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Long-term resveratrol administration reduces metabolic disturbances and lowers blood pressure in obese Zucker rats

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ABSTRACT

Resveratrol is a natural polyphenolic stilbene derivative found in several human diet components that possess important and wide-ranging effects in biological systems including anticancer, anti-inflammatory, antioxidant, cardio-protective, and anti-ageing actions and beneficial properties against metabolic diseases. This study addresses the effects of long-term administration of resveratrol on several functional alterations arising from the metabolic syndrome experimental model of obese Zucker rats, and the possible mechanisms involved. The high plasma concentrations of triglycerides, total cholesterol, free fatty acids, insulin and leptin found in obese Zucker rats were reduced in obese rats that received resveratrol. Furthermore, the elevated hepatic lipid content was significantly lower in obese rats treated with resveratrol, an effect which was related to the increased phosphorylation of 5′-AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) in the liver of these animals. Resveratrol treatment also improved the inflammatory status peculiar to this model, as it increased the concentration of adiponectin and lowered tumor necrosis factor-α production in the visceral adipose tissue (VAT) of obese Zucker rats. Moreover, chronic intake of resveratrol enhanced VAT eNOS expression among obese Zucker rats. These effects parallel the activation of AMPK and inhibition by phosphorylation of ACC in this tissue. The raised systolic blood pressure and reduced aortic eNOS expression found in obese Zucker rats were significantly improved in the resveratrol-treated obese rats. In conclusion, resveratrol improved dyslipidemia, hyperinsulinemia, hyperleptinemia and hypertension in obese Zucker rats, and produced anti-inflammatory effects in VAT, effects that seem to be mediated by AMPK activation.

1. Introduction

Type 2 diabetes and obesity are pathologies with a high prevalence in Western societies. Metabolic syndrome is a constellation of several specific abnormalities including abdominal obesity, insulin resistance, dyslipidemia and hypertension [1]. It is directly associated with an increased risk of developing cardiovascular diseases [2,3], which are the major causes of premature mortality in type 2 diabetes patients. In fact, metabolic syndrome is a greater detriment

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Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, 5′-AMP-activated protein kinase; eNOS, endothelial nitric oxide synthase; FFA, free fatty acids; LC, lean control rats group; LR, lean rats treated with resveratrol group; NO, nitric oxide; NOx, nitrates plus nitrites; OC, obese control rats group; OR, obese rats treated with resveratrol group; p-ACC, phospho-ACC; p-AMPK, phospho-AMPK (Thr172); SBP, systolic blood pressure; TNF-α, tumor necrosis factor-α.

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than cancer to our society's overall health. In order to address this problem, there has been increasing effort to find solutions that prevent and reverse metabolic syndrome. Managing the disorders clustered in this syndrome is of great relevance to prevent and to reduce the risk of all of these pathologies. Furthermore, obesity and insulin resistance have recently been linked to a low-grade chronic inflammatory response characterized by increased macrophage infiltration, altered cytokine production, and activation of the inflammatory signaling pathway in adipose tissue. Thus, pharmacological agents and natural products able to reduce inflammatory activity possess anti-diabetic properties.

The AMP-activated protein kinase (AMPK) system acts a sensor of cellular energy charge which is activated by AMP but also by phosphorylation by one or more upstream kinases at a threonine residue [4–6]. When it is activated, AMPK phosphorylates and inhibits acetyl-CoA carboxylase (ACC), leading to an increase in fatty acid oxidation and inhibiting fatty acid synthesis [7]. AMPK is activated by a number of pathological stresses, including hypoxia, oxidative stress, glucose deprivation, as well as exercise and dietary hormones, such as leptin and adiponectin [5,8,9]. Lately, strategies targeting AMPK activation have received increasing interest regarding the prevention and treatment of type 2 diabetes and other disorders associated with metabolic syndrome [10].

Various natural molecules found in the human diet have been described as potential agents to treat diabetes and hyperlipidemia [11–14]. Furthermore, recent studies have shown that some of these compounds strongly stimulate hepatic AMPK [11–13], an action that may help to explain some of the antihyperlipidemic effects of these natural derivatives and that provides an avenue for ameliorating hyperlipidemia and accelerated atherosclerosis in diabetes [11].

Resveratrol (trans-3,4′,5-trihydroxystilbene) (Fig. 1) is a natural polyphenolic compound synthesized in a large number of plant species including some components of the human diet, such as mulberries, peanuts, grapes, and red wines. It is responsible for the beneficial effect of regular wine consumption in moderate amounts [15]. The positive effects of resveratrol in biological systems are wide-ranging. It acts as a cancer chemoprevention agent [16], a powerful anti-inflammatory factor and an antioxidant agent [17,18]. Its cardiovascular properties, including inhibition of platelet aggregation and promotion of vasodilation by enhancing the production of NO [19] have also been described [15,20] and recent studies have reported its beneficial effects against ageing and metabolic diseases [21–24]. Resveratrol has been described as a potent activator of AMPK in several tissues and cell lines, i.e., HepG2 [24], C2C12 myotubes [25] neuronal cell lines and neurons [26].

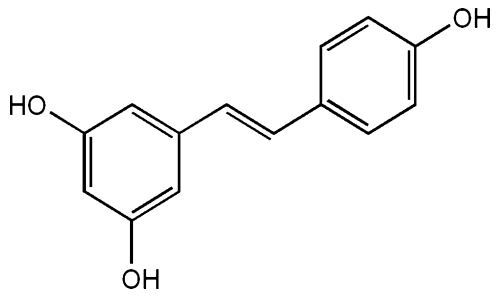

Fig. 1 – Chemical structure of resveratrol.

