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ORIGINAL ARTICLE

Effects of resveratrol and its analogue pterostilbene, on NOV/CCN3 adipokine in adipose tissue from rats fed a high-fat high-sucrose diet

J. Trepiana[1] · S. Gómez-Zorita[1,2,3] · Alfredo Fernández-Quintela[1,2,3] · M. González[4] · M. P. Portillo[1,2,3]

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Abstract

Nephroblastoma overexpressed protein, also called NOV/CCN3, is an adipokine which is present in various tissues and recently linked to obesity. The objective of the study was to determine the effect of resveratrol and pterostilbene on NOV/CCN3 in adipose tissue from rats fed an obesogenic diet. Thirty-six male Wistar rats were split into four groups ($n = 9$): fed a standard diet (CC), high-fat high-sucrose (HFS) diet supplemented with resveratrol (RSV; 30 mg/kg/day) or with pterostilbene (PT; 30 mg/kg/day), or without phenolic supplementation (HFS). Rats were sacrificed after 6 weeks of treatment, and adipose tissue (white and brown) from different anatomical locations were dissected. Then, *Nov/ccn3* gene and protein expression and the adipogenic genes, *Ucp-1* and *Pgc-1a*, expressions were studied. Increased weight of white adipose tissues was found in rats fed the HFS diet. Whereas resveratrol-treated rats showed reduced internal and total adipose tissue weights, pterostilbene-treated rats showed reduced subcutaneous, internal and total adipose depots. *Nov/ccn3* gene expression decreased in epididymal and interscapular brown depot in rats fed HFS diet when compared with the control group. Regarding the phenolic compounds, resveratrol prompted a *Nov/ccn3* gene expression increase in epididymal fat tissue, whereas pterostilbene reduced its protein expression compared with the obese group. However, these phenolic compounds did not affect NOV/CCN3 expression in brown depot. NOV/CCN3 seems to be involved in weight changes in epididymal adipose tissue under obesogenic feeding, but not in subcutaneous, acting as a protective mechanism counteracting the fattening effect of the diet. To our knowledge, this is the first study analyzing whether NOV/CCN3 is involved in the anti-obesity effect of resveratrol and pterostilbene. Our results suggest that this is not the case.

Keywords NOV/CCN3 · Resveratrol · Pterostilbene · Phenolic compounds · Adipose tissue · Obesity

Introduction

Obesity, defined as an excess of fat accumulation in white adipose tissue, induces changes in the adipose tissue microenvironment, by increasing adipocyte number (hyperplasia) and/or size (hypertrophy), which compromises the activity of this tissue. Moreover, epidemiological studies have demonstrated the association between obesity and a chronic low-grade inflammation state [27], which is linked to changes in the production of cytokines by adipose tissue. Indeed, adipose tissue is an important endocrine organ, which releases adipokines, a kind of cytokines, which act as modulators of metabolic processes. The amounts of adipokines secreted by adipose tissue are depot-specific [19]. Regarding the role of adipokines in obesity, these proteins regulate mainly appetite and energy expenditure, and secondarily, they affect insulin sensitivity, oxidative capacity, and lipid uptake. In recent years, the most newly discovered adipokines are apelin, fibroblast growth

J. Trepiana and S. Gómez-Zorita equally contributed to this manuscript and should be considered co-first authors.

✉ Alfredo Fernández-Quintela
alfredo.fernandez@ehu.eus

[1] Nutrition and Obesity Group, Department of Nutrition and Food Science, University of the Basque Country (UPV/EHU) and Lucio Lascaray Research Institute, 48940 Vitoria, Spain

[2] Biomedical Research Networking Centres, Physiopathology of Obesity and Nutrition (CIBERobn), Institute of Health Carlos III, 28029 Madrid, Spain

[3] Department Nutrición y Bromatología, Facultad de Farmacia, Paseo de la Universidad 7, 01006 Vitoria, Spain

[4] Nutrition and Food Science Department, Faculty of Biochemistry and Biological Sciences, National University of Litoral and National Scientific and Technical Research Council (CONICET), 3000 Santa Fe, Argentina

factor-21, neuroregulin-4, omentin, vaspin, cardiotrophin-1, TWEAK, and NOV/CCN3 [8, 9].

NOV/CCN3 (nephroblastoma overexpressed) protein is a member of an incipient family of six regulatory proteins (CCN1–6). They show a multimodular structure with a N-terminal secretory signal peptide followed by four conserved domains sharing identity with insulin-like growth factor binding proteins (IGFBPs), Von Willebrand factor, thrombospondin, and a cystein knot domain [12]. The *Nov* gen was originally described as a target for myeloblastosis-associated virus in avian nephroblastomas, which represent a unique model of Wilm's tumor [23]. Nowadays, it is well known that this protein is detected in various human tissues and organs, such as adipose tissue, adrenal cortex, kidney, muscle, and central nervous system, as well as in macrophages, cerebrospinal fluid, and plasma [20, 24, 26]. This family of proteins plays important roles regulating cell adhesion, mitogenesis, inflammation, migration, cell survival, differentiation, angiogenesis, tumorigenesis, or fibrosis [11, 14].

