in the brain by increasing SIRT1 functionality after chronic intracerebroventricular infusion of RSV. Furthermore, genetic overexpression of SIRT1 in mice reduces food intake and modulates energy expenditure [82]. All in all, our data indicate that RSV improves leptin sensitivity in obesity, but the exact mechanisms involved,

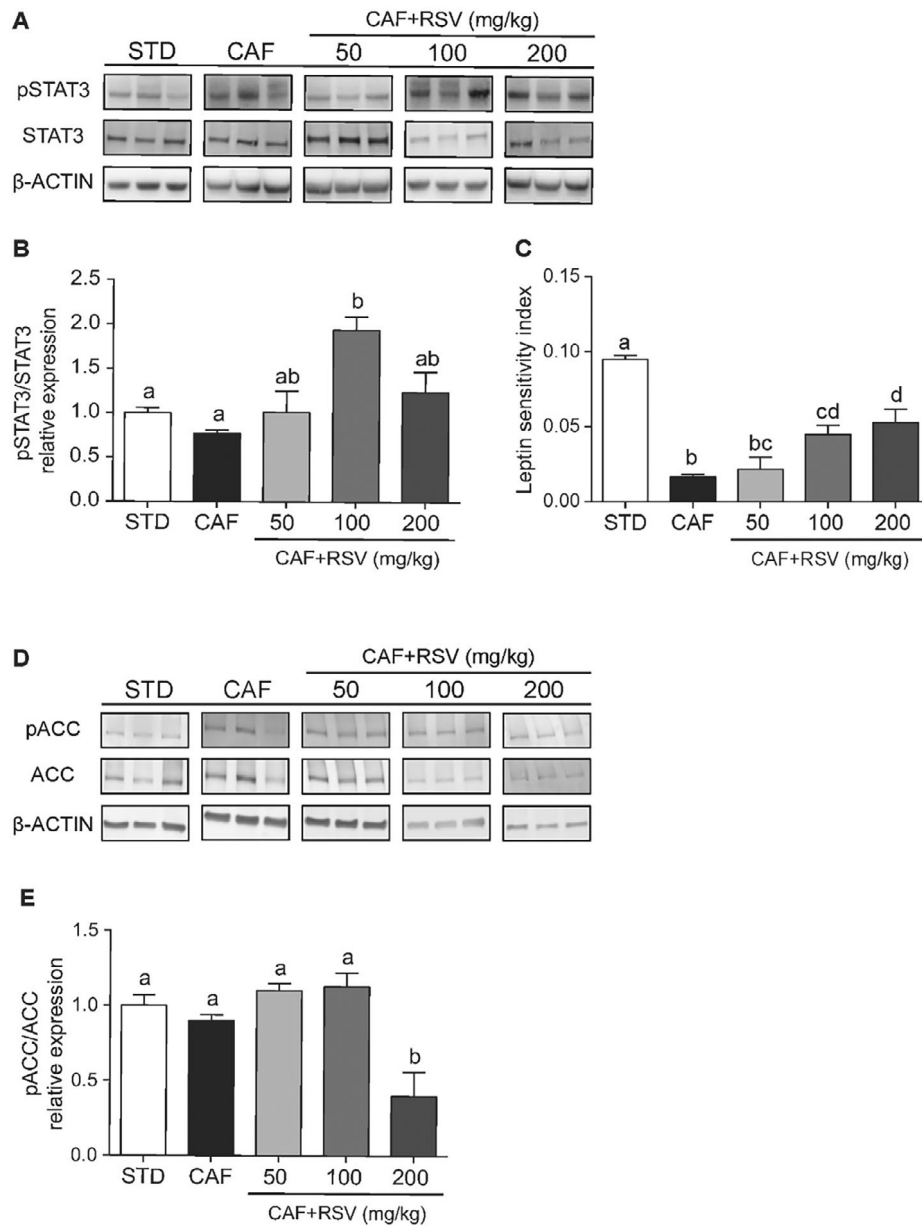


FIGURE 5 | Protein expression of factors involved in leptin signaling in the hypothalamus. Expression levels of pSTAT3 assessed by immunoblot (A), pSTAT3 representation of average expression per group (B), pSTAT3/serum leptin ratio (C), expression levels of pACC assessed by immunoblot (D), and pACC/ACC representation of average expression per group (E). Intensity signals of each protein were normalized by β -Actin. Animals were fed a STD or a CAF for 9 weeks. After this, STD group was supplemented with vehicle ($n = 6$) and the CAF group ($n = 18$) was divided in three groups. Animals daily received either a low dose of 50 mg/kg (CAF+50), a intermediate dose of 100 mg/kg (CAF+100), or a high dose of 200 mg/kg (CAF+200) of resveratrol for a period of 22 days. Values are mean $\pm$ SEM of six animals per group. Different letters indicate significant differences ($p \leq 0.05$) assessed by one-way ANOVA statistical analysis followed by multiple comparisons tests Tukey or Dunnett's T3 according to Levene's test.

including the functional roles of SOCS3 and SIRT1, need further investigation.

