RESEARCH ARTICLE

Delipidating effect of resveratrol metabolites in 3T3-L1 adipocytes

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Scope: Due to the low bioavailability of resveratrol, determining whether its metabolites exert any beneficial effect is an interesting issue.

Methods and results: 3T3-L1 maturing pre-adipocytes were treated from day 0 to day 8 of differentiation and mature adipocytes for 24 h on day 12 with 1, 10, or 25 μM of resveratrol or its metabolites. Triacylglycerols were assessed by spectrophotometry and gene expression by real time RT-PCR. Resveratrol, *trans*-resveratrol-4′-*O*-glucuronide and *trans*-resveratrol-3-*O*-sulfate reduced triacylglycerol content in maturing pre-adipocytes at 25 μM. In mature adipocytes, both resveratrol and its glucuronide metabolites, though not sulfate metabolite, reduced triacylglycerol content, although resveratrol was more effective than them. Resveratrol and the three metabolites reduced C/EBPβ mRNA levels. *Trans*-resveratrol-3-*O*-sulfate also reduced C/EBP-α, peroxisome proliferator-activated receptor γ (PPAR-γ), and lipoprotein lipase (LPL) expression. In mature adipocytes, resveratrol increased ATGL, CPT-1, deacetylase sirtuin 1 (SIRT-1), and PGC1-α expression. *Trans*-resveratrol-3-*O*-glucuronide reduced mRNA levels of FASN and increased those of SIRT-1. *Trans*-resveratrol-4′-*O*-glucuronide increased HSL and SIRT-1 mRNA levels. *Trans*-resveratrol-3-*O*-sulfate did not change gene expression.

Conclusion: The present study shows for the first time the delipidating effect of (i) resveratrol metabolites in maturing pre-adipocytes and (ii) glucuronide metabolites in mature adipocytes. This suggests that both resveratrol and resveratrol metabolites may be involved in the anti-obesity effect of this polyphenol.

Keywords:
3T3-L1 maturing pre-adipocytes and mature adipocytes / Delipidation / Glucuronide metabolites / Resveratrol / Sulfate metabolites

1 Introduction

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) is a phytolaexin polyphenolic compound occurring naturally in various plants, including grapes, berries and peanuts, produced in response to stress, as a defence mechanism against fungal, viral, bacterial infections and damage from exposure to ultraviolet radiation [1, 2].

A remarkable range of biological functions has been ascribed to this molecule. For example, it acts as a cancer chemoprevention agent, a powerful anti-inflammatory factor, and an antioxidant [3, 4]. Its cardiovascular properties have also been described [5]. More recently, resveratrol has been proposed as a potential anti-obesity compound. It seems to mimic the

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Abbreviations: ACC, acetyl-CoA carboxylase; **ATGL**, adipose triglyceride lipase; **C/EBP**, CCAAT-enhancer-binding protein; **FASN**, fatty acid synthase; **HSL**, hormone sensitive lipase; **LPL**, lipoprotein lipase; **PGC1**, peroxisome proliferated-activated receptor co-activator 1; **PPARγ**, peroxisome proliferator-activated receptor γ; **RSV**, *trans*-resveratrol; **SIRT-1**, deacetylase sirtuin 1

effects of energy restriction, thus leading to reduced body fat and improved insulin sensitivity [6–16].

Most resveratrol undergoes rapid and extensive metabolism into enterocytes, before entering blood. Furthermore, it undergoes rapid first-pass metabolism in the liver [17, 18]. Consequently, resveratrol bioavailability is very low and only a small proportion reaches plasma. The concentrations of glucuronide and sulfate metabolites are relatively higher [17, 19, 20]. For instance, it has been reported that the plasma concentration of this polyphenol after a dietarily relevant 25 mg oral dose of resveratrol is only in the nanomolar range compared with the micromolar range of its metabolites [17, 21, 22]. The proportions of glucuronide and sulfate metabolites depend on the tissue [23] and the species [24]. Juan et al. [23] observed that 1 min after intravenous *trans*-resveratrol administration, *trans*-resveratrol glucuronide and sulfate were found in rat plasma in percentages of 33 and 8%, respectively. In general, glucuronides have been reported to be the main metabolites detected in rodents, whereas sulfates are more abundant in humans [25]. With regard to tissue distribution, Juan et al. [23] reported that glucuronide conjugate concentrations were clearly higher than those of sulfate conjugates in testes and the liver, but not in the lungs.

Initially, conjugation is intended for xenobiotic and endogenous inactivation of molecules in order to decrease their cellular permeability and to make them more easily eliminated. Thus, it seems unlikely that glucuronide and sulfate resveratrol metabolites could be as active as resveratrol [7]. However, despite the low concentrations of resveratrol found in tissues, the above-mentioned in vivo studies, as well as others devoted to analyze other beneficial effects of this polyphenol, have reported significant effects [26]. This may suggest that some resveratrol metabolites could in fact show biological activities, or that resveratrol metabolites are converted back to resveratrol in target organs via glucuronidases and sulfatases. Thus, considerable controversy exists as to whether resveratrol is the active molecule in vivo.

Recently, a working group on resveratrol research has been created as a result of the 1st International Conference on Resveratrol and Health (www.resveratrol2010.com) held in Denmark in 2010. This group has published a paper in which recommendations for research on resveratrol for the coming years are proposed [27]; one of them is "to elucidate the biological effects of resveratrol metabolites".