Interestingly, Pakradouni et al. showed the association between NOV/CCN3 and obesity, reporting a relationship between plasma NOV/CCN3 concentration and BMI or fat mass [20]. Moreover, they found a positive correlation between NOV/CCN3 protein and C-reactive protein (CRP), which suggests that this adipokine is linked to obesity-related inflammation. On the contrary, Lin et al. suggested that NOV/CCN3 could have an anti-inflammatory effect, because its overexpression inhibited the cytokine-mediated induction of vascular adhesion molecule-1 (VCAM-1) in endothelial cells and reduced the monocyte adhesion [15]. Moreover, there are evidences suggesting the role of NOV/CCN3 on glycemic control [22]. Regarding adipogenesis, it is also noteworthy that, when in 3T3-L1 pre-adipocytes NOV/CCN3 expression was suppressed by NOV/CCN3 siRNA, the expression of adipogenic genes was increased [17].

Nowadays, there is an increasing interest in using phenolic compounds, as potential active molecules, for the prevention and treatment of obesity. Resveratrol, a polyphenol included in the group of stilbenes, and its derivative pterostilbene that is metabolized in a lesser extent, have emerged as useful compounds for decreasing body fat accumulation in adipose tissue of rodents [10, 25]. However, as far as we know, only a study has reported that NOV/CCN3 is upregulated in response to a high-fat high-sucrose (HFS) diet, and this effect is reverted by resveratrol supplementation. These results have been found in vascular smooth muscle of non-human primates [18]. However, there are not available data regarding the influence of both phenolic compounds on NOV/CCN3 adipokine production in white adipose tissue. In this scenario, we were interested in the study of the effect of resveratrol and its methoxy derivative, pterostilbene, on changes induced by a high-fat high-sucrose diet in NOV/CCN3 expression in epididymal and subcutaneous white adipose tissues and brown adipose tissue from rats fed an obesogenic diet.

Materials and methods

Animal, diets, and experimental design

The experiment was conducted with 36 6-week-old male Wistar rats with an initial body weight of 180 ± 2 g purchased from Harlan Ibérica (Barcelona, Spain) and performed in accordance with the University of the Basque Country's Guide for the Care and Use of Laboratory Animals (Reference protocol approval CUEID CEBA/30/2010). The rats were individually housed in polycarbonate cages (Tecniplast Gazzada, Buguggiate, Italy) and placed in an air-conditioned room (22 ± 2 °C) with a 12-h light–dark cycle (lights off at 9:00 a.m.). After a 6-day adaptation period, rats were randomly divided into four experimental groups of nine animals each. One group (control group; CC) was fed a commercial standard diet (TD.06416), which provided 3.7 kcal/g and 10% of calories as fat. The other three groups, high-fat high-sucrose group (HFS), resveratrol-treated group (RSV), and pterostilbene-treated group (PT), were fed a commercial high-fat high-sucrose diet (obesogenic diet; TD.06415), which provided 4.6 kcal/g and 45% of kcal as fat. Pterostilbene (99.9% purity) was synthesized according to published procedures [13]. Resveratrol and pterostilbene were added to the diet daily, in amounts that ensured a dose of 30 mg/kg body weight/day. Resveratrol was a generous gift from Monteloeder (Elche, Spain). All animals had free access to food and water. Food intake and body weight were measured daily. At the end of the total experimental period (6 weeks), rats from the four experimental groups were sacrificed after 12 h of fasting, under anesthesia (chloral hydrate), by cardiac exsanguination. White adipose tissue from different regions (subcutaneous, SC; epididymal, EPI; perirenal, and mesenteric) and interscapular brown adipose tissue (IBAT) were dissected and weighed, and then immediately frozen in liquid nitrogen. All samples were stored at − 80 °C until analysis. Percentage of fat mass was calculated as follows: Fat mass (%) = (fat content (g)/body weight (g)) × 100.

Extraction and analysis of RNA and semi-quantification by reverse transcription-polymerase chain reaction (real-time RT-PCR)

Total RNA was isolated from 100 mg of SC, EPI, and IBAT adipose tissues samples using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In the following step, DNase treatment (Applied Biosystems, Foster City, CA, USA) was carried out to remove any

contamination with genomic DNA. The yield and quality of the purified RNA was determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A total of 1.5 μg of RNA were reverse-transcribed into complementary DNA (cDNA) using the iScript cDNA Synthesis kit (BioRad, Hercules, CA, USA) according to the manufacturer's protocol. Reactions were incubated initially at 25 °C for 5 min, subsequently at 42 °C for 30 min, and finally at 85 °C for 5 min.

Relative mRNA levels were quantified using a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA) in the presence of SYBR® Green master mix (Applied Biosystems, Foster City, CA, USA). The PCR reagent mixture consisted of 4.75 μL aliquot of each diluted complementary DNA sample in a 12.5-μL reaction volume for PCR amplification. Sequences of primers are given in Table 1, and the concentration of the primers were as follows: 300 nM for *nov/ccn3*, uncoupling protein 1 (*Ucp1*) and *18Sr*; 900 nM for peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (*Pgc-1a*); and 600 nM for the rest of the genes. The PCR parameters were as follows: start at 50 °C for 2 min, denaturation at 95 °C for 10 min, denaturation at 95 °C for 15 s, annealing temperatures, and number of cycles are specified in Table 1, and in all the cases, the extension temperature was 60 °C. mRNA levels in all the samples were normalized to the values of *18Sr*, and the results were expressed as fold changes of the threshold cycle (Ct) value relative to the controls using the $2^{-\Delta\Delta Ct}$ method [16]. The specificity of a quantitative PCR assay was confirmed by dissociation curve.