Our data show most effects at the highest dose tested in existing obesity, indicating that high-dose supplementation would be overall most effective in achieving beneficial metabolic health effects. High-dose supplementation may raise safety concerns. However, the 200 mg RSV/kg body weight is within a dose range that is acceptable for rodents and that would be tolerated by humans. In rats, 300 mg RSV/kg of body weight has been reported as the maximum tolerated dose without detrimental

effects [83]. As mentioned above, based on a body surface area (BSA) calculation [34], the human equivalent dose to 200 mg RSV/kg for an obese adult would not exceed 5 g per day. Studies supplementing such high doses of RSV state that it was well tolerated except for some subjects that experimented mild episodic intestinal discomfort [84, 85]. The fact that RSV has different effects in a large range of concentrations emphasizes that this compound affects different signaling pathways, and, therefore, it is essential to know the suitable dose in each case to achieve beneficial effects [86]. Further research concerning oral dosage of RSV should be conducted in in vivo models of

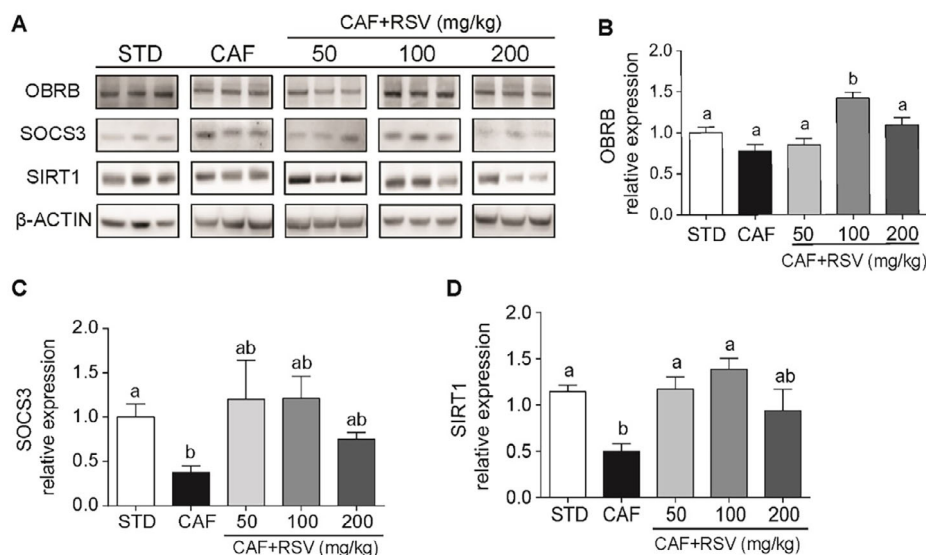


FIGURE 6 | Protein expression of factors involved in leptin signaling in the hypothalamus. Expression levels leptin receptor OBRB, SOCS3, and SIRT1 assessed by immunoblot (A), OBRB representation of average expression per group (B), SOCS3 representation of average expression per group (C), and SIRT1 representation of average expression per group (D). Intensity signals of each protein were normalized by β -Actin. Animals were fed a STD or a CAF for 9 weeks. After this, the STD group was supplemented with vehicle ($n = 6$) and the CAF group ($n = 18$) was divided in three groups. Animals daily received either a low dose of 50 mg/kg (CAF+50), an intermediate dose of 100 mg/kg (CAF+100), or a high dose of 200 mg/kg (CAF+200) of resveratrol for a period of 22 days. Values are mean $\pm$ SEM of six animals per group. Different letters indicate significant differences ($p \leq 0.05$) assessed by one-way ANOVA statistical analysis followed by multiple comparisons tests Tukey or Dunnett's T3 according to Levene's test.

diet-induced obesity before recommending its consumption to humans. As it is reviewed by Fernández-Quintela et al. [28], several works have shown that RSV does not always follow a dose-response pattern when exerting anti-obesity effects.

5 | Concluding Remarks

A high dose of RSV administrated to rats with established obesity has beneficial effects on obesity by inducing a healthier expansion of visceral WAT to match the energy excess of the CAF diet and also by improving leptin sensitivity in the hypothalamus. Therefore, the supplementation of regular diets with RSV could be a good approach against obesity.

Acknowledgments

In memory of Prof. Cinta Bladé, who sadly passed away on April 8, 2019. She served as the Doctoral Thesis supervisor for the first two authors of this paper.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.