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Resveratrol enhances fatty acid oxidation capacity and reduces resistin and Retinol-Binding Protein 4 expression in white adipocytes☆

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Abstract

Resveratrol is a naturally occurring polyphenol known to affect energy metabolism and insulin sensitivity in mice and lipogenic gene expression in adipocytes. Here, we sought to get further insight into the impact of resveratrol on adipocyte biology by studying its effects on oxidative metabolism and the expression of the insulin resistance-related adipokines resistin and Retinol-Binding Protein 4 (RBP4) in mature adipocytes. Effects were assessed in 3T3-L1 adipocytes and in adipocytes derived from primary mouse embryonic fibroblasts (MEF). Besides reducing triacylglycerol content and the mRNA levels of lipogenic genes, resveratrol treatment resulted in both models in increased mRNA levels of carnitine palmitoyltransferase 1 (a rate-limiting enzyme in mitochondrial fatty acid oxidation), reduced mRNA levels of receptor interacting protein 140 (a suppressor of oxidative metabolism), and signs of enhanced flux through the fatty acid beta-oxidation pathway. In primary MEF-derived adipocytes, the treatment also increased mitochondrial DNA content and the mRNA levels of subunit II of cytochrome oxidase (a component of the mitochondrial respiratory chain) and of uncoupling protein 1. Expression of resistin and RBP4 was reduced in both adipocyte models following resveratrol treatment. The results indicate that resveratrol directly acts in mature white adipocytes to favor a remodeling toward increased oxidative capacity and reduced lipogenesis, while down-regulating two putative insulin resistance factors. These results constitute novel insights into resveratrol action in adipocytes that add to the potential of this food phytochemical and its synthetic analogues for the control of obesity and related metabolic disorders.

Keywords: Resveratrol; White adipocytes; Fatty acid oxidation; Retinol binding protein 4; Resistin; Receptor interacting protein 140

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is a naturally occurring polyphenol present in grapes, berries, peanuts and other food vegetables. Resveratrol has many remarkable effects in mammals believed to be largely due to its ability to activate silent information regulation 2 homolog 1 (SIRT1) [1], a sirtuin enzyme that catalyzes NAD^+-dependent protein deacetylation according to the cell's nutritional status [2]. The bioactivity of resveratrol includes effects on energy metabolism and related aspects. In mice on high fat diets, supplementation with resveratrol has been shown to increase mitochondrial content/activity in skeletal muscle [3], brown adipose tissue (BAT) [3] and liver [4], to enhance whole body basal energy expenditure [3], to protect against the development of diet-induced obesity [3] and to improve metabolic disturbances such insulin resistance and hepatosteatosis [3–5]. Resveratrol treatment also reduced abdominal fat content and improved metabolic disturbances in a genetic rodent model of obesity, the obese Zucker rat [6]. Effects of resveratrol on energy metabolism and metabolic health are most likely SIRT1-mediated, as similar changes have been described in transgenic mice overexpressing SIRT1 [7,8] and in rodents treated with synthetic SIRT1 activators [9–11], and they have been linked to the ability of SIRT1 to deacetylate and thereby activate peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) [3,12], an essential cofactor in mitochondrial biogenesis and function driving metabolic rate [13].

Direct effects on white adipose tissue (WAT) are likely to contribute to the systemic changes elicited by resveratrol treatment/SIRT1 activation in vivo. Expression of SIRT1 is low in adipose tissue of humans and rodents with obesity [14,15], and resveratrol treatment has been shown to inhibit adipogenesis in maturing white preadipocytes [16–18] and to enhance fat mobilization [14,16,19] and reduce lipogenic gene expression [20], insulin-dependent glucose

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Table 1
Nucleotide sequences of primers used for PCR amplification

Gene	Primer sequence	Accession number
COX-II	5′-aagacgccacatcccctatt-3′ 5′-cttcagtatcattggtgccct-3′	AF378830
CPT1-L	5′-acaaattatgtgagtgactgg-3′ 5′-gatcccagaagacgaatagg-3′	NM_013495
GLUT4	5′-ggcatgcgtttccagtatgt-3′ 5′-gcccctcagtcattctcatc-3′	NM_009204
PGC-1α	5′-catttgatgcactgacagatgga-3′ 5′-ccgtcaggcatggaggaa-3′	AF049330
PPARγ	5′-agaccactcgcattcctttg-3′ 5′-tcgcactttggtattcttgg-3′	NM_011145.2
RBP4	5′-actggggtgtagcctccttt-3′ 5′-ggtgtcgtagtccgtgtcg-3′	NM_011255
Resistin	5′-ttccttttcttccttgtccctg-3′ 5′-ctttttcttcacgaatgtccc-3′	NM_022984
RIP140	5′-cggcctcgaaggcgtgg-3′ 5′-aaacgcacgtcagtatcgtc-3′	NM_173440.2
UCP1	5′-ggcattcagaggcaaatcag-3′ 5′-gcattgtaggtccccgtgta-3′	NM_009463.2
β-Actin	5′-tacagcttcaccaccacagc-3′ 5′-tctccagggaggaagaggat-3′	NM_007393.2
LRP10	5′-actgcacctgggttatcctg-3′ 5′-gggagattggtggctgta-3′	NM_022993

uptake [20] and lipogenesis from glucose [19] in fully differentiated fat cells. Effects of resveratrol/SIRT1 activation in white adipocytes have been linked to the repression of peroxisome proliferator-activated receptor γ (PPARγ) [16,20], a transcription factor essential for adipogenesis and for the maintenance of the characteristics of mature adipocytes [21]. Whether resveratrol can elicit an increase in oxidative metabolism in white adipocytes as it does in other cell types remains, however, largely unexplored.

Resveratrol also has an impact on the secretory function of adipose tissue. In particular, resveratrol was shown to reduce proinflammatory cytokine expression in cultured adipocytes [22–25] and in rodent WAT [6]. Because adipose tissue inflammation is tightly linked to systemic insulin resistance [26], resveratrol-induced down-regulation of the adipose expression of cytokines such as interleukin 6 [22,24,25], monocyte chemoattractant protein-1 [23], tumor necrosis factor α [6] and plasminogen activator inhibitor-1 [22] may conceivably contribute to the insulin sensitizing effect elicited by resveratrol supplementation in vivo [3,4,6]. In addition, resveratrol was shown to up-regulate the expression of adiponectin — an adipokine with well-established antidiabetic and anti-inflammatory action — both in cultured adipocytes [15] and in WAT of obese Zucker rats [6].