In this line, Halliwell observed that the antioxidant activity of resveratrol metabolites was lower than that of resveratrol [28]. Hoshino et al. [29] tested the activity of five sulfate metabolites in a set of assays associated with cancer chemoprotective activity and demonstrated that two of them were able to inhibit the activity of COX with nearly the same efficacy as resveratrol, but that in general their cytotoxicity toward cancer cells appeared to be reduced compared to resveratrol itself [29]. Furthermore, Calamini et al. [30] showed that 4′-*O*-sulfate metabolite was a potent inhibitor of both COX-1 and COX-2, only slightly less than the parent compound. In contrast, resveratrol-3-*O*-sulfate and -3-*O*-glucuronide were found to be only weak inhibitors of both enzymes. Moreover, resveratrol-3-*O*-sulfate and 4′-*O*-sulfate stimulated deacetylase sirtuin 1 (SIRT-1) activity to the same extent as resveratrol [30]. Delmas et al., found no effect of resveratrol glucuronide metabolites on colon cancer cell growth [26]. With regard to anti-inflammatory effects, sulfate conjugates have been reported to modulate inflammation pathways in vitro with similar efficacy to the parent compound in some cases, while glucuronide conjugates were inactive in vitro at concentrations up to 300 μM [29, 30]. Thus, the literature shows that the activity of resveratrol metabolites depends on the function analyzed. As far as we know, no data have yet been published concerning the effects of resveratrol metabolites on lipid metabolism. This is an important issue to well understand the effects of resveratrol on obesity.

Following the resveratrol working group recommendations, the aim of the present study was to determine whether the following resveratrol phase II metabolites, *trans*-resveratrol-3-*O*-glucuronide, *trans*-resveratrol-4′-*O*-glucuronide, and *trans*-resveratrol-3-*O*-sulfate, show delipidating effect in 3T3-L1 maturing and mature adipocytes and to compare this effect with that of the parent compound.

2 Materials and methods

2.1 Reagents

DMEM was purchased from GIBCO (BRL Life Technologies, Grand Island, NY, USA). *Trans*-resveratrol (98% purity), *trans*-resveratrol-3-*O*-glucuronide (95% purity), *trans*-resveratrol-4′-*O*-glucuronide (95% purity) and *trans*-resveratrol-3-*O*-sulfate (98% purity) were provided by Bertin Pharma (Montigny le Bretonneux, France).

2.2 Experimental design

The 3T3-L1 pre-adipocytes, supplied by American Type Culture Collection (Manassas, VA, USA), were cultured in DMEM containing 10% fetal calf serum. Two days after confluence (day 0), the cells were stimulated to differentiate with DMEM containing 10% fetal calf serum, 10 μg/mL insulin, 0.5 mM isobutylmethylxanthine (IBMX), and 1 μM dexamethasone for 2 days. On day 2, the differentiation medium was replaced by FBS/DMEM medium (10%) containing 0.2 μg/mL insulin. This medium was changed every 2 days until cells were harvested (day 8 in the case of maturing pre-adipocytes and day 12 in the case of mature adipocytes). At day 12, greater than 90% of cells developed mature adipocytes with visible lipid droplets. All media contained 1% penicillin/streptomycin (10 000 U/mL), and the media for differentiation and maturation contained 1% (v/v) of biotin and panthothenic acid. Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere.

2.3 Cell treatment

Maturing pre-adipocytes grown in 6-well plates were incubated with either 0.1% ethanol (95%) (control group) or with *trans*-resveratrol, *trans*-resveratrol-3-*O*-glucuronide, *trans*-resveratrol-4′-*O*-glucuronide or *trans*-resveratrol-3-*O*-sulfate, all of them at 1, 10, and 25 μM (diluted in 95% ethanol) during the adipogenic phase from day 0 to day 8 of differentiation. The medium was changed every two days. On day 8, the culture supernatant was removed and cells were used for triacylglycerol determination and RNA extraction.

Mature adipocytes grown in 6-well plates were also incubated with either 0.1% ethanol (95%) (control group) or with *trans*-resveratrol, *trans*-resveratrol-3-*O*-glucuronide, *trans*-resveratrol-4'-*O*-glucuronide, or *trans*-resveratrol-3-*O*-sulfate, all of them at 1, 10, and 25 μM (diluted in 95% ethanol) on day 12 after differentiation. After 24 h, supernatant was removed and cells were used for triacylglycerol determination and RNA extraction.

2.4 Measurement of triacylglycerol content in maturing and mature adipocytes

For triacylglycerol extraction, treated cells (maturing and mature adipocytes) were washed extensively with PBS and incubated three times with 500 μL of hexane/isopropanol (2:1). The total volume was then evaporated by vacuumed centrifugation and the pellet was resuspended in 200 μL Triton X-100 in 1% distilled water. Afterwards, triacylglycerols were disrupted by a sonicator and the content was measured by Infinity Triglycerides reagent (Thermo Scientific, Rockford, IL, USA). For protein determinations, cells were lysed in 0.3N NaOH, 0.1% SDS. Protein measurements were performed using the BCA reagent (Thermo Scientific).

2.5 Extraction and analysis of RNA and quantification by real-time RT-PCR

RNA samples were extracted from cells by using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The integrity of the RNA extracted from all samples was verified and quantified using an RNA 6000 Nano Assay (Thermo Scientific, Wilmington, DE, USA). RNA samples were then treated with DNase I kit (Applied Biosystems, Foster City, CA, USA) to remove any contamination with genomic DNA.