Protein expression measurements by Western blot

All tissues (100 mg) were homogenized in 500 μL of cellular PBS buffer (pH 7.4) for adipose tissues, containing nuclease inhibitors, 100 mM phenylmethylsulfonyl fluoride, and 100 mM iodoacetamide. The homogenates were centrifuged (800×*g*, 5 min, 4 °C), and the protein concentration was measured following the Bradford method [6] using bovine serum albumin as standard. Immunoblot analyses were performed using 30 μg of protein from SC homogenate, 20 μg from EPI homogenate, and 40 μg from IBAT homogenate. NOV/CCN3 was separated by electrophoresis in a 10% SDS-polyacrylamide gel and transferred on to PVDF membranes. Then, the membranes were blocked with 5% casein 0.5% BSA PBS-Tween buffer for 1.5 h. Subsequently, they were divided into two parts (one for tubulin determination and the other for NOV/CCN3) and incubated overnight at 4 °C with appropriate antibodies: anti-NOV/CCN3 (1:1000) and anti-tubulin (1:1000; Cell Signaling Technology, Danvers, Massachusetts, USA). Bound antibodies were visualized by using the Clarity™ Western ECL Blotting Substrate (BioRad, Hercules, CA, USA) and quantified by a ChemiDoc MP imaging system (BioRad, Hercules, CA, USA). For densitometric quantification, levels of NOV/CCN3 protein was normalized by tubulin.

Statistical analysis

Results are presented as mean ± standard error of the means. Statistical analysis was performed using SPSS 24.0 (SPSS, Chicago IL, USA). All the parameters are normally distributed

Table 1 Primer sequences and PCR conditions for RT-PCR amplification of each gene studied

Gene	Sense primer sequence	Antisense primer sequence	Annealing temperature and no. of cycles
Nov/ccn3	5′-CTACAGAGTGGAGCGCGTGTT-3′	5′-GGAAGATTCCTGTTGGTGACCC-3′	SC 56.6 °C, 45 cycles; EPI 61.7 °C, 40 cycles; IBAT 59.8 °C, 45 cycles
Cebpb	5′-CAAGCTGAGCGACGAGTACA-3′	5′-CAGCTGCTCCACCTTCTTCT-3′	EPI 60 °C, 40 cycles
Srebf1	5′-GGAGCCATGGATTGCACATT-3′	5′-GCTTCCAGAGAGGAGCCCAG-3′	SC 64 °C, 45 cycles; EPI 60 °C, 40 cycles
Cebpa	5′-GAGTCGGCCGACTTCTACG-3′	5′-GTCTCGTGCTCGCAGATGC-3′	EPI 64 °C, 45 cycles
Pparg	5′-CTGTCGGTTTCAGAAGTGCCT-3′	5′-AGCTGGTCGATATCACTGGAG-3′	SC 56.6 °C, 45 cycles; EPI 60 °C, 40 cycles
Acc	5′-GGACCACTGCATGGAATGTTA-3′	5′-TGAGTGACTGCCGAAACATCT-3′	EPI 60 °C, 45 cycles
Ucp1	5′-ATCTCGGCTGGCTTGATGAC-3′	5′-ATTCTGACCTTCACCACCTCTG-3′	IBAT 60 °C, 40 cycles
Pgc-1a	5′-ACCCACAGGATCAGAACAAAC-3′	5′-GACAAATGCTCTTTGCTTTAxTTGC-3′	IBAT 60 °C, 40 cycles
18Sr	5′- CATCGAGCAGGTCTGTTCCC-3′	5′-TAGATTGGCTTGACGGACTTGG-3′	SC, EPI, IBAT 60 °C, 40 cycles

18Sr 18S ribosomal, *Acc* acetyl-CoA carboxylase, *Cebpa* CCAAT/enhancer-binding protein alpha, *Cebpb* CCAAT/enhancer-binding protein beta, *EPI* epididymal adipose tissue, *IBAT* interscapular brown adipose tissue, *Nov/ccn3* nephroblastoma overexpressed, *Pgc-1a* peroxisome proliferator-activated receptor-gamma coactivator 1 alpha, *Pparg* peroxisome proliferator-activated receptor gamma, *SC* subcutaneous adipose tissue, *Srebf1* sterol regulatory element-binding transcription factor 1, *Ucp1* uncoupling protein 1

according to the Shapiro–Wilk's test. Then, comparisons between each treatment with the controls were analyzed by using one-way ANOVA followed by the Newman–Keuls *post hoc* test. Pearson's r correlations were used to assess the potential associations between NOV/CCN3 and genes related to adipogenesis and thermogenesis. Statistical significance was set-up at the $P < 0.05$.