In this work, we sought to get further insight into the impact of resveratrol on adipocyte biology by studying its effects on aspects not yet addressed, namely, its impact on adipocyte oxidative metabolism/fatty acid oxidation and the expression of resistin and retinol binding protein 4 (RBP4), two adipokines that act as insulin resistance factors in mice [27,28] and may also be related to insulin resistance and inflammation in humans [29,30]. Effects of resveratrol were assessed both in 3T3-L1 murine adipocytes, a well-established white adipocyte cell model, and in mature adipocytes derived from primary mouse embryonic fibroblasts (MEF), which are increasingly been used as white adipocyte model cells. The two models were used in view of previously published differences between them in basal adipokine expression [31] and the expression of energy metabolism-related genes [32].

2. Methods and materials

2.1. Cell culture and differentiation

3T3-L1 cells (American Type Culture Collection, LGC Deselaers, Barcelona, Spain) were grown to confluence in basal medium — Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) with 50 IU/ml penicillin (Sigma), 50 μg/ml streptomycin (Sigma) and 2 mM L-glutamine (Sigma) — supplemented with 10% new born calf serum (Linus, Madrid, Spain). Two days after the cells reached confluence (referred as Day 0), they were induced to differentiate in basal medium containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 1 μM dexamethasone (DEX; Sigma), 0.5 mM methylisobutylxanthine (MIX; Sigma) and 1 μg/ml insulin (Sigma) for 48 h, followed by 48 h in basal medium containing 10% FBS and 1 μg/ml insulin. The cells were subsequently refed every other day fresh basal medium supplemented with 10% FBS (without insulin). Primary MEFs were prepared and cultured as described previously [33]. MEFs on passage 3-5 were grown to confluence in AmnioMAX-C100 basal medium (Invitrogen) supplemented with 7.5% AmnioMAX-C100 supplement (Invitrogen), 7.5% FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine. For differentiation, 2-day postconfluent cells were treated (Day 0) with growth medium containing 1 μM DEX, 0.5 mM MIX, 5 μg/ml insulin and 0.5 μM rosiglitazone (BioVision, Mountain View, CA, USA) for 48 h; the cells were subsequently refed every other day fresh medium containing 5 μg/ml insulin and 0.5 μM rosiglitazone. Two different lots of 3T3-L1 cells and MEFs from two different embryos were used in the study. In both cell systems, adipogenesis was monitored by morphological examination of the cells for lipid accumulation. trans-Resveratrol (Sigma, catalogue number R5010) was added together with fresh medium when more than 90% of the cells had acquired the adipose phenotype (on day 8 of culture), at the final doses and for the times indicated in the figure legends. For treatment, stock solutions of resveratrol in dimethyl sulfoxide (DMSO) as vehicle were prepared at 1000× the desired final working concentrations, diluted 1:1000 with culture medium and added to the cells. Control cells received culture medium containing an equal volume of DMSO (0.1%). For cytotoxicity assays, mature adipocytes were incubated with either 0.1% DMSO or resveratrol (5–100 μM) for 24 h after which lactate dehydrogenase activity in the culture medium was measured using a commercial kit (Roche Diagnostics, Mannheim, Germany).

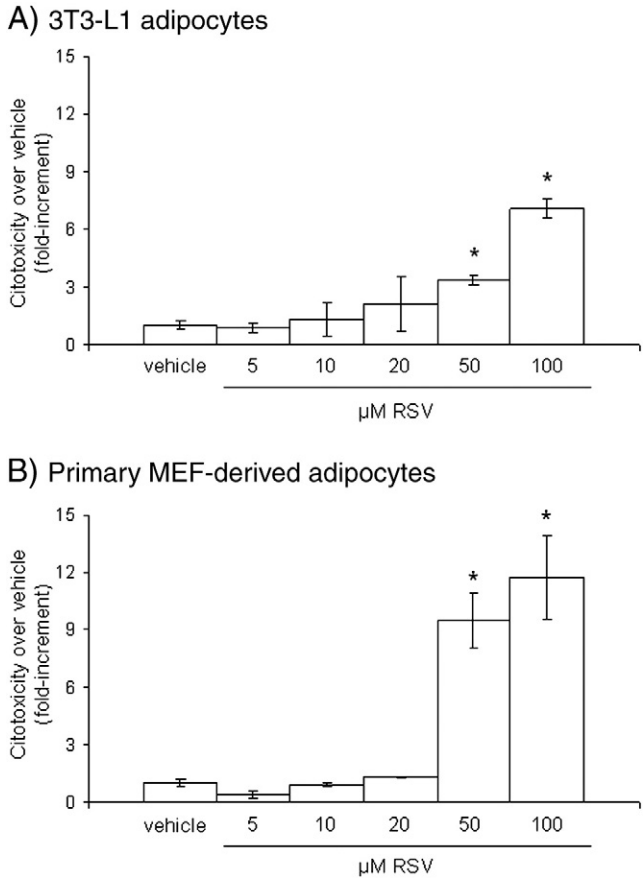

Fig. 1. Cytotoxicity of resveratrol in the adipocyte cell models used. Cytotoxicity was assessed in 3T3-L1 adipocytes (A) and primary MEF-derived adipocytes (B) differentiated in 12-well plates by measuring lactate dehydrogenase activity in the culture medium following a 24-h treatment with the indicated resveratrol (RSV) concentration or vehicle (0.1% DMSO). For each cell model, data are expressed relative to the mean value of the vehicle-treated cells, which was set at 1, and are the mean±S.E.M. of $n=12$ wells per treatment condition, distributed in three separate experiments (4 wells per treatment condition per experiment). Student's t test significance: *, $P<.05$ versus vehicle.

2.2. Gene-expression and mitochondrial DNA content analysis

Total RNA and DNA were extracted from cultured cells using Trizol reagent (Invitrogen) according to the supplier's instructions. For cDNA synthesis, 0.25 μg total RNA was denatured at 65°C for 10 min prior to being reverse transcribed using murine leukemia virus reverse transcriptase (MuLV RT, Perkin-Elmer, Madrid, Spain) in the presence of 50 pmol of random primers, in a Perkin-Elmer 2400 Thermal Cycler. Polymerase chain reaction (PCR) analysis on cDNA and DNA were carried out on a StepOnePlus Real-Time PCR System (Applied Biosystems). Sense and antisense primers used were designed with specific primer analysis software Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA), and their specificity analyzed by the ENTREZ and BLAST databases utilities (National Center for Biotechnology Information, Bethesda, MD, USA). The sequences of the primers used are shown in Table 1. Relative gene-expression was calculated using the $2^{\Delta\Delta Ct}$ method [34] with β-actin and low-density lipoprotein receptor-related protein 10 (LRP10) [35] as internal controls; normalization to each of these two genes gave similar results, and mean values were taken for representation in Figs. 2, 3 and 5. As a measure of mitochondrial content, the mitochondrial DNA to nuclear DNA ratio was determined by real-time PCR quantification of mitochondrial Cox1 DNA and nuclear18S DNA as previously described [36].