One microgram of total RNA in a total reaction volume of 20 μL was reverse transcribed using the iScript cDNA Archive Kit (Applied Biosystems) according to the manufacturer's protocols. Reactions were incubated initially at 25°C for 10 min and subsequently at 37°C for 120 min and 85°C for 5 min.

Relative CCAAT enhancer-binding proteins β and α (C/EBPβ and C/EBPα), peroxisome proliferator-activated receptor γ (PPARγ), and lipoprotein lipase (LPL) mRNA levels in maturing pre-adipocytes and relative adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), carnitine palmitoyltransferase 1(CPT-1), LPL, fatty acid synthase (FASN), acetyl CoA carboxylase (ACC), SIRT-1, and peroxisome proliferated-activated receptor γ co-activator 1α (PGC1α) mRNA levels in mature adipocytes were quantified using Real-Time PCR with an iCycler™ – MyiQ™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA). β-actin mRNA levels were similarly measured and served as the reference gene. The PCR reagent mixture consisted of 1 μL of each cDNA (10 pmol/μL), SYBR® Green Master Mix (Applied Biosystems) and the upstream and downstream primers (300 nM each, except in the case of C/EBPβ and C/EBPα whose primer concentration was 600 nM). Specific primers were synthesized commercially (Tib Molbiol, Berlin, Germany for 3T3-L1 and Eurofins MWG Operon, Ebersberg, Germany for SGBS cells) (Table 1).

PCR parameters were as follows: initial 2 min at 50°C, denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s (except in the case of C/EBPβ and C/EBPα where the annealing was at 68.4°C and 66.4°C, respectively), and extension at 60°C for 30 s. All sample mRNA levels were normalized to the values of 18S and the results expressed as fold changes of threshold cycle (Ct) value relative to controls using the $2^{-\Delta\Delta Ct}$ method [31].

2.6 Statistical analysis

Results are presented as mean ± SEM. Statistical analysis was performed using SPSS 19.0 (SPSS, Chicago, IL, USA). Comparisons between each treatment with the control were analyzed by Student's *t*-test. Statistical significance was set up at the $p < 0.05$ level.

3 Results and discussion

3.1 Effects of resveratrol and its metabolites on triacylglycerol content in 3T3-L1 maturing and mature adipocytes

As explained in the Introduction section, following ingestion, most resveratrol undergoes rapid metabolism resulting in up to a 20-fold higher concentration of circulating conjugates, and less than 1% of the parent compound [17, 25]. This is a matter of concern for scientists because this fact could represent a major obstacle to considering resveratrol as an efficient functional ingredient.

Bearing this in mind, enhancement of the bioavailability of resveratrol is the subject of extensive biotechnology research. Different systems have been proposed for this purpose:

Table 1. Primers for PCR amplification of each gene studied by SYBR® Green RT-PCR

	Sense primer	Antisense primer
ATGL	5′-CAC TTT AGC TCC AAG GAT GA-3′	5′-TGG TTC AGT AGG CCA TTC CT-3′
HSL	5′-GGT GAC ACT CGC AGA AGA CAA TA-3′	5′-GCC GCC GTG CTG TCT CT-3′
CPT1-b	5′- AGA ACA CTC ATG GGC AGA TGCT-3′	5′- TAC CTT TCA CCT GGG CTA CAC G-3′
LPL	5′-CAG CTG GGC CTAACT TTG AG-3′	5′-CCT CTC TGC AAT CAC ACG AA-3′
ACC	5′-GGA CCA CTG CAT GGA ATG TTA A-3′	5′-TGA GTG ACT GCC GAA ACA TCT C-3′
FASN	5′-AGC CCC TCA AGT GCA CAG TG-3′	5′-TGC CAA TGT GTT TTC CCT GA-3′
SIRT1	5′-GAC GAC GAG GGC GAG GAG-3′	5′-ACA GGA GGT TGT CTC GGT AGC-3′
PGC1α	5′-CCA AAG CTGA AGC CCT CTT GC-3′	5′-GTT TAG TCT TCC TTT CCT CGT GTC C-3′
C/EBPβ	5′-GAG CGA CGA GTA CAA GAT GCG-3′	5′-GCT GCT CCA CCT TCT TCT GC-3′
C/EBPα	5′-TTC CTC CGG CTA AGA CTT AGC C-3′	5′-CAG GGG TGT GTG TAT GAA CTG G-3′
PPARγ	5′-ATT CTG GCC CAC CAA CTT CGG-3′	5′-TGG AAG CCT GAT GCT TTA TCC CCA-3′
18S	5′-GTG GGC CTG CGG CTT AAT-3′	5′-GCC AGA GTC TCG TTC GTT ATC-3′

ATGL = adipose triglyceride lipase, HSL = hormone sentitive lipase, CPT-1 = carnitine palmitoyltransferase-1, LPL = lipoprotein lipase, ACC = acetyl-CoA carboxylase, FASN = fatty acid synthase, SIRT1 = deacetylase sirtuin 1, PGC-1 α = peroxisome proliferator-activated receptor gamma coactivator, C/EBPβ and C/EBPα = CCAAT-enhancer-binding protein beta and alpha

combinations of resveratrol with other molecules able to inhibit resveratrol metabolism, such as flavonoids [32], or modifications in the chemical structure, such as the introduction of methoxy groups [25].