The main aim of the present study was to examine the effects of the chronic daily administration of resveratrol on the disturbances present in metabolic syndrome, and to analyze the mechanisms involved in its effects. The dose of resveratrol used is equivalent to that used by humans as a diet supplement. For this purpose, we used the obese Zucker rat, a widely used animal model of obesity and type 2 diabetes that presents many of the human metabolic syndrome features. These animals display insulin resistance, dyslipidemia, hyperinsulinemia [2,27] and, in some colonies, hypertension that develops by 4–5 months of age [28]. On the contrary, their lean littermates are insulin-sensitive, normoinsulinemic, normotensive, and present a normal lipid profile and glucose tolerance. Furthermore, this model presents a loss of functional mutation in the leptin receptor [29,30]. Because some of the metabolic changes caused by resveratrol mimic those observed in response to AMPK activation, we hypothesized that AMPK activation might be an important mediator of resveratrol actions in this model.

2. Materials and methods

This study was carried out in accordance with the European Union guidelines for animal care and protection.

2.1. *Reagents*

Trans-resveratrol and all chemicals were obtained from Sigma Chemicals (Madrid, Spain).

2.2. *Animals and experimental protocol*

Fourteen obese male Zucker rats and 14 lean heterozygous littermates at the age of 13 weeks (Charles River Laboratories, Barcelona, Spain) were housed two or three per cage at a constant temperature (24 ± 1 °C), with a 12 h dark/light cycle and free access to tap water and food. Rats were allowed to adapt to these conditions for 2 weeks, before the beginning of the experimental protocol.

Obese and lean rats were randomly assigned to two groups of seven animals. One of them received a daily dose of resveratrol (10 mg/kg of body weight) ($n = 7$), mixed in the vehicle (1 ml water), while the other one received just the vehicle ($n = 7$). The rats were treated orally by gavage for 8 weeks. Henceforth, the obese and lean groups given resveratrol are referred to as OR and LR, respectively, and the groups given the vehicle (control groups) are designated as OC and LC. Administration of resveratrol was stopped 2 days before the end of the experiments, in order to study its long-term consequences without the involvement of its acute administration effects. Over the experimental period, the rats had free access to tap water and diet, and food and water intake was measured daily.

2.3. Blood pressure measurements

Systolic blood pressure (SBP) was determined weekly, in the morning, in conscious, pre-warmed, restrained rats by tail-cuff plethysmography (digital pressure meter, LE 5000, Letica S.A., Barcelona, Spain). At least seven determinations were made in every session and the mean of the lowest three values within 5 mmHg was taken as the SBP value.

2.4. Samples collection and storage

At the end of the experimental period, rats were fasted overnight, blood was obtained from the tail vein to analyze biochemical parameters, and animals were sacrificed. The epididymal and retroperitoneal adipose tissue (visceral adipose tissue, VAT) were removed and weighed for evaluation of abdominal fat content. The liver was also removed and weighed, as well as abdominal aorta. All tissue samples were frozen until analysis. Plasma was obtained by blood centrifugation at 2000 × *g* for 15 min, aliquoted and frozen.

2.5. Plasma analytical procedures

Plasma glucose, triglycerides, and total cholesterol concentrations were measured by colorimetric methods using Spinreact kits (Spinreact, S.A., Girona, Spain). Plasma free fatty acids (FFA) concentration was determined using a Wako NEFA C test kit (Wako Chemicals, Richmond, VA). Plasma insulin concentration was quantified using a rat insulin enzyme immunoassay kit (Spibio, Montigny le Bretonneux, France). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin levels as a measure of insulin resistance [31]. Plasma leptin concentration was determined using a rat leptin EIA kit (Assay Designs, Inc., Ann Arbor, MI, USA).

2.6. Measurement of lipid content in the liver

Total lipids were extracted according to the Bligh and Dyer method [32]. Briefly, 1.25 g of tissues were homogenized with 3.75 ml of chloroform–methanol (1:2 by volume) using a Heidolph polytron. The homogenates were vigorously vortexed for 15 min, mixed with 1.25 ml of chloroform and then with 1.25 ml of water, and centrifuged briefly at 3000 rpm to separate the phases. Next, the lower phase was transferred to another tube, and the residue was mixed with 1.88 ml of chloroform for the second-step vortex and centrifugation, and the lower phase obtained by the centrifugation was mixed with the first chloroform phase in the same tube. After evaporation with nitrogen gas at 55 °C, the lipid extract was dissolved in 2 ml of 2-propanol. Triglycerides and total cholesterol were assayed by the enzymatic quantitative method described in plasma analytical procedures.

2.7. Adiponectin, nitrates plus nitrites and tumor necrosis factor-α (TNF-α) production by adipose tissue

Visceral adipose tissue obtained from each rat was homogenized in phosphate buffer saline. Homogenates were incubated at 37 °C for 20 min, and centrifuged at 15,000 × *g* for 15 min. Adiponectin concentration in VAT was also measured using a mouse/rat adiponectin ELISA kit (B-Bridge International, Inc., Mountain View, CA, USA). Nitrate plus nitrite levels in plasma, as markers for the activity of NO synthase and NO biosynthesis, were determined by means of an assay based on the determination of nitrite, using the Griess reaction. Nitrate was measured as nitrite after enzymatic conversion by nitrate reductase as previously described [33]. The levels of TNF-α in the tissue supernatants were determined using an ELISA kit (Diaclone, Inc., Besançon, France) specific for rat TNF-α.

2.8. eNOS expression by Western blot in aorta and visceral adipose tissue

The eNOS Western blots from VAT and aorta were performed as described elsewhere [14]. 50 μg of protein of VAT and 40 μg of protein of aortic homogenates from each sample were separated by SDS-polyacrylamide (8%) gel electrophoresis in a mini-gel system (Bio-Rad Laboratories, S.A., Madrid, Spain), and then transferred electrophoretically onto nitrocellulose membranes overnight. Relative amounts of proteins were ascertained by staining membranes with Ponceau S. After blocking the filters in 5% non-fat dry milk–Tris-buffered saline-0.1% Tween 20 (TBST), they were incubated with a mouse anti-eNOS monoclonal antibody (BD Transduction Laboratories, San Jose, CA, USA), diluted at 1:2500 in 5% albumin–TBST. The filters were then washed five times for 10 min in TBST and incubated respectively with secondary peroxidase conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, USA) diluted at 1:2000 in 5% non-fat dry milk–TBST. All incubations were performed at room temperature for 2 h. After washing membranes, antibody binding was detected by an ECL system (PerkinElmer, LAS, Boston, USA). Films were scanned and densitometric analysis was performed on the scanned images using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com).