Results

Adipose tissue weights

Rats fed a high-fat high-sucrose diet (HFS) showed higher subcutaneous and internal adipose tissues weights than rats fed a standard diet. Resveratrol prevented the increase in epididymal adipose tissue weight, as well as the weight of internal depots or total adipose tissue weight. However, pterostilbene prevented this increase in the case of subcutaneous adipose tissue and when the weights of either the internal or all the dissected white adipose tissues were pooled. The prevention of body fat gain induced by both phenolic compounds was partial because adipose tissue weights in rats treated with these compounds were higher than those in the control rats fed a standard diet (Table 2). No significant changes were found in interscapular brown adipose tissue (IBAT).

Gene and protein determinations in white adipose tissue

In epididymal adipose tissue rats fed the HFS diet showed decreased *Nov/ccn3* gene expression (0.37-fold change) when compared with the CC group (Fig. 1a). A tendency towards reduced values was also observed in protein expression (− 70%, $P = 0.07$) (Fig. 1b). As far as phenolic compound supplementation is concerned, resveratrol significantly increased *Nov/ccn3* gene expression (7-fold change) versus HFS diet. However, pterostilbene-treated rats showed lower NOV/CCN3 protein expression (− 53%) when compared with this group.

When adipogenic genes were analyzed, we observed that HFS diet induced a significant decreased in sterol regulatory element-binding transcription factor 1 (*Srebf1*), CCAAT/enhancer-binding protein alpha (*Cebpa*), peroxisome proliferator-activated receptor gamma (*Pparg*), and acetyl-CoA carboxylase (*Acc)* levels, whereas CCAAT/enhancer-binding protein beta (*Cebpb*), which is implicated in the early state of adipogenesis, was not changed (Fig. 2). With regard to resveratrol and pterostilbene supplementation, both phenolic compounds prevented this effect. Moreover, in resveratrol-treated rats, a tendency towards increased *Cebpb* levels (2.6-fold change, $P = 0.068$) and significantly higher levels of *Pparg* (5.2-fold change) were observed when data were compared with rats under the obesogenic feeding (Fig. 2).

A positive correlation was revealed by Pearson r analysis between *Nov/ccn3* gene expression and *Cebpb* (r = 0.648, $P = 0.000$), *Srebf1* (r = 0.771, $P = 0.000$), *Cebpa* (r = 0.478, $P = 0.014$), *Pparg* (r = 0.854, $P = 0.000$), and *Acc* (r = 0.495, $P = 0.009$) gene expression levels.

In subcutaneous adipose tissue, NOV/CCN3 gene and protein expressions were not modified by HFS feeding (Fig. 3a, b). When resveratrol or pterostilbene was added to the diet, NOV/CCN3 gene and protein expressions remained unchanged with regard to HFS group. In this tissue, only results concerning *Srebf1* and *Pparg* gene expressions are presented. The other three adipogenic genes were also analyzed but, under our experimental conditions, we did observe that the sensitivity of the RT-PCR was not high enough to provide

Table 2 Final body weight, food intake, and white and brown adipose tissue weights (expressed as percentage of total body weight) of rats fed on the experimental diets for 6 weeks

	CC	HFS	RSV	PT
Total body weight (g)	334 ± 8[b]	389 ± 9[a]	362 ± 5[c]	= 376 ± 9[ac]
Food intake (g/day)	17.0 ± 0.4	17.6 ± 0.3	16.5 ± 0.6	17.3 ± 0.3
Adipose tissue weight (% BW)				
SC	2.7 ± 0.1b	4.5 ± 0.4a	4.1 ± 0.1a	3.1 ± 0.2b
EPI	2.0 ± 0.1b	4.0 ± 0.1a	3.4 ± 0.1c	3.6 ± 0.3ac
PERI	2.5 ± 0.2b	3.9 ± 0.2a	3.5 ± 0.1a	3.3 ± 0.2ab
MES	0.7 ± 0.0b	1.4 ± 0.0a	1.4 ± 0.0a	1.2 ± 0.1a
Internal AT	5.2 ± 0.3b	9.3 ± 0.3a	8.3 ± 0.1c	8.2 ± 0.4c
Total AT	7.9 ± 0.4b	13.8 ± 0.6a	12.4 ± 0.2c	11.6 ± 0.8c
IBAT	0.9 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1

Values are the means ± SEM. Values in the same row with different lowercase letters are significantly different at $P < 0.05$

BW body weight, *SC* subcutaneous adipose tissue, *EPI* epididymal adipose tissue, *PERI* perirenal adipose tissue, *MES* mesenteric adipose tissue, *AT* adipose tissue, *IBAT* interscapular brown adipose tissue