2.3. Adipokine analysis

Mature adipocytes were treated with varying concentrations of resveratrol or vehicle (0.1% DMSO) along with fresh medium for 24 h, after which media were collected and analyzed for RBP4 and resistin content. RBP4 protein levels were determined by immunoblotting as previously described [31]. In brief, aliquots of collected media (25 μl) were boiled in Laemmli sample buffer containing 20% 2-mercaptoethanol, fractionated in sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and electrotransferred onto polyvinylidene fluoride (PVFD) membranes (Bio-Rad, Madrid, Spain). Black amide B10 staining provided visual evidence for correct loading and transfer of proteins. After blocking, membranes were incubated with polyclonal anti-mouse RBP4 antibody raised in rabbits (Axxora, San Diego, CA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was used as secondary antibody (Amersham Biosciences, Buckinghamshire, UK). The immunocomplexes were revealed using an enhanced chemiluminiscence detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were exposed to Hyperfilm ECL (Amersham Biosciences, Barcelona, Spain). Resistin protein levels accumulated in the culture medium were determined using an ELISA kit (Phoenix Pharmaceutical, Belmont, CA, USA).

2.4. Measurement of fatty acid beta-oxidation

After exposure of differentiated adipocytes to resveratrol (20 μM) or vehicle (0.1% DMSO) during 20 h, medium was removed and cells were incubated for 4 h in fresh medium containing 0.2 mM L-carnitine (Sigma) and 200 μM [^{14}C(U)] palmitate (0.1 μCi/ml, from Perkin Elmer, Boston, MA, USA) in the continued presence of resveratrol or vehicle. Incubations were terminated by acidification with 6 M HCl. Acid-soluble products in 2 ml of the medium were extracted by addition of 1 ml cold 1 M $HClO_4$. After centrifugation (10 min, 1800 g), radioactivity in the supernatant was measured by scintillation counting. DNA content in parallel cultures of vehicle- and resveratrol-treated cells was analyzed using a PCR method [36] and found to be unaffected by resveratrol.

2.5. Other analysis

The effect of resveratrol on adipocyte triacylglycerol content was analyzed after 72 h of treatment where fresh medium and resveratrol were added to the cells every

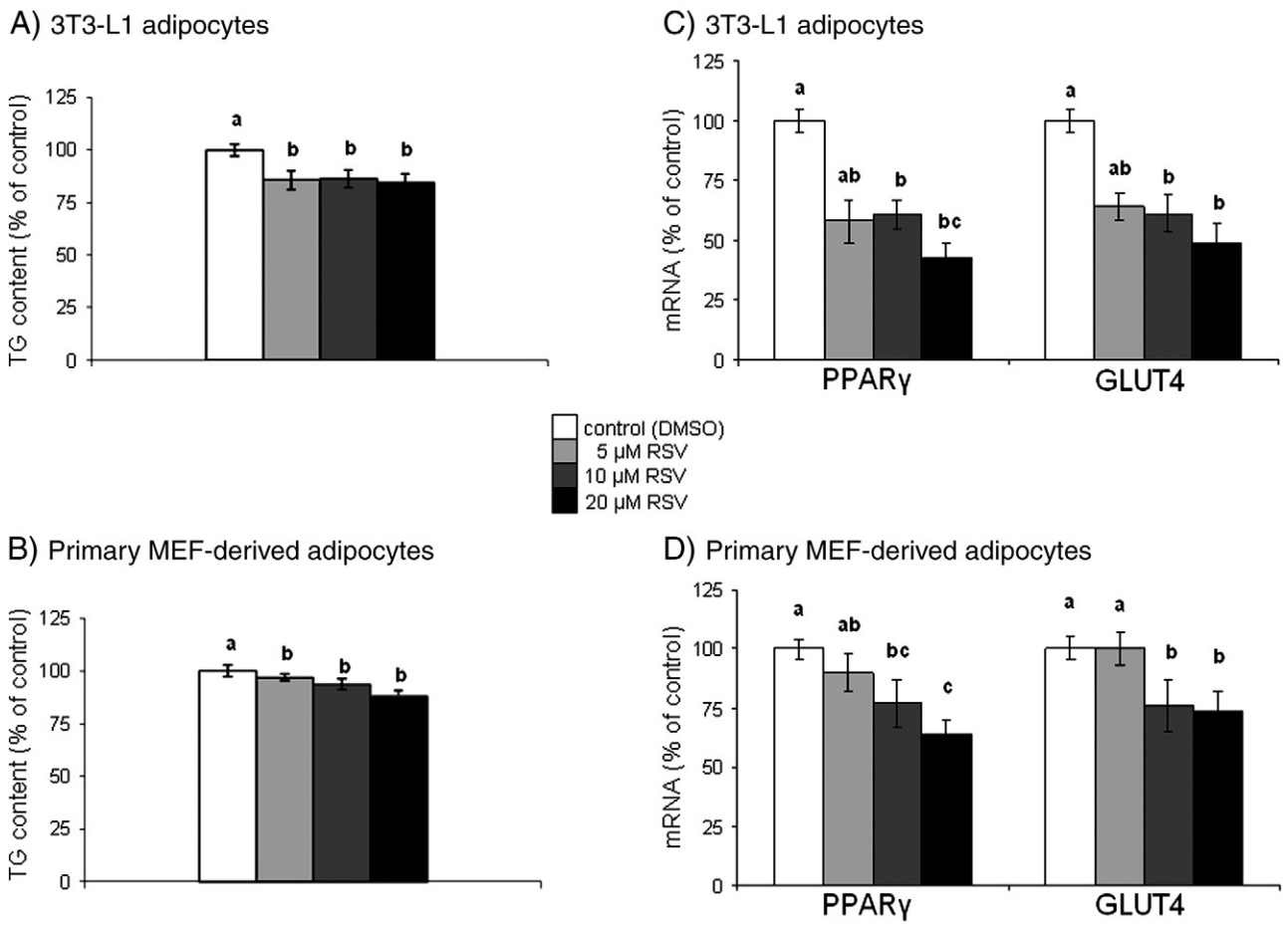

Fig. 2. Effects of resveratrol treatment on triacylglycerol content and PPARγ and GLUT4 expression levels in mature adipocytes. (A and B) 3T3-L1 adipocytes and primary MEF-derived adipocytes differentiated in 6-well plates were treated with vehicle (0.1% DMSO, control cells) or RSV at the indicated concentrations for 3 days with replacement of medium with fresh RSV every 24 h, following which cellular triacylglycerol (TG) content was determined. (C and D) 3T3-L1 adipocytes and primary MEF-derived adipocytes differentiated in 12-well plates were treated with vehicle or RSV at the indicated concentrations for 24 h before harvesting and total RNA extraction. Gene-expression was determined by real time PCR and was normalized to the expression of β-actin and LRP10. Data are expressed relative to the mean value of the vehicle-treated cells, which was set at 100%, and are the mean±SEM of $n=9$ (A, B) or $n=12$ (C, D) wells per treatment condition, distributed in at least three separate experiments (3 wells per treatment condition per experiment). Bar values not sharing a common letter are statistically different by LSD post hoc comparison following one-way ANOVA ($P<.05$).