2.9. AMPK and ACC activity in liver and visceral adipose tissue

VAT samples obtained from each rat were homogenated in modified RIPA buffer (50 mmol/L Tris–HCl, pH 7.4, 1% Triton X-100, 0.2% sodium deoxycholate, 0.2% sodium dodecylsulfate (SDS), 1 mmol/L sodium ethylenediaminetetraacetate, 1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/ml of aprotinin, 5 μg/ml of leupeptin). Liver samples were homogenated in buffer (20 mmol/L Tris–HCl, pH 8.0, 1% (v/v) Nonidet P-40, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin). Homogenates were centrifuged at 14,000 rpm for 10 min at 4 °C. Protein concentrations in homogenates were measured by the bicinchoninic acid protein assay. 30 μg of each sample were subjected to 10% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes by wet transfer at 90 V for 2 h. The membranes were blocked with 5% (w/v) non-fat dry milk in TBST for 2 h at room temperature and subsequently blotted with the appropriate antibodies in 5% (w/v) non-fat dry milk–TBST. The

antibodies anti-AMPK, anti-phospho-AMPK (Thr172) (anti-p-AMPK), anti-ACC, and anti-phospho-ACC (anti-p-ACC) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) and were used at manufacturer-recommended dilutions (1:1000). Incubations with primary antibodies were performed overnight at 4 °C. Following incubation, membranes were washed three times with TBST for 10 min each, before incubation for 2 h at room temperature with secondary peroxidase conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, USA) diluted at 1:2000 in 5% non-fat dry milk–TBST. Membranes were then washed five times with TBST for 10 min each, and the bound antibodies were visualized by an ECL system (PerkinElmer, LAS, Boston, USA). Phosphorylated AMPK and ACC were quantified by densitometric analysis as described above, and normalized to the levels of endogenous AMPK and ACC protein respectively. Phosphorylation intensity of AMPK and ACC was expressed relative to the control level.

2.10. Statistical analysis

Results are expressed as mean ± standard error of mean (SEM) of measurements. For statistical analysis, two-way ANOVA with Bonferroni's post test was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com), with statistical significance set at $P < 0.05$.

3. Results

3.1. Food intake, body weight, and organ weights

The average daily food intake throughout the experimental period was significantly greater among the obese Zucker rats than among their lean littermates (Table 1), and resveratrol did not modify this parameter in either rat strain.

The body weight of obese Zucker rats was significantly higher ($P < 0.00001$) than that of their lean littermates when the treatment began. Chronic oral administration of resveratrol did not produce changes in body weight in either rat strain (Table 1).

The liver weight of the obese animals was much higher when compared with their lean littermates ($P < 0.001$) (Table 1). Eight weeks of resveratrol administration did not affect this parameter in either rat strain (Table 1).

The obese animals were, as expected, characterized by significant obesity and a much higher content of abdominal fat (epididymal and retroperitoneal adipose tissue) compared with the lean animals ($P < 0.00001$) (Table 1). In the obese rats, resveratrol treatment was associated with a significantly lower content of abdominal fat ($P < 0.01$ vs. OC), while no effects were observed in the lean rats (Table 1).

3.2. Effects of resveratrol on plasma parameters

Fasting plasma glucose was significantly higher in the obese Zucker rats before treatment compared with the lean controls ($P < 0.001$) (Fig. 2A). Similarly, fasting plasma insulin was approximately 3-fold higher in the obese animals than in the lean animals ($P < 0.05$) (Fig. 2B). After 4 weeks of resveratrol administration, the OR rats presented the same fasting levels of circulating glucose as the lean animals, and showed significantly reduced fasting plasma insulin concentration compared with the values observed in the OC rats ($P < 0.001$) (Fig. 2A, B). As expected, the OC rats had greater insulin resistance, expressed as HOMA-IR, than did the LC rats. This parameter was significantly improved in the OR rats ($P < 0.001$ vs. OC) (Fig. 2D). All these effects were maintained after 8 weeks of treatment.

Obese Zucker rats have hyperleptinemia, as this experimental model is characterized by genetic error in leptin receptor gene expression [29,30]. As expected, this alteration was present among the obese rats before the experiment began. Although no differences were observed in this parameter in the obese rats treated with resveratrol for 4 weeks, by the end of the experimental period, plasma leptin concentration was significantly reduced in the OR rats ($P < 0.01$ vs. OC), (Fig. 2C).

Plasma levels of triglycerides, FFA and total cholesterol were higher in the OC than in the lean rats before the treatment began ($P < 0.01$) (Table 2). With respect to the OC rats, the plasma concentrations of triglycerides, FFA and total cholesterol decreased by 31.6%, 26.2% and 29.7%, respectively ($P < 0.05$ vs. OC) among the OR rats. However, among the lean rats no differences were found between the rats given resveratrol and their controls (Table 2).

3.3. Effects of resveratrol on liver parameters

Likewise, by the end of the experimental period the content of triglycerides and total cholesterol in the liver had increased

Table 1 – Food intake, body and organ weight in lean and obese Zucker rats that received vehicle (control) or resveratrol (10 mg/kg/day) for 8 weeks.