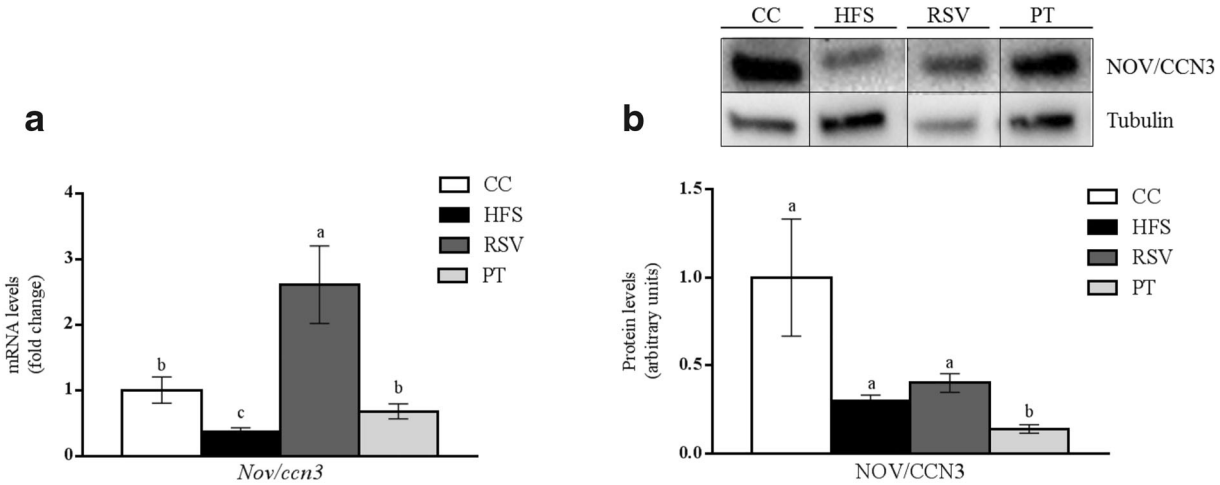

Fig. 1 Gene (**a**) and protein (**b**) expression of NOV/CCN3 in epididymal white adipose tissue from rats treated with standard diet (CC), high-fat high-sucrose diet (HFS), HFS supplemented with resveratrol (RSV), and HFS supplemented with pterostilbene (PT) for 6 weeks. The western blot bands shown are representative of 9 samples/group. Data are means ± SEM. Values not sharing a common letter are significantly different ($P < 0.05$)

accurate data in this adipose tissue depot. The obesogenic diet significantly increase *Srebf1* gene expression (2.1-fold change) compared with the control group, and this rise was prevented by both resveratrol and pterostilbene supplementation (Fig. 4a). Regarding its downstream target gene *Pparg*, no significant changes were observed in HFS or RSV groups. By contrast, pterostilbene supplementation induced a significant decrease (Fig. 4b). No significant correlations were observed between these genes and *Nov/ccn3*.

Gene and protein NOV/CCN3 expressions in interscapular brown adipose tissue

A significant decrease (0.3-fold change) was observed in *Nov/ccn3* gene expression in rats fed an obesogenic diet compared with the control group (Fig. 5a). In addition, a reduction (− 50%) was also detected at the protein level, but this change did not reach statistical significance (Fig. 5b). Resveratrol or pterostilbene administration did not prevent the reduction induced by the HFS diet.

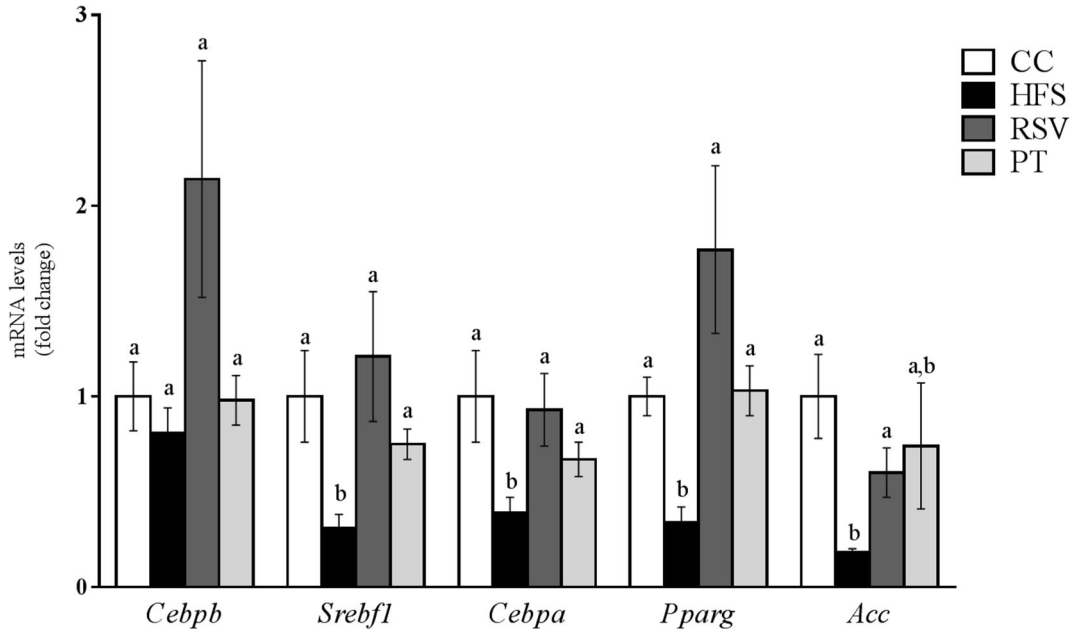

Fig. 2 Gene expression of *Cebpb*, *Srebf1*, *Cebpa*, *Pparg*, and *Acc* in epididymal white adipose tissue from rats treated with standard diet (CC), high-fat high-sucrose diet (HFS), HFS supplemented with resveratrol (RSV), and HFS supplemented with pterostilbene (PT) for 6 weeks. Data are means ± SEM. Values not sharing a common letter are significantly different ($P < 0.05$). *Cebpb:* CCAAT/enhancer-binding protein beta; *Srebf1:* sterol regulatory element-binding transcription factor 1; *Cebpa:* CCAAT/enhancer-binding protein alpha; *Pparg:* peroxisome proliferator-activated receptor gamma; *Acc:* acetyl-CoA carboxylase