24 h. For quantification of triacylglycerol content, cellular lipids were stained with Oil Red O (Sigma), followed by extraction of the dye with isopropanol and spectrophotometric quantification at 510 nm.

2.6. Statistics

Data are presented as mean±S.E.M. Statistical significance was assessed by two-tailed Student's *t* test or one-way analysis of variance (ANOVA) followed by least significance difference (LSD) post hoc comparison. Results were considered statistically significant when $P<.05$.

3. Results

3.1. Cytotoxicity of resveratrol in the adipocyte cell models used

Resveratrol treatment has been reported to decrease viability of mature 3T3-L1 adipocytes partly by increasing apoptosis [18,37,38]. We therefore analyzed the cytotoxicity of a 24 h treatment with varying resveratrol doses in our adipocyte working models by the lactate dehydrogenase method [39] (Fig. 1). At concentrations up to 20 μM, resveratrol did not elicit higher cytotoxicity than the vehicle (0.1% DMSO), whereas at 50 μM and 100 μM, it had a significant cytotoxic effect, which was especially evident in the primary MEF-derived adipocytes. In the remaining experiments, resveratrol was used at final concentrations ranging from 5 to 20 μM.

3.2. Resveratrol treatment reduces triacylglycerol content and PPARγ and glucose transporter 4 (GLUT4) mRNA expression levels in mature adipocytes

We examined whether previously reported effects of resveratrol treatment in fully differentiated adipocytes were reproduced in our working cell models as a mean of validating them. In accordance with previous findings in 3T3-L1 adipocytes [16], exposure to 5–20 μM resveratrol for 3 days triggered a modest, yet significant, reduction in intracellular triacylglycerol content both in mature 3T3-L1 adipocytes (15% reduction at 20 μM; $P<.05$) and primary MEF-derived adipocytes (12% reduction at 20 μM; $P<.05$) (Fig. 2A and B, respectively). Resveratrol treatment has been reported to inhibit PPARγ gene-expression and to increase proteasome-dependent PPARγ protein degradation in mature 3T3-L1 adipocytes [20]. In keeping, we found that exposure to resveratrol for 24 h led to decreased PPARγ mRNA levels both in mature 3T3-L1 adipocytes (39% reduction at 10 μM; $P<.001$) and in primary MEF-derived adipocytes (23% reduction at 10 μM; $P<.05$) (Fig. 2C and D). Inhibition of PPARγ [21,40] and resveratrol treatment [20] both have been shown to result in reduced GLUT4 expression in mature 3T3-L1 adipocytes. In keeping, we found a down-regulatory effect of resveratrol treatment on GLUT4 mRNA levels both in 3T3-L1 adipocytes (39% reduction at 10 μM; $P<.001$) and in primary MEF-derived adipocytes (24% reduction at 10 μM; $P<.05$) (Fig. 2C and D).

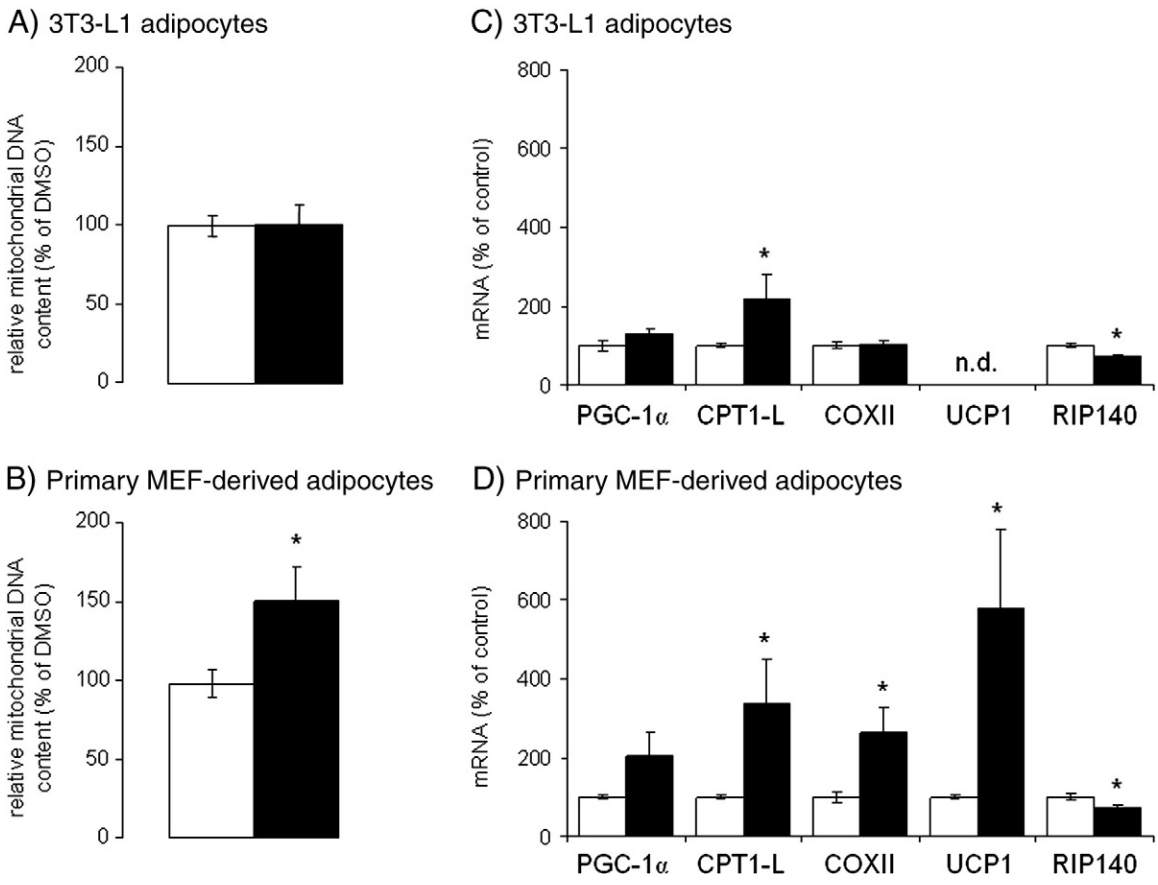

Fig. 3. Effects of resveratrol treatment on oxidative and thermogenic capacity in mature adipocytes. 3T3-L1 adipocytes and primary MEF-derived adipocytes differentiated in 12-well plates were treated with vehicle (0.1% DMSO, open bars) or 20 μM resveratrol (RSV, black bars) for 24 h before harvesting and total RNA and DNA extraction. The mitochondrial DNA to nuclear DNA ratio (A, B) was determined by a real time PCR method, as described in Methods. Gene-expression (C, D) was analyzed by real time PCR and was normalized to the expression of β-actin and LRP10. Data are expressed relative to the mean value of the vehicle-treated cells, which was set at 100%, and are the mean±S.E.M. of $n=9$ (A, B) or $n=12$ (C, D) wells per treatment condition, distributed in at least three separate experiments (3 wells per treatment condition per experiment). Student's *t* test significance: *$P<.05$ versus vehicle; n.d. (non-detectable), UCP1 mRNA levels in 3T3-L1 adipocytes were very low both in control and RSV-treated cells and could not be consistently measured using the analytical method applied.