Experimental groups*	LC ($n = 7$)	LR ($n = 7$)	OC ($n = 7$)	OR ($n = 7$)
Average food intake (g/day)	20.7 ± 0.1^{b}	20.4 ± 0.2^{b}	22.6 ± 0.6^{a}	22.5 ± 0.5^{a}
Initial body weight (g)	340.3 ± 7.0^{b}	339.4 ± 5.6^{b}	418.9 ± 6.0^{a}	416.9 ± 8.0^{a}
Final body weight (g)	438.0 ± 9.7^{b}	419.7 ± 8.4^{b}	535.4 ± 7.5^{a}	520.0 ± 11.0^{a}
Liver weight (g)	14.3 ± 0.4^{b}	13.0 ± 0.6^{b}	24.7 ± 1.2^{a}	24.1 ± 1.1^{a}
Fat content (g)	7.2 ± 0.5^{c}	7.2 ± 0.9^{c}	21.3 ± 0.4^{a}	19.1 ± 0.5^{b}

Values are expressed as mean ± SEM. Values within a row without a common letter differ significantly, $P < 0.05$.

* Experimental groups: lean control group (LC), lean group treated with resveratrol (LR), obese control group (OC) and obese group treated with resveratrol (OR).

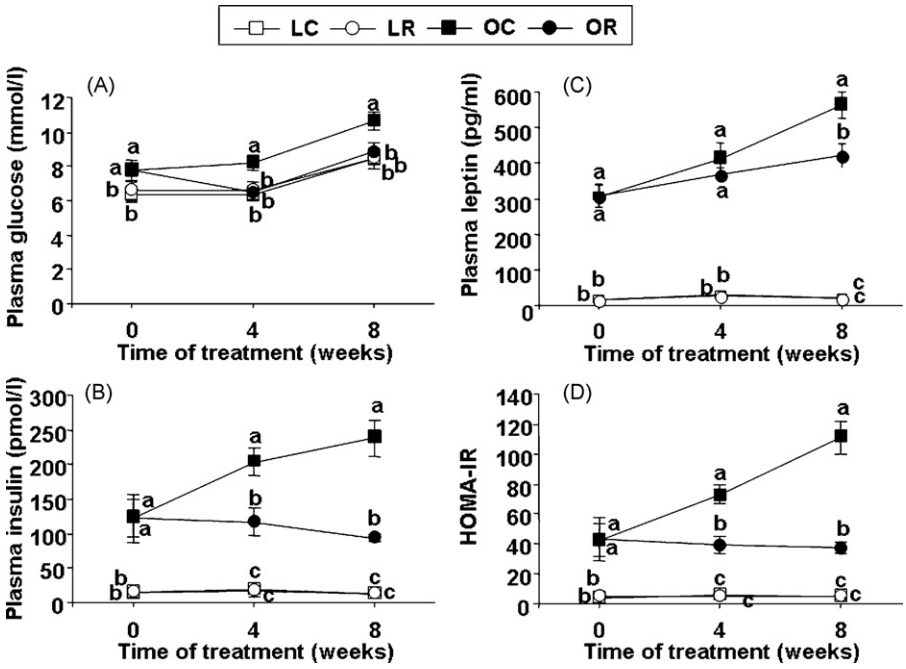

Fig. 2 – Effects of resveratrol on circulating concentration of glucose (A), insulin (B), and leptin (C) and homeostasis model assessment of insulin resistance (HOMA-IR) (D) in lean and obese Zucker rats before and after 4 or 8 weeks of treatment. Rats received vehicle (control) or resveratrol (10 mg/kg/day). Experimental groups: LC, lean control rats; LR, lean rats treated with resveratrol; OC, obese control rats; OR, obese rats treated with resveratrol. HOMA-IR: fasting glucose mmol/L × fasting insulin pmol/L/22.5. Data are expressed as means ± SEM ($n = 7$). Means at a time without a common letter differ, $P < 0.05$.

more than 2-fold in the obese rats compared with the lean rats ($P < 0.00001$), and treatment with resveratrol significantly lowered these levels by approximately 45% and 47% respectively (Fig. 3A) ($P < 0.00001$ vs. OC).

We also analyzed the liver expression of AMPK, p-AMPK, ACC and p-ACC by the Western blot method. Although there were no differences between the groups in AMPK or ACC expression in the liver (Fig. 3B) compared with the lean control rats, Thr172 phosphorylation of AMPK was dramatically decreased in obese Zucker rat livers by approximately 50% ($P < 0.001$) (Fig. 3C). Likewise, inhibition of AMPK activation was confirmed by decreased phosphorylation of ACC in the livers of the obese rats (Fig. 3D). In the obese Zucker rats treated with resveratrol (10 mg/kg/day), phosphorylation of AMPK had increased approximately 2-fold ($P < 0.001$) (Fig. 3C) and phosphorylation of ACC had risen by 35.3% ($P < 0.01$) with respect to the OC rats (Fig. 3D).

3.4. *Effects of resveratrol on visceral adipose tissue parameters*

VAT NOx levels increased 1.67-fold in the OC rats ($P < 0.01$ vs. LC rats) (Fig. 4A). In the OR rats this parameter was 38% lower than among the OC group ($P < 0.01$). Adiponectin

Table 2 – Lipid concentration in plasma and liver of lean and obese Zucker rats that received vehicle (control) or resveratrol (10 mg/kg/day) for 8 weeks.