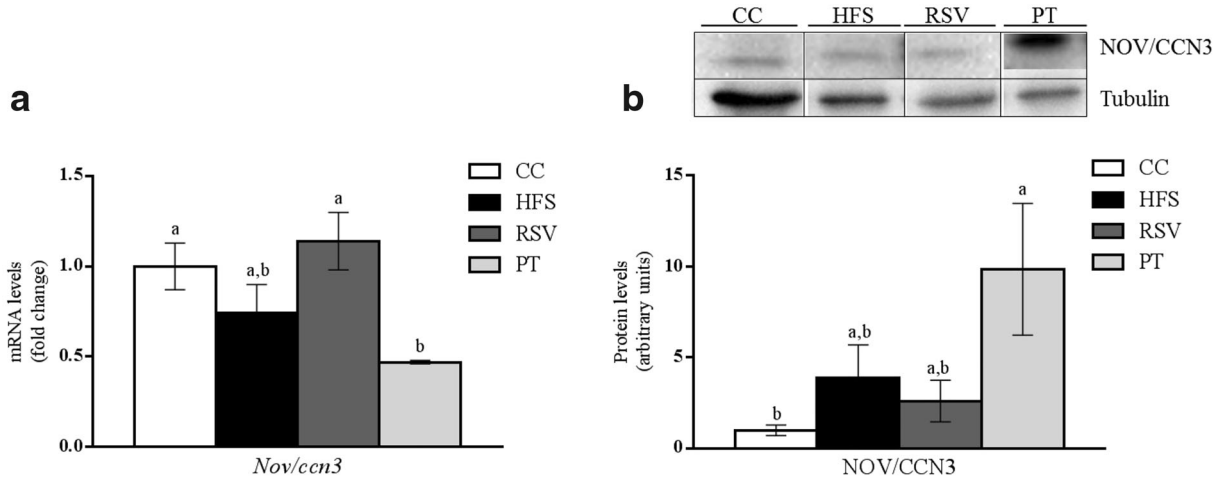

Fig. 3 Gene (**a**) and protein (**b**) expression of NOV/CCN3 in subcutaneous white adipose tissue from rats treated with standard diet (CC), high-fat high-sucrose diet (HFS), HFS supplemented with resveratrol (RSV), and HFS supplemented with pterostilbene (PT) for 6 weeks. The western blot bands shown are representative of 9 samples/group. Data are means ± SEM. Values not sharing a common letter are significantly different ($P < 0.05$)

As IBAT is specialized in thermogenesis, we analyzed the expression of *Ucp1* and *Pgc-1a*, two key genes in this metabolic process. The HFS diet prompted a significant decrease in *Ucp1* (Fig. 5c) and *Pgc-1a* (Fig. 5d) gene expression levels. This effect was prevented by resveratrol but not by pterostilbene. There were positive correlations between *Nov/ccn3* and *Ucp1* (r = 0.370, $P = 0.050$) and *Pgc-1a* (r = 0.619, $P = 0.001$).

Discussion

Obesity is becoming a growing public health problem in Western countries due to its high prevalence, not only in adults but also in children. At present, the scientific community is looking for active biomolecules showing beneficial effects on obesity prevention and/or treatment. In this context, the fat-lowering effect for resveratrol [25] and pterostilbene [10] has been reported. Although several mechanisms of action have been described, more research is needed to totally clarify this issue [2]. The effect of resveratrol and pterostilbene on NOV/CCN3 production in adipose tissue have been scarcely studied so far. Thus, in the present study, we were interested in analyzing the effects of resveratrol and its methoxy derivative, pterostilbene, on NOV/CCN3 adipokine expression in adipose tissues from rats fed an obesogenic diet.

As expected, we observed that rats fed a HFS diet for 6 weeks showed higher white adipose tissue weights than rats fed a standard diet. The effect of the phenolic compounds on adipose tissues was different depending on their anatomical location. Thus, resveratrol partially prevented epididymal tissue weight increase, whereas pterostilbene prevented subcutaneous adipose tissue growth induced by the HFS diet. It has been reported that subcutaneous and internal white adipose tissues show different metabolic features [21], and consequently different responses to dietary interventions. In the