To properly address the issue of potential solutions to increase resveratrol bioavailability, it is important to know whether resveratrol metabolites show biological activities. In this context, the present study focussed on the effects of several phase II resveratrol metabolites, *trans*-resveratrol-3-*O*-glucuronide, *trans*-resveratrol-4′-*O*-glucuronide and *trans*-resveratrol-3-*O*-sulfate, on lipid metabolism in isolated 3T3-L1 maturing pre-adipocytes and mature adipocytes because, as far as we know, these potential effects have not been reported yet. In order to clearly characterize the delipidating effect of resveratrol metabolites, three concentrations were used: 1, 10, and 25 μM. Of these, 25 μM is one of the most commonly used in in vitro studies performed to analyze the effects of resveratrol on adipocytes [33–35]. As far as we know, a concentration as low as 1 μM has never been used in studies devoted to determining the effects of resveratrol on lipid metabolism in adipocytes. Thus, this is an original aspect of the present study. This concentration was used because it is closer to in vivo plasma values reported in humans and rodents.

Biological events leading to obesity include changes in adipocyte number, achieved through a complex interplay between proliferation and differentiation of pre-adipocytes, and changes in lipid metabolism in mature adipocytes. It has been described that resveratrol inhibits both pre-adipocyte differentiation [34–38] and lipid accumulation in mature adipocytes [13, 39, 40]. Thus, in the present study, we were interested in the potential effects of resveratrol metabolites on adipogenesis, as well as on triacylglycerol metabolism in mature adipocytes.

With regard to 3T3-L1 maturing pre-adipocytes, exposure to 1 and 10 μM of either *trans*-resveratrol or resveratrol metabolites did not modify triacylglycerol content (Fig. 1A and B). By contrast, at a concentration of 25 μM, resveratrol, *trans*-resveratrol-4′-*O*-glucuronide and *trans*-resveratrol-3-*O*-sulfate led to a significant reduction in this lipid species (−13.0%, −13.8%, and −20.0%, respectively). *Trans*-resveratrol-3-*O*-glucuronide induced a reduction of 11.3% but this effect did not reach statistical significance (Fig. 1C). The results concerning resveratrol are in good accordance with those previously reported by other authors who observed no effect with 10 or 12.5 μM of this polyphenol and a significant reduction in triacylglycerol content with 20 or 25 μM [35, 38, 41].

Exposure of mature 3T3-L1 adipocytes to 1 μM resveratrol for 24 h triggered a significant reduction in intracellular triacylglycerol content (−52.7%). *Trans*-resveratrol-3-*O*-glucuronide showed a tendency toward lower values at this dose ($p = 0.07$), and the other two metabolites did not show any delipidating effect (Fig. 2A). At a dose of 10 μM, resveratrol and the glucoronide metabolites showed delipidating effects (−60.0%, −48.9%, and −32.9%, respectively). By contrast, *trans*-resveratrol-3-*O*-sulfate did not induce significant changes. The effect of resveratrol was significantly higher than that of the metabolites (Fig. 2B). At the highest dose (25 μM), additional delipidating effects were not observed (Fig. 2C).

These results demonstrate that both maturing pre-adipocytes and mature adipocytes are targets for glucuronide metabolites. By contrast, the analyzed sufate metabolite seems to be only effective in pre-adipocytes. When comparing the effects of reveratrol with those of its metabolites, it can be observed that both resveratrol and its metabolites only showed the delipidating effect at the highest dose used (25 μM) in maturing pre-adipocytes, whereas in mature adipocytes the delipidating effect of resveratrol was found at a lower dose (1 μM) than that of glucuronide metabolites (10 μM). Moreover, in maturing pre-adipocytes, the percentage of tricylglycerol reduction was similar in resveratrol and the three analyzed metabolites, whereas in mature adipocytes the delipidating effect of resveratrol was stronger than that of glucuronide metabolites ($p < 0.05$ when comparing resveratrol to