Experimental groups*	LC ($n = 7$)	LR ($n = 7$)	OC ($n = 7$)	OR ($n = 7$)
Plasma triglycerides, mmol/L				
Before	0.49 ± 0.01^{b}	0.47 ± 0.04^{b}	4.75 ± 0.20^{a}	4.62 ± 0.34^{a}
8 weeks	0.67 ± 0.05^{c}	0.63 ± 0.06^{c}	5.25 ± 0.39^{a}	3.48 ± 0.18^{b}
Plasma cholesterol, mmol/L				
Before	2.84 ± 0.29^{b}	2.98 ± 0.21^{b}	5.03 ± 0.58^{a}	5.12 ± 0.42^{a}
8 weeks	3.5 ± 0.3^{c}	3.3 ± 0.3^{c}	7.5 ± 0.4^{a}	5.3 ± 0.3^{b}
Plasma FFA, mmol/L				
Before	0.66 ± 0.06^{b}	0.71 ± 0.06^{b}	0.96 ± 0.04^{a}	0.92 ± 0.08^{a}
8 weeks	0.92 ± 0.09^{b}	0.87 ± 0.07^{b}	1.42 ± 0.16^{a}	1.04 ± 0.06^{b}

Values are expressed as mean ± SEM. Values within a row without a common letter differ significantly, $P < 0.05$.

* Experimental groups: lean control group (LC), lean group treated with resveratrol (LR), obese control group (OC) and obese group treated with resveratrol (OR).

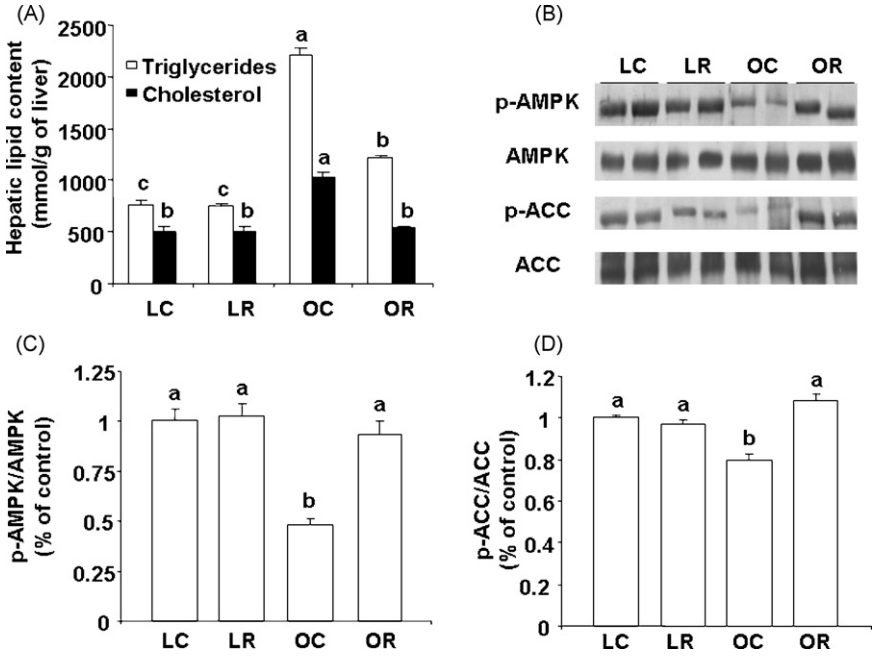

Fig. 3 – Effects of long-term resveratrol treatment in hepatic alterations of obese Zucker rats. Hepatic content of triglycerides and total cholesterol (A) in lean and obese Zucker rats receiving vehicle (control) or resveratrol (10 mg/kg/day) for 8 weeks. AMPK, phospho-AMKP (Thr172) (p-AMPK), ACC and phospho-ACC (p-ACC) protein expression in the liver of lean and obese Zucker rats receiving vehicle (control) or resveratrol (10 mg/kg/day) for 8 weeks (B). 30 μg of protein of liver homogenates were resolved by SDS-PAGE and probed using antibodies directed against AMPK, p-AMPK, ACC and p-ACC. Immunoblots are representative duplicates of liver homogenates of five different rats for each group of treatment. Densitometric analysis of AMPK (C) and ACC phosphorylation (D) in the liver of lean and obese rats given vehicle or resveratrol (10 mg/kg/day) for 8 weeks. Levels of p-AMPK were normalized to the total AMPK and compared to the LC group measurements which were assigned a value of 1.0 (C). Values are expressed as means ± SEM ($n = 5$). Levels of p-ACC were normalized to the total ACC and compared to the LC group measurements which were assigned a value of 1.0 (D). Values are expressed as means ± SEM ($n = 5$). Experimental groups: LC, lean control rats; LR, lean rats treated with resveratrol; OC, obese control rats; OR, obese rats treated with resveratrol. Means without a common letter differ, $P < 0.05$.

concentration in VAT was about 3.5-fold lower among the OC rats than among the LC rats ($P < 0.0001$). A daily intake of resveratrol for 8 weeks increased VAT adiponectin concentration in the obese rats by 38% ($P < 0.01$ vs. OC rats) (Fig. 4B). No differences in this parameter were observed among the lean rats groups. TNF-α production by VAT was 2.9-fold higher in the obese Zucker rats than in the lean rats ($P < 0.001$) (Fig. 4C). The daily administration of 10 mg/kg of resveratrol for 8 weeks reduced the production of this inflammatory cytokine by 30% in the obese rats ($P < 0.01$ vs. OC).

We found that eNOS protein expression was down-regulated by 60% in the VAT of the OC rats compared with the LC rats ($P < 0.0001$) (Fig. 5). Chronic administration of resveratrol increased eNOS protein expression in the VAT of the obese Zucker rats 2.2-fold ($P < 0.0001$ vs. OC), but produced no effects on the expression of either protein among the lean rats (Fig. 5).

Thr172 phosphorylation of AMPK was significantly reduced in obese Zucker rat VAT compared with the lean control rats (Fig. 6A, C). Similarly, inhibition of AMPK activation was confirmed by a reduction of 35% in phosphorylation of ACC in the VAT of the obese rats (Fig. 6B, D) ($P < 0.01$ vs. LC). In obese Zucker rats treated with resveratrol (10 mg/kg/day), phosphorylation of both AMPK (Fig. 6A, C) and ACC (Fig. 6B, D) increased 1.6-fold in VAT ($P < 0.05$ vs. OC), confirming the activation of AMPK by resveratrol in the VAT of the obese Zucker rats.