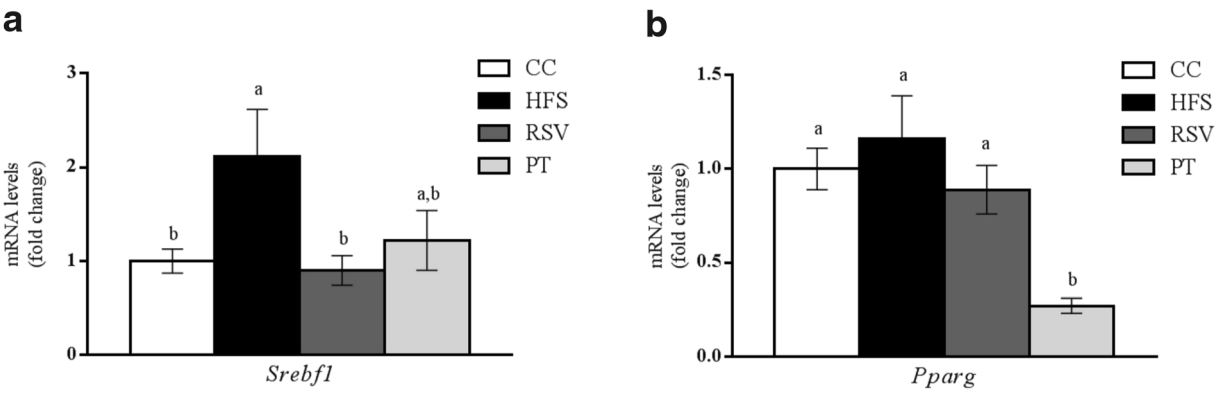

Fig. 4 Gene expression of *Srebf1* (**a**) and *Pparg* (**b**) in subcutaneous white adipose tissue from rats treated with standard diet (CC), high-fat high-sucrose diet (HFS), HFS supplemented with resveratrol (RSV), and HFS supplemented with pterostilbene (PT) for 6 weeks. Data are means ± SEM. Values not sharing a common letter are significantly different ($P < 0.05$). *Srebf1:* sterol regulatory element-binding transcription factor 1; *Pparg:* peroxisome proliferator-activated receptor gamma

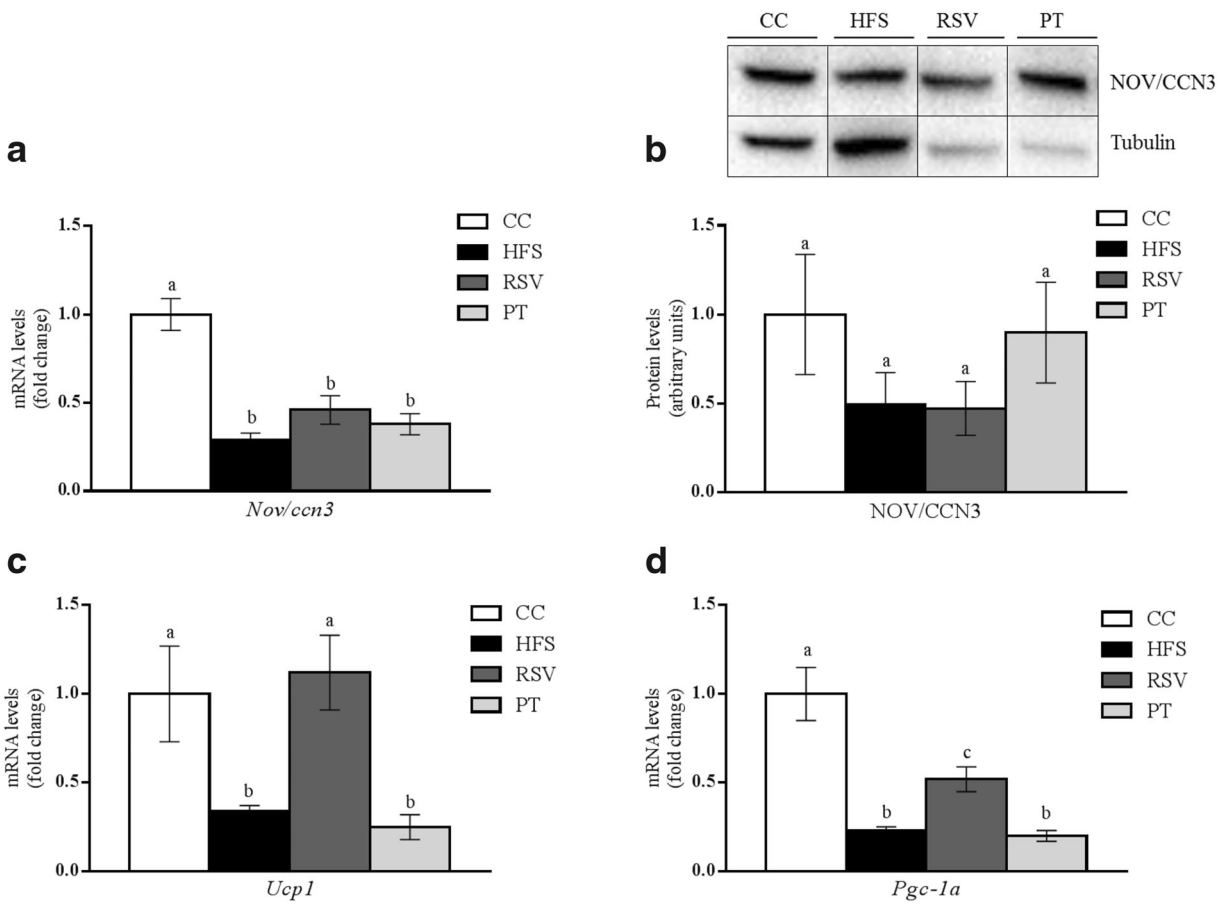

Fig. 5 Gene (**a**, **c**, **d**) and protein (**B**) expression of NOV/CCN3, *Ucp1* and *Pgc-1a* in IBAT tissue from rats fed a standard diet (CC), high-fat high-sucrose diet (HFS), HFS supplemented with resveratrol (RSV), and HFS supplemented with pterostilbene (PT) for 6 weeks. The western blot bands shown are representative of nine samples/group. Data are means ± SEM. Values not sharing a common letter are significantly different ($P < 0.05$). *Ucp1:* uncoupling protein 1; *Pgc-1a:* peroxisome proliferator-activated receptor-gamma coactivator 1 alpha