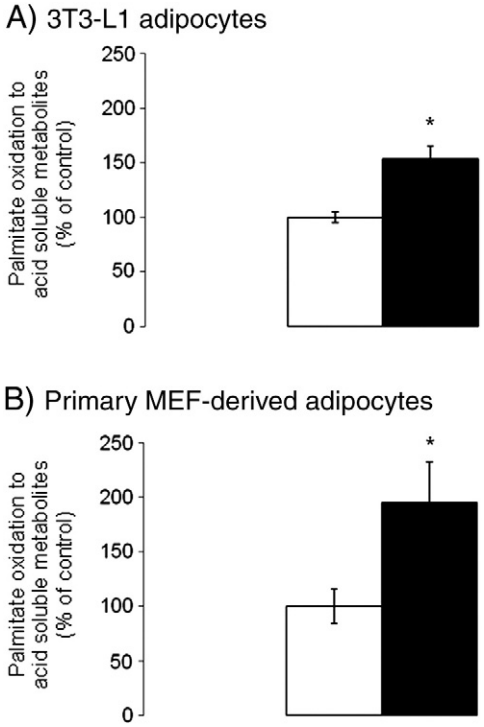

Fig. 4. Effect of resveratrol treatment on palmitate oxidation to acid soluble metabolites in mature adipocytes. 3T3-L1 adipocytes (A) and primary MEF-derived adipocytes (B) differentiated in 6-well plates were treated with vehicle (0.1% DMSO, open bars) or 20 μM resveratrol (RSV, black bars) for 20 h. The medium was then changed and the cells incubated with [^{14}C(U)] palmitate in the continued presence of vehicle or RSV for 4 h. ^{14}C-labeled acid soluble metabolites were measured as described in Methods. Data are expressed relative to the mean value of the vehicle-treated cells, which was set at 100%, and are the mean±S.E.M. of n=9 (A) or n=12 (B) wells per treatment condition, distributed in at least three separate experiments (three wells per treatment condition per experiment). Student's t test significance: *P<.05 versus vehicle.

3.3. Resveratrol treatment increases oxidative and thermogenic capacity in mature adipocytes

We next examined the effects of resveratrol on mitochondrial DNA content and the mRNA expression levels of several genes related to mitochondria biogenesis and function. In primary MEF-derived adipocytes, a 24 h treatment with 20 μM resveratrol resulted in a significant increment in mitochondrial DNA levels (50% increase) (Fig. 3B) and the mRNA levels of key genes related to oxidative metabolism and thermogenesis, namely: liver type carnitine palmitoyltransferase 1 (CPT1-L), which is necessary for mitochondrial import of fatty acids, the rate-limiting step in mitochondrial long-chain fatty acid oxidation (2.4-fold increase); subunit II of cytochrome oxidase (COX-II), a component of the mitochondrial respiratory chain (1.6-fold increase); and uncoupling protein 1 (UCP1), a mitochondrial uncoupler whose activity may drive enhanced substrate oxidation (4.8-fold increase) (Fig. 3D). PGC-1α mRNA levels were not significantly affected, whereas the mRNA levels of receptor-interacting protein 140 (RIP140) — a corepressor for many nuclear receptors that acts as a major suppressor of oxidative metabolism in white adipocytes [41,42] — were significantly reduced by 25% (Fig. 3D). In 3T3-L1 adipocytes, treatment with 20 μM resveratrol induced a significant 1.2-fold increase in CPT1-L mRNA levels and a significant 28% reduction in RIP140 mRNA levels, yet it did not affect the other parameters analyzed (Fig. 3A and C). Lower resveratrol concentrations tested (5 and 10 μM) did not significantly affect the expression of the oxidative genes assayed in either adipocyte cell model (results not shown). The production of acid soluble metabolites from exogenously administered, uniformly labeled ^{14}C-palmitate was enhanced by pre-exposure to 20 μM resveratrol, by 50% in the 3T3-L1 adipocytes and by 90% in the primary MEF-derived adipocytes (Fig. 4).

3.4. Resveratrol treatment reduces resistin and RBP4 expression in mature adipocytes

3T3-L1 adipocytes in the untreated state displayed a consistent expression of resistin mRNA yet very low or undetectable levels of RBP4 mRNA (this work and [31]). Treatment with 5 to 20 μM resveratrol reduced resistin mRNA levels in 3T3-L1 adipocytes (Fig. 5A) as well as the accumulation of resistin in the culture medium in contact with the cells (Fig. 5B). In primary MEF-derived adipocytes, expression of both resistin and RBP4 was consistently detectable in the untreated state (this work and [31]), and exposure to 5–20 μM resveratrol decreased the cellular mRNA levels of both adipokines (Fig. 5C), as well as their accumulation in the culture medium (Fig. 5D and E).

4. Discussion

The strong association between obesity and insulin resistance implicates the adipocyte as an important link in the pathophysiology of these two disorders. A dysregulated secretion of adipokines is a feature of adipose tissue in obesity and insulin resistant states (reviewed in Ref. [30]). In addition, even if fatty acid oxidation is not a major pathway in adipocytes, a number of studies have shown mitochondrial dysfunction and reduced expression of genes involved in fatty acid oxidation and thermogenesis in adipose tissue from obese and diabetic animal models and humans [43–47]. These and other results have led to the concept that increases in oxidative capacity in white adipocytes may represent a target for treatment of obesity and insulin resistance [48,49]. In cell and animal models, increases in oxidative metabolism in white adipocytes have been demonstrated following certain genetic manipulations, such as forced expression of PGC-1α [50] or inactivation of the retinoblastoma protein [51,52], and after exposure to certain bioactive food components, such as vitamin A derivatives and polyunsaturated fatty acids of marine origin [31,53–57]. In this report, we show that resveratrol directly acts on mature murine white adipocytes triggering changes in gene-expression and related biochemical parameters consistent with both reduced lipogenesis and enhanced substrate oxidation. We also present first evidence of an inhibitory effect of resveratrol on the adipose expression of resistin and RBP4, two adipokines that antagonize insulin action in mice and may also be related to insulin resistance and inflammation in humans [27–30].