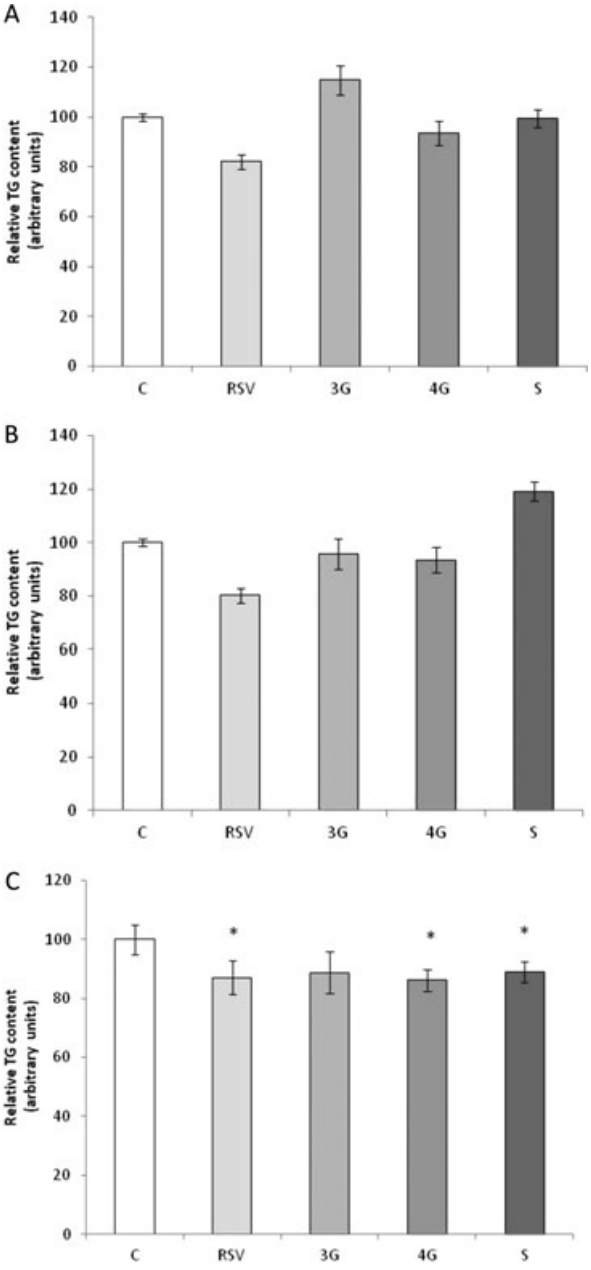

Figure 1. Triacylglycerol amounts in 3T3-L1 maturing preadipocytes treated from day 0 to day 8 with 1 (A), 10 (B) or 25 μM (C) *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4′-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (3S; S). Values are means ± SEM. Comparisons between each treatment with the control were analyzed by Student's *t*-test. The asterisks represent differences versus the control (*$p < 0.05$).

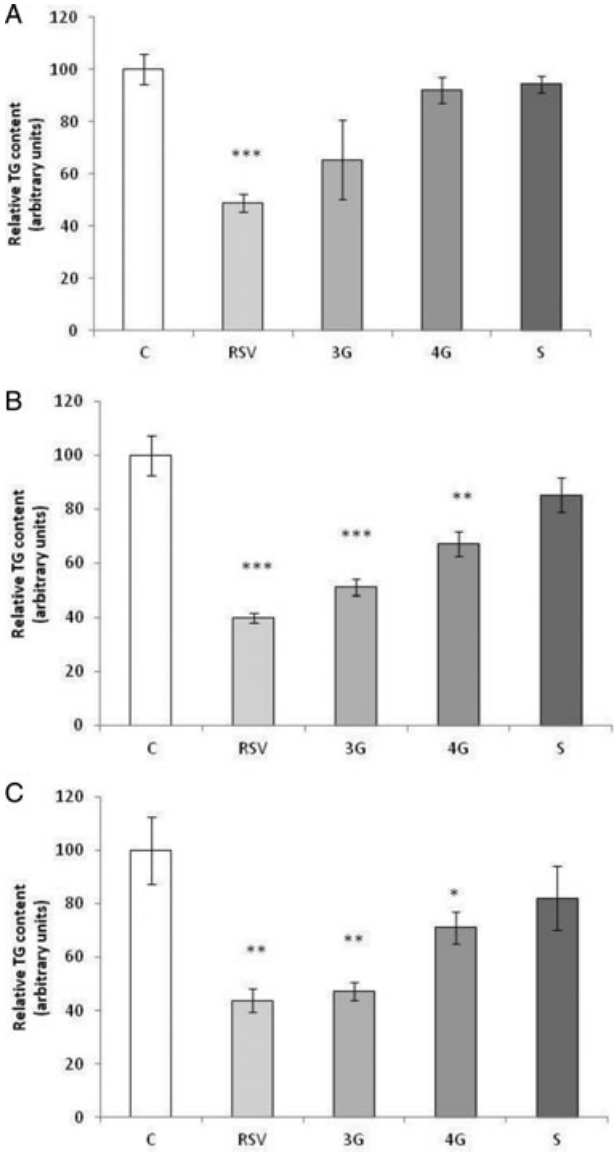

Figure 2. Triacylglycerol amounts in 3T3-L1 mature adipocytes treated for 24 h with 1 (A), 10 (B), or 25 μM (C) *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4′-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (3S; S). Values are means ± SEM. Comparisons between each treatment with the control were analyzed by Student's *t*-test. The asterisks represent differences versus the control (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

trans-resveratrol-3-*O*-glucuronide and $p < 0.01$ when comparing resveratrol to *trans*-resveratrol-4′-*O*-glucuronide).

These results as a whole suggest that even if resveratrol, the parent compound, seems to be mostly responsible for the body-fat lowering effects observed when this polyphenol is orally administered in vivo, the analyzed metabolites can contribute to this effect.

3.2 Effects of resveratrol and its metabolites on gene expression in 3T3-L1 maturing and mature adipocytes

It has been demonstrated that C/EBPβ, PPARγ, and C/EBPα are transcriptional factors involved in adipogenesis. At the

very early stage of differentiation (day 1–2 post-confluence), which corresponds to the period of mitotic clonal expansion, C/EBPβ expression is increased. This change, in turn, triggers high-level expression of PPARγ, which is considered the master coordinator of adipocyte differentiation. C/EBP1α is induced during later stages of differentiation and cooperates with PPARγ [42].