3.5. *Systolic blood pressure*

At the beginning of the experimental period, the obese Zucker rats showed moderate but significantly higher SBP values than those of the lean Zucker rats ($P < 0.0001$) (Fig. 7). During the experimental period, SBP in the obese control rats remained unchanged while in the obese rats that received oral resveratrol, these values were significantly decreased ($P < 0.01$) from the first week of administration, with a reduction of 79.1% by the end of the experimental period ($P < 0.0001$) (Fig. 7). The administration of resveratrol did not affect SBP among the lean rats.

3.6. *eNOS expression in aortic rings*

eNOS protein expression was 5-fold lower ($P < 0.0001$) in the aortic rings of the OC rats compared to the corresponding values for the LC rats (Fig. 8). Chronic administration of resveratrol increased aortic expression of this protein 3.7-fold in the obese Zucker rats ($P < 0.00001$ vs. OC).

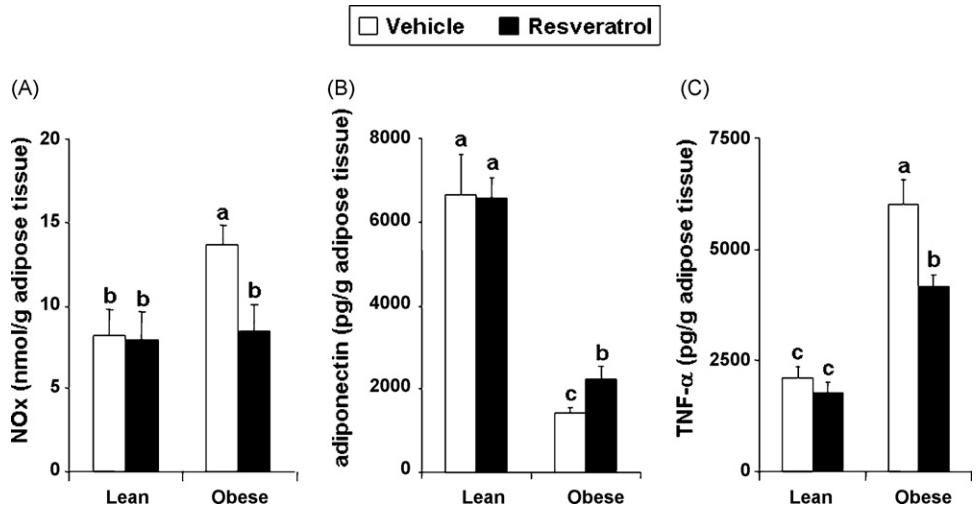

Fig. 4 – Effects of resveratrol on inflammatory and anti-inflammatory markers secreted by adipose tissue in lean and obese Zucker rats. Adipose tissue nitrates plus nitrites (NOx) (A), adiponectin (B) and TNF-α (C) secretion in lean and obese Zucker rats receiving vehicle (control) or resveratrol (10 mg/kg/day) for 8 weeks. Data are expressed as means ± SEM ($n = 7$). Means without a common letter differ, $P < 0.05$.

4. Discussion

In the current study, the obese (fa/fa) Zucker rat was used as an experimental model of metabolic syndrome. Daily oral administration of resveratrol to obese Zucker rats for 8 weeks reduces dyslipidemia, hyperinsulinemia, and hyperleptinemia that feature this experimental model. Such effects were accompanied by a reduction in the inflammatory status characteristic of this model, while an increase in AMPK activity was found in the liver and VAT of obese rats treated with resveratrol. An important reduction in SBP was also observed in the obese Zucker rats treated with resveratrol, an effect that is related to enhanced eNOS expression in the aorta.

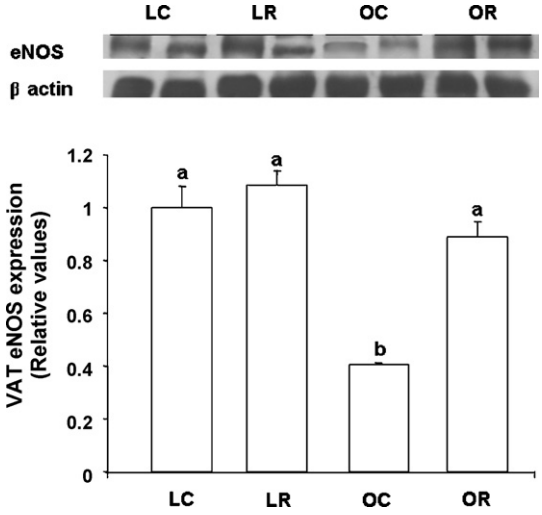

Fig. 5 – eNOS protein expression in the visceral adipose tissue (VAT) of lean and obese Zucker rats given vehicle (control) or resveratrol (10 mg/kg/day) for 8 weeks. 50 μg of protein of VAT homogenates were resolved by SDS-PAGE and probed using an antibody directed against eNOS. Immunoblots are representative duplicates of VAT homogenates of five different rats for each group of treatment. Experimental groups: LC, lean control rats; LR, lean rats treated with resveratrol; OC, obese control rats; OR, obese rats treated with resveratrol. Relative values are expressed as means ± SEM ($n = 5$), and they indicate the values from densitometric analysis normalized to β-actin, relative to LC measurements, which were assigned a value of 1.0. Means without a common letter differ, $P < 0.05$.