In mature white adipocytes in culture, reduction of triacylglycerol content and promotion of fat mobilization following exposure to resveratrol has been reported and linked to PPARγ repression [16,20]. Our results are in line with these previous reports, yet they extend them by providing evidence suggesting that activation of oxidative metabolism can also contribute to the resveratrol-induced reduction in adipocyte lipid content. Thus, exposure of adipocytes to resveratrol oppositely affected the gene-expression levels of a recognized suppressor of oxidative metabolism (RIP 140) and a rate-limiting enzyme for mitochondrial fatty acid oxidation (CPT1-L), down-regulating the former and up-regulating the latter. In concord with these gene-expression changes, resveratrol-treated adipocytes displayed signs of increased flux through the fatty acid beta-oxidation pathway, and a reduced triacylglycerol content following long-term treatment. The significance of these results is reinforced by the fact that they were reproduced in two different adipocyte cell models, namely 3T3-L1 adipocytes and primary MEF-derived adipocytes. Furthermore, in the latter model, increases in mitochondrial DNA

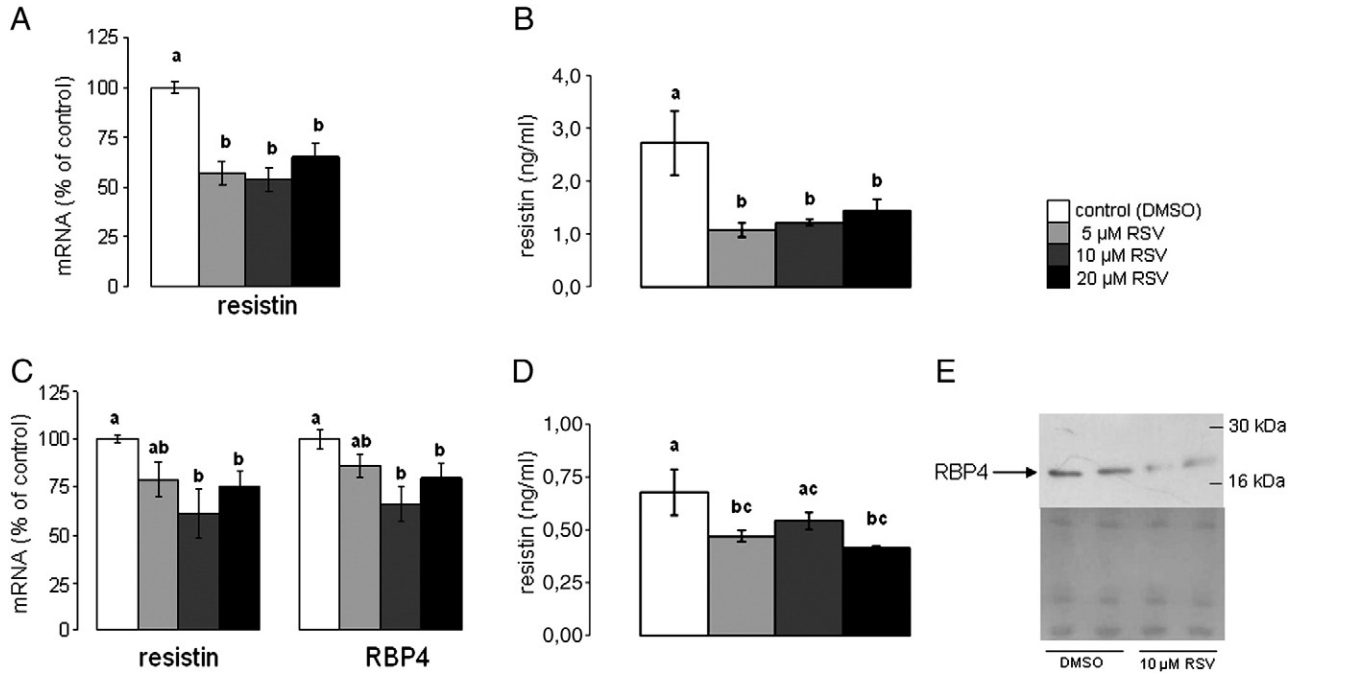

Fig. 5. Effect of resveratrol treatment on resistin and RBP4 expression in mature adipocytes. 3T3-L1 adipocytes (A and B) and primary MEF-derived adipocytes (C-E) were treated with vehicle (0.1% DMSO) or the indicated RSV concentrations along with fresh medium for 24 h before total cellular RNA extraction or culture medium collection. Resistin and RBP4 mRNA levels (A, C) were analyzed by real time PCR and normalized to the expression of β-actin and LRP10. mRNA data are expressed relative to the mean value of the vehicle-treated cells, which was set at 100%, and are the mean±S.E.M. of $n=12$ wells per treatment condition, distributed in four separate experiments (3 wells per treatment condition per experiment). RBP4 mRNA could not be consistently measured in 3T3-L1 adipocytes due to very low expression levels. Levels of resistin accumulated in the culture medium (B and D) were assessed using an ELISA kit, and the data are the mean±S.E.M. of $n=6$ wells per treatment condition, distributed in two separate experiments (three wells per treatment condition per experiment). Levels of RBP4 accumulated in the culture medium of primary MEF-derived adipocytes were assessed by immunoblotting (E); a representative immunoblot is shown, together with a section of the black amide B10-stained blot showing equal loading and blotting of proteins (25 μl of noncondensed culture medium per lane). The cells were differentiated in 12-well plates for analysis of effects on mRNA levels and in six-well plates for analysis of effects on protein accumulation in medium. Bar values not sharing a common letter are statistically different by LSD post hoc comparison following one-way ANOVA ($P<.05$).

content and COX-II and UCP1 mRNA expression levels were also apparent following resveratrol treatment. UCP1 is a brown adipocyte-specific molecular marker, yet its expression has been shown to be inducible in primary MEF-derived adipocytes, but not 3T3-L1 adipocytes [54], following certain treatments, in particular retinoid administration [32]. Enhanced mitochondrial function and fatty acid oxidation has been previously reported in skeletal muscle, BAT and liver of resveratrol-supplemented mice, yet effects on WAT metabolism were not addressed in those in vivo studies [3,4]. Our results might, however, be in line with a previous report pointing to increased expression of marker genes of oxidative metabolism in WAT of mice following in vivo treatment with a synthetic SIRT1 activator (SRT1729) [10].