The expression of genes related to adipogenesis was analyzed in maturing pre-adipocytes treated with 25 μM of resveratrol or resveratrol metabolites, because of the three concentrations studied this was the only one which led to significant reduction in triacylglycerol content. Resveratrol and the three studied metabolites significantly reduced mRNA levels of C/EBPβ. As far as C/EBPα, PPARγ, and LPL expressions are concerned only *trans*-resveratrol-3-*O*-sulfate led to a significant reduction (Fig. 3). Data in the literature concerning the effect of resveratrol on adipogenesis are controversial. In several studies performed in 3T3-L1, pre-adipocytes the expression of both PPARγ and C/EBPα was reduced, but in others they remained unchanged when using similar doses [34, 35, 38, 41].

The amount of triacylglycerols stored in mature adipocytes, and hence in adipose tissue, results from the balance among lipid mobilization and oxidation and fatty acid uptake from circulating triacylglycerols and de novo lipogenesis. Thus, the effects of resveratrol metabolites on genes involved in these metabolic pathways were also assessed in mature adipocytes. For this purpose, we chose the dose of 10 μM because at 1 μM only resveratrol showed a delipidating effect and at 25 μM the effects were similar to those induced by 10 μM.

The selected enzyme genes were the two main lipases, ATGL and HSL, the oxidative enzyme, CPT-1, the enzyme that allows adipose tissue to uptake fatty acids from circulating triacylglycerols, LPL, and two lipogenic enzymes, ACC and FASN. Moreover, the deacetylase SIRT-1 and PGC-1α genes were included in the analysis.

Resveratrol significantly increased the expression of ATGL and CPT-1b. This suggests that the polyphenol can activate lipolysis and the oxidative pathways. The two glucuronide metabolites showed a trend toward increased values of ATGL ($p = 0.08$ in the case of *trans*-resveratrol-3-*O*-glucuronide and $p = 0.09$ in the case of *trans*-resveratrol-4′-*O*-glucuronide). *Trans*-resveratrol-4′-*O*-glucuronide also increased HSL lipase expression. *Trans*-resveratrol-3-*O*-glucuronide, unlike resveratrol or the other glucoronide metabolite, significantly reduced FASN expression. This could suggest that while *trans*-resveratrol-3-*O*-glucuronide could be acting at both increasing lipolysis and decreasing lipogenesis, *trans*-resveratrol-4′-*O*-glucuronide could be mainly acting on the lipolytic pathway. T*rans*-resveratrol-3-*O*-sulfate did not induce significant changes in gene expression (Figs. 4 and 5).

Resveratrol has been proposed to drive its beneficial effects by targeting and activating the NAD^+-dependent protein deacetylase SIRT-1, although results have been inconsistent [7, 43–46]. Several authors have also showed that

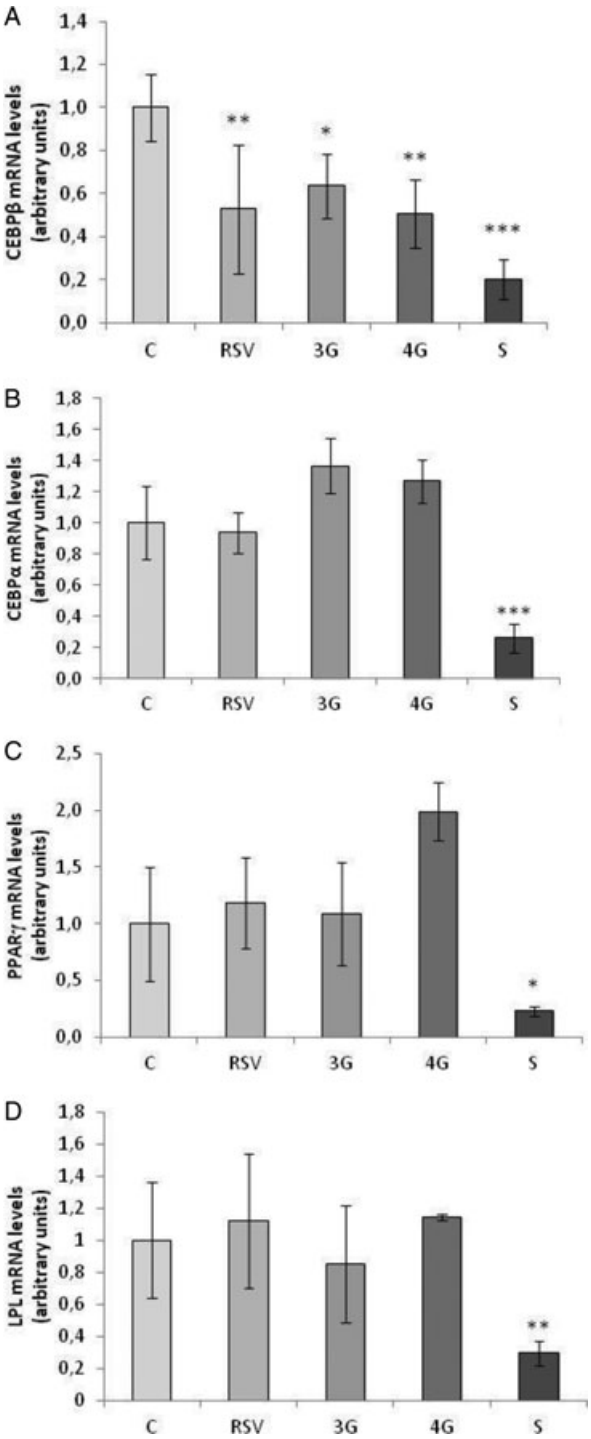

Figure 3. Effects of 25 μM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4′-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (3S; S) on the expression of C/EBP1β (A), C/EBP1α (B), PPARγ (C) and LPL (D) in 3T3-L1 maturing pre-adipocytes treated from day 0 to day 8. Values are means ± SEM. Comparisons between each treatment with the control were analyzed by Student's *t*-test. The asterisks represent differences versus the control ($^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