Although no significant differences were observed in the final body weight between the obese groups, the administration of resveratrol was associated with a modest but non significant decrease in overall body weight compared to the control group, an effect that is not due to a lower diet intake as resveratrol did not modify the average daily food intake in either rat strain. Resveratrol has recently been shown to protect mice from many detrimental effects of diet-induced obesity. Lagouge et al. reported that resveratrol prevents diet-induced obesity [34]. Another study shows that mice fed a high-calorie diet plus resveratrol are slightly lighter than those fed a high-calorie diet during the initial months of treatment [21]. In both studies, weight gain is significantly attenuated in mice that are given high doses of resveratrol. This fact suggests that a longer treatment with resveratrol could produce a slight but significant reduction in body weight. Our results show that resveratrol administration ameliorates insulin resistance in obese Zucker rats after 4 weeks of treatment, while hyperleptinemia does not decrease until 8 weeks. We hypothesize that these effects are previous and would lead finally to a decrease in body weight.

Liver weight remained unchanged in the resveratrol-treated animals. This is an important finding, in view of the fact that long-term exposure to other AMPK activators, such as AICAR, produces a moderate increase in the liver weight of animals [35,36] that has been considered a potential side-effect of this kind of molecule, and which has not been observed in the chronic treatment with resveratrol.

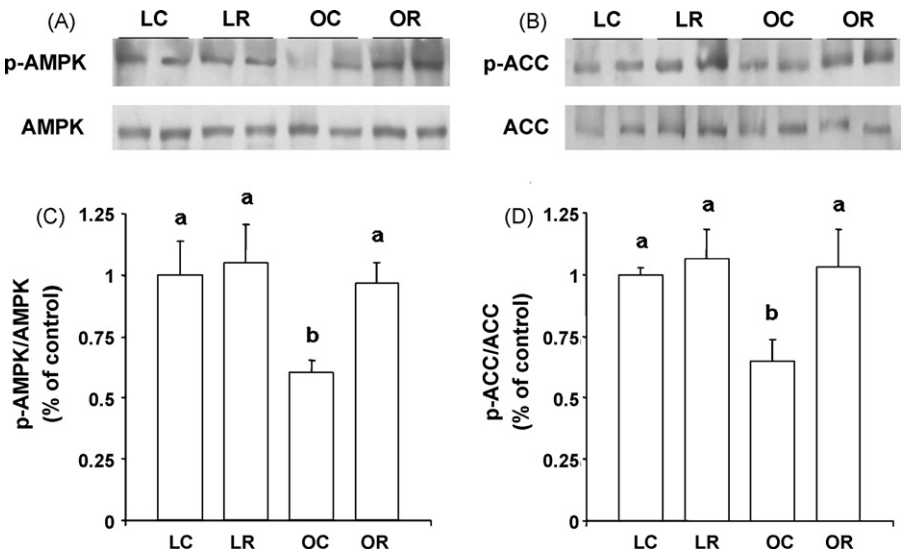

Fig. 6 – AMPK, phospho-AMKP (Thr172) (p-AMPK) (A), ACC and phospho-ACC (p-ACC) (B) protein expression in the visceral adipose tissue (VAT) of lean and obese rats given vehicle or resveratrol (10 mg/kg/day) for 8 weeks. 30 μg of protein of VAT homogenates were resolved by SDS-PAGE and probed using antibodies directed against AMPK, p-AMPK, ACC and p-ACC. Immunoblots (A and B) are representative duplicates of VAT homogenates of five different rats for each group of treatment. Densitometric analysis of AMPK (C) and ACC phosphorylation (D) in the VAT of lean and obese rats given vehicle or resveratrol (10 mg/kg/day) for 8 weeks. Levels of phospho-AMPK were normalized to the total AMPK and compared to the LC group measurements which were assigned a value of 1.0 (C). Values are expressed as means ± SEM (n = 5). Levels of phospho-ACC were normalized to the total ACC and compared to the LC group measurements which were assigned a value of 1.0 (D). Values are expressed as means ± SEM (n = 5). Experimental groups: LC, lean control rats; LR, lean rats treated with resveratrol; OC, obese control rats; OR, obese rats treated with resveratrol. Means without a common letter differ, $P < 0.05$.

In our experiment, long-term resveratrol administration leads to a decrease in abdominal fat content. This result suggests that there is an increased degradation of adipose tissue. In fact, resveratrol significantly activated AMPK in the adipose tissue of our animals, eventually producing the phosphorylation and inhibition of ACC which leads to an increase in fatty acid oxidation and to the inhibition of fatty acid synthesis. This effect, together with other events taking place outside the adipose tissue, could explain the decrease in fat mass as well as the lower FFA circulating levels in OR rats that would contribute to ameliorate lipid metabolism. In fact, excessive FFA mobilization from adipose tissue, whether central or peripheral, leads to a marked plasma FFA increase and ectopic deposition of triglycerides in muscle and liver, resulting in insulin resistance [37]. Moreover, circulating FFA play an important role in stimulating hepatic triglyceride production [38,39]. Plasma FFA enter the hepatocytes and are either oxidized or esterified. An enhanced esterification is accompanied by increased triglycerides production and secretion [38]. AMPK activity is decreased in tissues of rats that lack or have a functionally deficient receptor [40]. Previous studies with human hepatocytes and type 1 diabetic LDL receptor deficient mice have shown that polyphenols strongly stimulate hepatic AMPK and reduce lipid accumulation, which in turn attenuates hyperlipidemia and atherosclerosis in diabetic mice [11]. Our results show that resveratrol exerts similar effects in obese Zucker rats, as it decreases FFA circulating levels, leading to decrease triglyceride and cholesterol production and accumulation in the liver, while enhancing AMPK activation and ACC inhibition. These actions of resveratrol contribute to ameliorate insulin resistance, which was already improved after 4 weeks of treatment, and also to reduce hyperleptinemia at a later stage.