Stimulatory effects of resveratrol treatment/SIRT1 activation on tissue fatty acid catabolism have been previously related to the activation of PGC-1α through its SIRT1-catalysed deacetylation [3,12] and the indirect activation of AMP-dependent protein kinase [10]. Our results are first to suggest that effects on RIP140 may also be involved. Interestingly, depletion of RIP140 has been shown to result in the up-regulation of CPT1 and UCP1 gene-expression, without changes in PGC-1α gene-expression, both in MEF-derived adipocytes [41] and in WAT of RIP140-knockout mice, which are lean and resistant to dietary obesity [58].

Resveratrol treatment or SIRT1 activation have been shown to improve insulin sensitivity in diet-induced and genetic animal models of obesity [3,4,6,10]. Our findings indicate inhibitory effects of resveratrol on the adipose expression of resistin and RBP4 that could contribute to a systemic insulin-sensitizing effect. In fact, effects on the secretory function of adipocytes — together with independent effects on other glucose responsive tissues such as skeletal muscle and the liver — might be particularly relevant for agents, such as resveratrol, capable of improving systemic insulin sensitivity coincident with decreased PPARγ expression and glucose uptake in adipocytes [20]. Reduction of resistin expression after resveratrol treatment might be secondary to resveratrol-induced PPARγ repression (this work and [16,20]), as in mice adipocyte-specific resistin gene-expression is under the control of PPARγ [59]. Of note, in the present work the effects of resveratrol down-regulating resistin and RBP4 expression in adipocytes were evidenced at lower doses than its effects on the expression of oxidative marker genes, mitochondrial DNA content and palmitate oxidation. This might be in line with findings in mice on high fat diets supplemented with resveratrol as, in those studies, reduced adiposity and enhanced whole body energy expenditure were evidenced following high resveratrol doses (400 mg/kg per day) [3], whereas an improvement in insulin sensitivity was already present at lower doses (22.4 mg/kg per day) that did not affect adiposity, body weight or body temperature of treated animals [4].

In summary, this study shows that resveratrol directly promotes a remodeling of mature white adipocytes towards increased capacity for oxidative metabolism and reduced lipogenesis, while down-regulating resistin and RBP4, two adipokines that have been implicated as insulin resistance factors. These results constitute novel insights into resveratrol action in adipocytes, and they add to the potential of this food phytochemical and its synthetic analogues in the control of obesity and related metabolic disorders.