present study, the increase in epididymal tissue size was accompanied by lower gene and protein expressions of NOV/CCN3. Due to high SEM values, the decrease in protein expression (− 70%, $P = 0.07$) did not reach statistical significance. By contrast, in subcutaneous adipose tissue, both gene and protein expression remained unchanged. These results support the idea that epididymal adipose tissue is metabolically more active than subcutaneous depot [4, 7].

By analyzing epididymal adipose tissue, Martinerie et al. observed that $NOV^{-/-}$ mice showed lower body weights than wild-type mice, when they were fed a high-fat diet [17], suggesting that the absence of NOV/CCN3 protects from excessive fat accumulation in adipose tissue. Taking these results into consideration, it can be proposed that the reduction in NOV/CCN3 observed in our study in rats fed the obesogenic diet can represent a protective mechanism to counteract adipose tissue expansion. Since Martinerie et al. also reported that NOV/CCN3 is strongly associated with adipocyte differentiation, and due to the fact that this process could be responsible in part for the reduction in adipose tissue weight, the expressions of genes involved in adipogenesis was measured. Our results showed that, as a whole, the analyzed adipogenic genes were significantly reduced. In addition, and in line with this study reported by Martinerie et al., negative correlations between *Nov/ccn3* and these genes were found. Consequently, it can be proposed that, in the rats fed the obesogenic diet, the reduction in adipogenesis is a compensatory mechanism in order to minimize obesity development. It could be proposed that changes in NOV/CCN3 were probably involved in the reduction of adipogenic gene expression. Nevertheless, further data, for instance the expression of adipogenic genes in $NOV^{-/-}$ mice, are needed to confirm this hypothesis.

A positive correlation between serum NOV/CCN3 and body mass index has been reported in humans [20]. Obviously, our results are not in good accordance with this study. Nevertheless, it should be taken into account that Pakradouni et al. measured NOV/CCN3 in plasma, not in

adipose tissue, and serum concentration of this adipokine results from the secretion of a great number of tissues, being important to discriminate the contribution of each tissue separately.

As far as phenolic compounds are concerned, on the one hand, resveratrol partially prevented epididymal adipose tissue expansion, but not subcutaneous adipose tissue growth. Although gene expression of *Nov/ccn3* was significantly increased, this change was no reflected in protein expression, perhaps due to posttranscriptional modifications, or to microRNA regulation. In fact, miRWalk database shows that *Nov/ccn3* can be a predicted target for several microRNAs (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/). These results suggest that NOV/CCN3 is not a target for this compound and thus, it does not participate in its anti-obesogenic effect. Interestingly, the decrease induced by the obesogenic feeding in adipogenic genes was completely prevented by resveratrol, probably because this phenolic compound was acting by preventing obesity and thus additional mechanisms were not needed. On the other hand, in good accordance with the lack of effect of pterostilbene on epididymal adipose tissue growth, this compound did not induce significant changes in *Nov/ccn3* gene expression, when compared with non-treated animals. Thus, although pterostilbene induced a small NOV/CCN3 protein expression reduction, it seems that this change was not functionally relevant.

Little is known about the role of NOV/CCN3 in white adipose tissue, and even less in brown adipose tissue. As far as we know, this is the first time NOV/CCN3 adipokine was detected in this tissue. It is well known that brown adipose tissue can dissipate high amounts of energy through heat production (thermogenesis), and that resveratrol [3] and pterostilbene [1] increase thermogenic and oxidative capacity of brown adipose tissue in obese rats. Thermogenesis occurs thanks to UCP1 protein, which is regulated by PGC-1a. This protein is located in the inner mitochondrial membrane of brown adipose tissue, and the proton gradient generated is dissipated through this protein liberating the energy as heat [5]. Thus, in our study, gene expression of these two proteins was measured.

The results showed that the obesogenic diet reduced *Ucp1* and *Pgc-1a* gene expression levels, in interscapular brown adipose tissue thus decreasing its thermogenic capacity, but no changes were observed in NOV/CCN3 protein expression. Resveratrol, but not pterostilbene, prevented these changes, but it did not induce changes in NOV/CCN3. Altogether, these results suggest that probably, NOV/CCN3 is not related to thermogenesis. Nevertheless, further studies are needed to clarify this issue.

In summary, NOV/CCN3 seems to be involved in weight changes observed in epididymal adipose tissue under obesogenic feeding, but not in subcutaneous, acting as a protective mechanism counteracting the fattening effect of the diet. As far as we know, this is the first study devoted to analyzing whether NOV/CCN3 is involved in the anti-obesity effect of resveratrol and pterostilbene. The results obtained suggest that this is not the case, but this is a preliminary study and further research is needed to gain more insight on this issue.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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