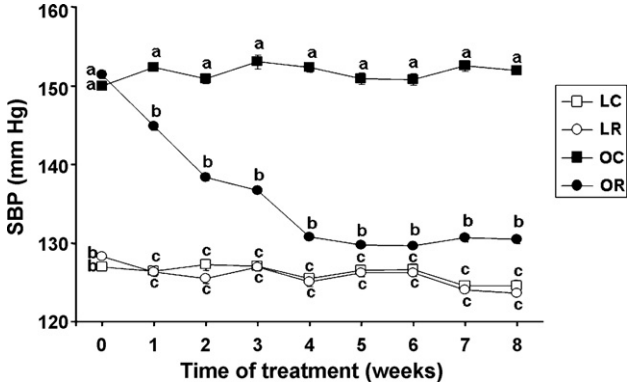

Fig. 7 – Evolution of systolic blood pressure (SBP), measured by tail-cuff plethysmography, in lean and obese Zucker rats receiving vehicle (control) or resveratrol (10 mg/kg/day) for 8 weeks. Experimental groups: LC, lean control rats; LR, lean rats treated with resveratrol; OC, obese control rats; OR, obese rats treated with resveratrol. Values are expressed as means ± SEM (n = 7). Means at a time without a common letter differ, $P < 0.05$.

Recent literature dealing with metabolic syndrome documents the involvement of an important inflammatory

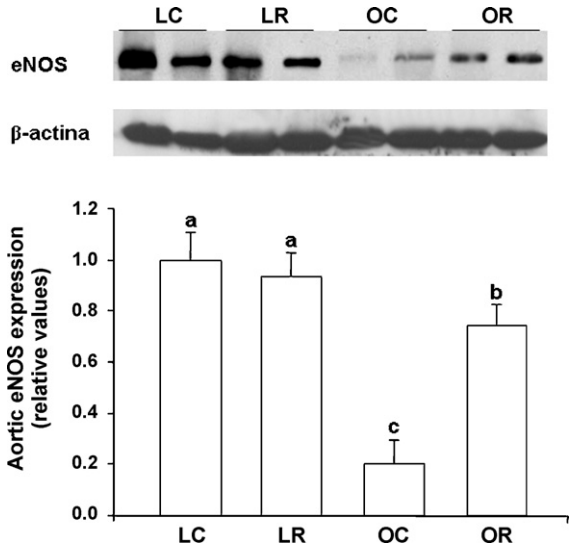

Fig. 8 – eNOS protein expression in VAT of lean and obese Zucker rats given vehicle (control) or resveratrol (10 mg/kg/day) for 8 weeks. 40 μg of protein of aortic homogenates were resolved by SDS-PAGE and probed using an antibody directed against eNOS. Immunoblots are representative duplicates of aortic homogenates of five different rats for each group of treatment. Experimental groups: LC, lean control rats; LR, lean rats treated with resveratrol; OC, obese control rats; OR, obese rats treated with resveratrol. Relative values are expressed as means ± SEM ($n = 5$), and they indicate the values from densitometric analysis normalized to β-actin, relative to LC measurements, which were assigned a value of 1.0. Means without a common letter differ, $P < 0.05$.

component in its etiopathology [41]. Obese and type 2 diabetes Zucker rats and human subjects are characterized by adipose tissue overproduction of pro-inflammatory cytokines such as TNF-α and by decreased plasma concentration of anti-inflammatory adipocytokines such as adiponectin ([14,42–44] and present results). Under normal conditions, the low amounts of NO produced by eNOS play a key role in the regulation of metabolic homeostasis, since mice lacking the eNOS gene develop metabolic syndrome [45]. Furthermore, eNOS expression is down-regulated by TNF-α in the adipose tissue of obese Zucker rats [46]. Hence, treatments that decrease eNOS down-regulation mediated by pro-inflammatory cytokines are expected to ameliorate the alterations found in metabolic syndrome [47]. Our results show that the chronic administration of resveratrol reduces adipose tissue TNF-α and NOx production in obese Zucker rats, while increasing plasma adiponectin concentration, resulting in an important anti-inflammatory effect. Moreover, this dose of resveratrol produces an enhancement of eNOS expression in the adipose tissue of obese Zucker rats, an effect that could be related to the reduction in TNF-α, since this cytokine inhibits eNOS expression in adipose tissue [46]. Furthermore, we observed increased phosphorylation of AMPK together with higher phosphorylation of ACC, confirming the activation of AMPK, in the VAT of the obese Zucker rats treated with resveratrol compared with the controls. Activation of AMPK by resveratrol would also mediate the enhancement of eNOS expression in the VAT of obese Zucker rats, an effect that has been described previously in endothelial cells [48].

Chronic administration of resveratrol produced a reduction in the SBP values of obese Zucker rats compared with the controls from the first week of intake. Beneficial cardiovascular properties of this polyphenol have been described previously [15,19,20]. Furthermore, the chronic administration of resveratrol enhances aortic eNOS expression in obese Zucker rats. This effect is related to the reduced SBP values of OR rats with respect to the OC rats. Resveratrol has been described as an efficient antihypertensive agent in an experimental model of hypertension in rats, when administered at the doses of 10 and 50 mg/kg of body weight [49]. This antihypertensive effect has been related to the beneficial properties of resveratrol on endothelial dysfunction. In the model of obese Zucker rats, as well as in subjects with insulin resistance, FFA are involved both in insulin resistance and in the inhibition of aortic eNOS activity through an oxidative mechanism [50,51]. The present results show that the chronic daily administration of resveratrol restores aortic expression of eNOS in obese Zucker rats, an effect which could explain the antihypertensive effects of this polyphenol in the insulin resistance model described, and which would be mediated by its antioxidant properties [15].

In conclusion, the present results show for the first time that daily chronic administration of 10 mg/kg of resveratrol reduces dyslipidemia, insulin resistance, hyperleptinemia and hypertension in the experimental model of metabolic syndrome of obese Zucker rats, these effects being accompanied by a reduction in the inflammatory status in VAT and the activation of AMPK in target tissues of metabolic syndrome such as liver and VAT. Our results corroborate the beneficial effects of resveratrol in the treatment of alterations involved in metabolic syndrome.

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