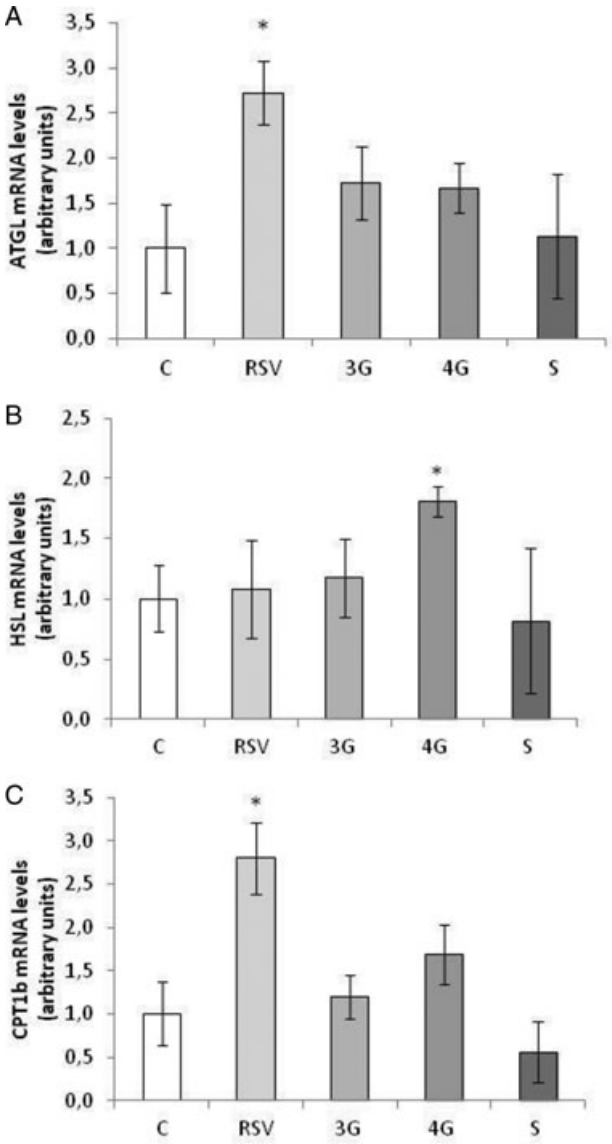

Figure 4. Effects of 10 μM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4′-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (3S; S) on the expression of ATGL (A), HSL (B), and CPT-1 (C) in 3T3-L1 mature adipocytes treated for 24 h. Values are means ± SEM. Comparisons between each treatment with the control were analyzed by Student's *t*-test. The asterisks represent differences versus the control (*$p < 0.05$).

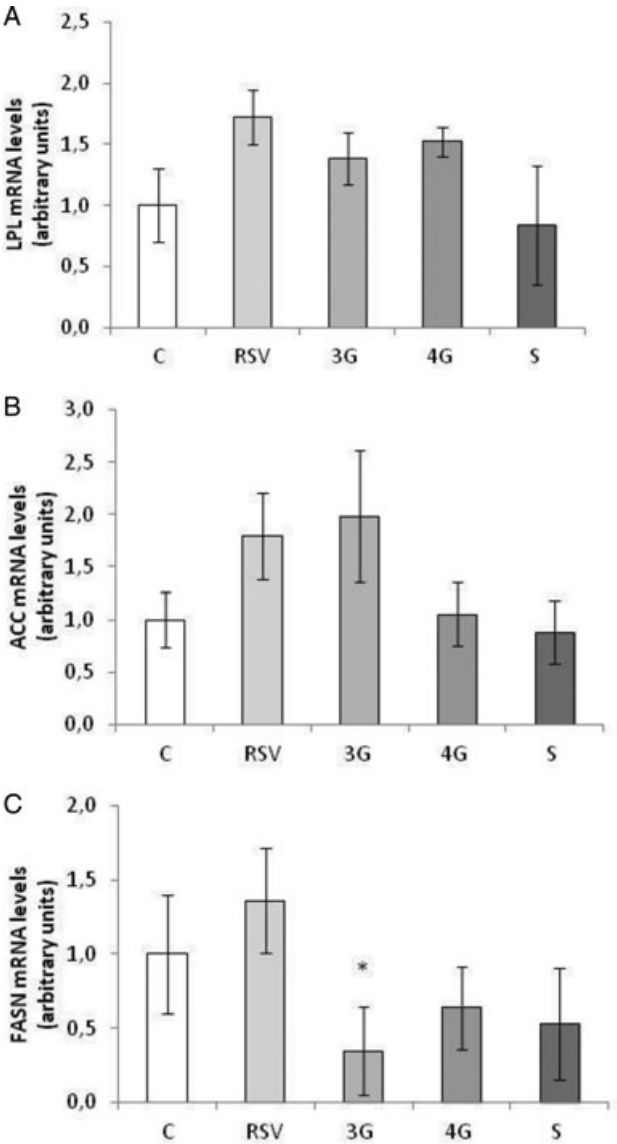

Figure 5. Effects of 10 μM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4′-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (3S; S) on the expression of LPL (A), ACC (B), and FASN (C) in 3T3-L1 mature adipocytes treated for 24 h. Values are means ± SEM. Comparisons between each treatment with the control were analyzed by Student's *t*-test. The asterisks represent differences versus the control (*$p < 0.05$).

resveratrol can increase the expression of this protein [37]. In the present study, resveratrol and its glucuronide metabolites, but not the sulfate metabolite significantly upregulated this gene. Resveratrol also increased the expression of PGC1α (Fig. 6).