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References

[1] Howitz KT, Bitterman KJ, Cohen HY, et al. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature 2003;425:191–6.
[2] Blander G, Guarente L. The Sir2 family of protein deacetylases. Annu Rev Biochem 2004;73:417–35.
[3] Lagouge M, Argmann C, Gerhart-Hines Z, et al. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell 2006;127:1109–22.
[4] Baur JA, Pearson KJ, Price NL, et al. Resveratrol improves health and survival of mice on a high-calorie diet. Nature 2006;444:337–42.
[5] Ahn J, Cho I, Kim S, Kwon D, Ha T. Dietary resveratrol alters lipid metabolism-related gene expression of mice on an atherogenic diet. J Hepatol 2008;49: 1019–28.
[6] Rivera L, Moron R, Zarzuelo A, Galisteo M. Long-term resveratrol administration reduces metabolic disturbances and lowers blood pressure in obese Zucker rats. Biochem Pharmacol 2009;77:1053–63.
[7] Bordone L, Cohen D, Robinson A, et al. SIRT1 transgenic mice show phenotypes resembling calorie restriction. Aging Cell 2007;6:759–67.
[8] Pfluger PT, Herranz D, Velasco-Miguel S, Serrano M, Tschop MH. Sirt1 protects against high-fat diet-induced metabolic damage. Proc Natl Acad Sci U S A 2008;105:9793–8.
[9] Milne JC, Lambert PD, Schenk S, et al. Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. Nature 2007;450:712–6.
[10] Feige JN, Lagouge M, Canto C, et al. Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation. Cell Metab 2008;8:347–58.
[11] Smith JJ, Kenney RD, Gagne DJ, et al. Small molecule activators of SIRT1 replicate signaling pathways triggered by calorie restriction in vivo. BMC Syst Biol 2009;3: 31.
[12] Gerhart-Hines Z, Rodgers JT, Bare O, et al. Metabolic control of muscle mitochondrial function and fatty acid oxidation through SIRT1/PGC-1alpha. Embo J 2007;26:1913–23.
[13] Puigserver P, Spiegelman BM. Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. Endocr Rev 2003;24:78–90.
[14] Pedersen SB, Olholm J, Paulsen SK, Bennetzen MF, Richelsen B. Low Sirt1 expression, which is upregulated by fasting, in human adipose tissue from obese women. Int J Obes (Lond) 2008;32:1250–5.
[15] Qiao L, Shao J. SIRT1 regulates adiponectin gene expression through Foxo1-C/enhancer-binding protein alpha transcriptional complex. J Biol Chem 2006;281: 39915–24.
[16] Picard F, Kurtev M, Chung N, et al. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature 2004;429:771–6.
[17] Bai L, Pang WJ, Yang YJ, Yang GS. Modulation of Sirt1 by resveratrol and nicotinamide alters proliferation and differentiation of pig preadipocytes. Mol Cell Biochem 2008;307:129–40.
[18] Rayalam S, Yang JY, Ambati S, Della-Fera MA, Baile CA. Resveratrol induces apoptosis and inhibits adipogenesis in 3T3-L1 adipocytes. Phytother Res 2008;22: 1367–71.
[19] Szkudelska K, Nogowski L, Szkudelski T. Resveratrol, a naturally occurring diphenolic compound, affects lipogenesis, lipolysis and the antilipolytic action of insulin in isolated rat adipocytes. J Steroid Biochem Mol Biol 2009;113:17–24.
[20] Floyd ZE, Wang ZQ, Kilroy G, Cefalu WT. Modulation of peroxisome proliferator-activated receptor gamma stability and transcriptional activity in adipocytes by resveratrol. Metabolism 2008;57:S32–8.
[21] Tamori Y, Masugi J, Nishino N, Kasuga M. Role of peroxisome proliferator-activated receptor-gamma in maintenance of the characteristics of mature 3T3-L1 adipocytes. Diabetes 2002;51:2045–55.
[22] Ahn J, Lee H, Kim S, Ha T. Resveratrol inhibits TNF-alpha-induced changes of adipokines in 3T3-L1 adipocytes. Biochem Biophys Res Commun 2007;364:972–7.
[23] Zhu J, Yong W, Wu X, et al. Anti-inflammatory effect of resveratrol on TNF-alpha-induced MCP-1 expression in adipocytes. Biochem Biophys Res Commun 2008;369:471–7.
[24] Gonzales AM, Orlando RA. Curcumin and resveratrol inhibit nuclear factor-kappaB-mediated cytokine expression in adipocytes. Nutr Metab (Lond) 2008;5: 17.
[25] Kennedy A, Overman A, Lapoint K, et al. Conjugated linoleic acid-mediated inflammation and insulin resistance in human adipocytes are attenuated by resveratrol. J Lipid Res 2009;50:225–32.
[26] Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest 2005;115:1111–9.
[27] Steppan CM, Bailey ST, Bhat S, et al. The hormone resistin links obesity to diabetes. Nature 2001;409:307–12.
[28] Yang Q, Graham TE, Mody N, et al. Serum retinol binding protein 4 contributes to insulin resistance in obesity and Type 2 diabetes. Nature 2005;436:356–62.
[29] Lazar MA. Resistin- and obesity-associated metabolic diseases. Horm Metab Res 2007;39:710–6.
[30] Rabe K, Lehrke M, Parhofer KG, Broedl UC. Adipokines and insulin resistance. Mol Med 2008;14:741–51.
[31] Mercader J, Granados N, Bonet ML, Palou A. All-trans retinoic acid decreases murine adipose retinol binding protein 4 production. Cell Physiol Biochem 2008;22:363–72.
[32] Mercader J, Palou A, Luisa Bonet M. Induction of uncoupling protein-1 in mouse embryonic fibroblast-derived adipocytes by retinoic acid. Obesity 2010;18: 655–62.
[33] Hansen JB, Jorgensen C, Petersen RK, et al. Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. Proc Natl Acad Sci U S A 2004;101:4112–7.
[34] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2[-Delta Delta C(T)] method. Methods 2001;25:402–8.
[35] Gabrielsson BG, Olofsson LE, Sjogren A, et al. Evaluation of reference genes for studies of gene expression in human adipose tissue. Obes Res 2005;13:649–52.
[36] Wu JJ, Roth RJ, Anderson EJ, et al. Mice lacking MAP kinase phosphatase-1 have enhanced MAP kinase activity and resistance to diet-induced obesity. Cell Metab 2006;4:61–73.
[37] Rayalam S, Della-Fera MA, Yang JY, Park HJ, Ambati S, Baile CA. Resveratrol potentiates genistein's antiadipogenic and proapoptotic effects in 3T3-L1 adipocytes. J Nutr 2007;137:2668–73.
[38] Yang JY, Della-Fera MA, Rayalam S, et al. Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. Life Sci 2008;82:1032–9.
[39] Decker T, Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. J Immunol Methods 1988;115:61–9.
[40] Liao W, Nguyen MT, Yoshizaki T, et al. Suppression of PPAR-gamma attenuates insulin-stimulated glucose uptake by affecting both GLUT1 and GLUT4 in 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab 2007;293:E219–27.
[41] Christian M, Kiskinis E, Debevec D, Leonardsson G, White R, Parker MG. RIP140-targeted repression of gene expression in adipocytes. Mol Cell Biol 2005;25: 9383–91.
[42] Powelka AM, Seth A, Virbasius JV, et al. Suppression of oxidative metabolism and mitochondrial biogenesis by the transcriptional corepressor RIP140 in mouse adipocytes. J Clin Invest 2006;116:125–36.
[43] Bottcher H, Furst P. Decreased white fat cell thermogenesis in obese individuals. Int J Obes Relat Metab Disord 1997;21:439–44.
[44] Oberkofler H, Dallinger G, Liu YM, Hell E, Krempler F, Patsch W. Uncoupling protein gene: quantification of expression levels in adipose tissues of obese and non-obese humans. J Lipid Res 1997;38:2125–33.
[45] Hammarstedt A, Jansson PA, Wesslau C, Yang X, Smith U. Reduced expression of PGC-1 and insulin-signaling molecules in adipose tissue is associated with insulin resistance. Biochem Biophys Res Commun 2003;301:578–82.
[46] Semple RK, Crowley VC, Sewter CP, et al. Expression of the thermogenic nuclear hormone receptor coactivator PGC-1alpha is reduced in the adipose tissue of morbidly obese subjects. Int J Obes Relat Metab Disord 2004;28:176–9.
[47] Choo HJ, Kim JH, Kwon OB, et al. Mitochondria are impaired in the adipocytes of type 2 diabetic mice. Diabetologia 2006;49:784–91.
[48] Maassen JA, Romijn JA, Heine RJ. Fatty acid-induced mitochondrial uncoupling in adipocytes as a key protective factor against insulin resistance and beta cell dysfunction: a new concept in the pathogenesis of obesity-associated Type 2 diabetes mellitus. Diabetologia 2007;50:2036–41.
[49] Frayn KN, Langin D, Karpe F. Fatty acid-induced mitochondrial uncoupling in adipocytes is not a promising target for treatment of insulin resistance unless adipocyte oxidative capacity is increased. Diabetologia 2008;51:394–7.
[50] Tiraby C, Tavernier G, Lefort C, et al. Acquirement of brown fat cell features by human white adipocytes. J Biol Chem 2003;278:33370–6.
[51] Dali-Youcef N, Mataki C, Coste A, et al. Adipose tissue-specific inactivation of the retinoblastoma protein protects against diabesity because of increased energy expenditure. Proc Natl Acad Sci U S A 2007;104:10703–8.
[52] Mercader J, Ribot J, Murano I, et al. Haploinsufficiency of the retinoblastoma protein gene reduces diet-induced obesity, insulin resistance, and hepatosteatosis in mice. Am J Physiol Endocrinol Metab 2009;297:E184–93.
[53] Felipe F, Bonet ML, Ribot J, Palou A. Modulation of resistin expression by retinoic acid and vitamin A status. Diabetes 2004;53:882–9.
[54] Mercader J, Madsen L, Felipe F, Palou A, Kristiansen K, Bonet ML. All-trans retinoic acid increases oxidative metabolism in mature adipocytes. Cell Physiol Biochem 2007;20:1061–72.
[55] Mercader J, Ribot J, Murano I, et al. Remodeling of white adipose tissue after retinoic acid administration in mice. Endocrinology 2006;147:5325–32.
[56] Flachs P, Horakova O, Brauner P, et al. Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce beta-oxidation in white fat. Diabetologia 2005;48:2365–75.
[57] Maeda H, Tsukui T, Sashima T, Hosokawa M, Miyashita K. Seaweed carotenoid, fucoxanthin, as a multi-functional nutrient. Asia Pac J Clin Nutr 2008;17(Suppl 1): 196–9.
[58] Leonardsson G, Steel JH, Christian M, et al. Nuclear receptor corepressor RIP140 regulates fat accumulation. Proc Natl Acad Sci U S A 2004;101:8437–42.
[59] Tomaru T, Steger DJ, Lefterova MI, Schupp M, Lazar MA. Adipocyte-specific expression of murine resistin is mediated by synergism between peroxisome proliferator-activated receptor gamma and CCAAT/enhancer-binding proteins. J Biol Chem 2009;284:6116–25.