The results obtained in the present study can help to better understand the results that we obtained in a previous study carried out in rats by using three doses of resveratrol (6, 30, and 60 mg/kg/day) [14]. We observed that this polyphenol reduced body fat at a dose of 30 mg/kg/day, but not at 6 mg/kg/day. Surprisingly, rats treated with 60 mg/kg/day resveratrol did not show further reduction as compared with those treated with 30 mg/kg/day. This means that a "plateau" was reached when the dose of resveratrol increased. Moreover, it was found in the same study that the amount of sulfate metabolites increased with the doses administered to rats. However, no differences in terms of glucuronide metabolite accumulation were found between rats treated with 30 or

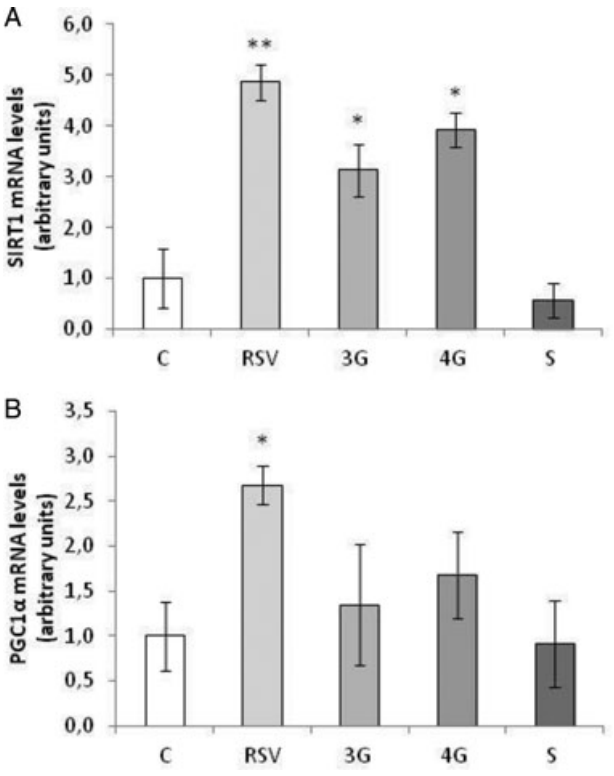

Figure 6. Effects of 10 μM *trans*-resveratrol (RSV), *trans*-resveratrol-3-*O*-glucuronide (3G), *trans*-resveratrol-4′-*O*-glucuronide (4G) or *trans*-resveratrol-3-*O*-sulfate (3S; S) on the expression of SIRT-1 (A) and PGC-1α (B) in 3T3-L1 mature adipocytes treated for 24 h. Values are means ± SEM. Comparisons between each treatment with the control were analyzed by Student's *t*-test. The asterisks represent differences versus the control (*$p < 0.05$; **$p < 0.01$).

60 mg/kg/day (submitted for publication). Thus, the lack of increase in glucuronides, active metabolites which may be responsible in part for the body fat-lowering effect of resveratrol, when the dose of this polyphenol increases from 30 to 60 mg/kg/day could help to explain the above-mentioned "plateau" effect. Moreover, the results in the present study show that in mature adipocytes there is no significant increase in the delipidating effect when the dose of glucuronide metabolites rose from 10 μM to 25 μM and that of resveratrol from 1 μM to 10 and 25 μM, meaning that in vitro there is also a "plateau" in the effect. Thus, this fact can also contribute to the described in vivo effect.

As stated before, a combination of resveratrol with other molecules able to inhibit resveratrol metabolism has been proposed as an option to increase resveratrol biovailability. It has been proved that some flavonoids are able to inhibit resveratrol metabolism. De Santi and co-workers observed that quercetin inhibited sulfated metabolites production from resveratrol [32]. The results obtained in the present study give support to the interest in this molecule combination since a decrease in the amount of ineffective metabolites increases the total amount of effective resveratrol + glucuronide metabolites and so the success of resveratrol treatment, at least in adult subjects in which adipogenesis is not an important process underlying obesity development.

4 Concluding remarks

In conclusion, the present study shows for the first time that among resveratrol metabolites, *trans*-resveratrol-4′-*O*-glucuronide and *trans*-resveratrol-3-*O*-sulfate induced similar delipidating effects to resveratrol in maturing pre-adipocytes and that both glucuronide metabolites (*trans*-resveratrol-3-*O*-glucuronide and *trans*-resveratrol-4′-*O*-glucuronide) show a delipidating effect, although lower than that of resveratrol, in mature adipocytes. Consequently, it may be suggested that both resveratrol and resveratrol metabolites are involved, to greater or lesser extents, in the anti-obesity effect of this polyphenol.

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