OBES SURG (2011) 21:356–361
DOI 10.1007/s11695-010-0251-7

BASIC SCIENCE RESEARCH

Resveratrol Upregulated SIRT1, FOXO1, and Adiponectin and Downregulated PPARγ1–3 mRNA Expression in Human Visceral Adipocytes

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Published online: 26 September 2010

Abstract
Background The SIRT1 enzyme is involved in adipose tissue (AT) lipolysis. FOXO1 is a protein that plays a significant role in regulating metabolism. Adiponectin is an adipokine, secreted by the AT, which has been considered to have an antiobesity function. PPARγ is one of the key actors in adipocytes differentiation. This study was undertaken to investigate whether resveratrol can regulate SIRT1, FOXO1, adiponectin, PPARγ1–3, and PPARβ/δ in human AT.
Methods The effects of resveratrol were analyzed in freshly isolated adipocytes prepared from visceral fat tissue samples obtained during bariatric surgery. Genes messenger ribonucleic acid (mRNA) levels were determined by qRT-PCR.
Results Ours results show that resveratrol modulates the studied genes, increasing SIRT1 (p=0.021), FOXO1 (p=0.001), and adiponectin (p=0.025) mRNA expression and decreasing PPARγ1–3 (p=0.003) mRNA in human visceral adipocytes.
Conclusions Resveratrol, *in vitro* and at low concentration, modulates genes that are related to lipid metabolism, possibly preventing metabolic disease in human visceral adipose tissue (VAT).

Keywords Adipocytes · SIRT1 · FOXO1 · Adiponectin · PPARγ1–3 · Resveratrol

Abbreviations

AT	Adipose tissue
VAT	Visceral adipose tissue
FOXO1	Forkhead/winged helix
SIRT1	Sirtuin 1
PPAR	Peroxisome proliferator-activated receptors
qRT-PCR	Quantitative real-time polymerase chain reaction

Introduction

Obesity is related to the metabolic syndrome, and visceral adipose tissue (VAT) has been proposed to mediate this relationship [1]. The study of adipose tissue (AT), particularly, adipocytes, is central to the understanding of metabolic abnormalities associated with the development of weight gain [2]. Adipocytes are involved in energy balance regulation by endocrine and nonendocrine mechanisms, which can be controlled by various hormones, cytokines, and nutrients [2]. During the last couple of years, several studies have used *in vitro* and *in vivo* systems to focus on various biological effects of resveratrol (3,5,4-trihydroxystilbene) [3]. Some of these results indicate that resveratrol mediated effects that are consistent with metabolic syndrome prevention in obese mice and 3T3-L1 adipocytes [4–6].

Sirtuin 1 (SIRT1) is one of the seven mammalian homologs of Sir2 family that catalyzes NAD^+-dependent protein

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deacetylation [7]. Scientists have been proposing that SIRT1 might be a target protein to prevent and control obesity and related diseases [2, 8]. In yeast, resveratrol significantly increases SIRT1 activity through an allosteric interaction, resulting in the increase of SIRT1 affinity for both NAD^+ and the acetylated substrate [9]. In mice on a high-calorie diet, resveratrol produces SIRT1-dependent effects that are consistent with improved cellular function and organism health [6].

Forkhead/winged helix1 (FOXO1) is a transcription factor found to inhibit adipogenesis [10]. Subauste and Burant [11] found that treatment with resveratrol was able to increase FOXO1 levels in 3T3-L1 adipocytes. Moreover, a recent study of Wang and Tong [10] shows that FOXO1 can function as a transrepressor of PPARγ in white AT through a direct protein–protein interaction.

Adiponectin is an adipocyte-derived protein that has antiobesity, antidiabetic and anti-inflammatory properties [12]. Rogers et al. [13] recently found that resveratrol treatment of ethanol-fed mice markedly increased serum adiponectin concentrations with prevention of hepatic fat accumulation. SIRT1 and FOXO1 are apparently involved in transcriptional regulation of adiponectin in mice AT [13].

The nuclear peroxisome proliferator-activated receptor (PPAR) family has been intensively study in the past several years [14]. PPAR family comprises three isotypes: PPARα, PPARγ, and PPARβ/δ [15]. The nuclear hormone receptor PPARγ is a central regulator of adipogenesis and plays a dominant role in fat tissue development [15]. As studied by Floyd et al. [5], resveratrol negatively modulates PPARγ protein levels in 3T3-L1 adipocytes. It has been reported that SIRT1 represses PPARγ in white AT by docking with its cofactors nuclear receptor co-repressor (NCoR) [8]. No studies have been done about resveratrol modulation on PPARβ/δ in human adipocytes.

To date, there are few reports linking dietary compounds to human adipocytes' genes expression in the literature. On the other hand, there are lots of papers citing VAT playing an important part in the metabolic syndrome [16]. Therefore, this article will focus of the modulation by resveratrol on SIRT1, FOXO1, adiponectin, PPARγ1–3, and PPARβ/δ relative messenger ribonucleic acid (mRNA) expression in isolated visceral adipocytes of morbidly obese patients. The results of this study will provide important information regarding visceral adipocytes and obesity modulation by resveratrol, as well as dietetic medical research.

Materials and Methods

Preparation of Human Isolated Adipocytes

VAT samples were obtained from morbidly obese patients (BMI≥40 kg/m^2) who underwent open Roux-en-Y gastric bypass (RYGBP) at Centro de Obesidade Mórbida of Hospital São Lucas of Pontifícia Universidade Católica do Rio Grande do Sul (COM/PUCRS, Brazil). The extensive clinical and laboratory data routinely collected for each patient is shown in Table 1. Patient's weights were stable for at least 1 year. No subjects were taking any medications affecting adipocyte metabolism. The study was approved by the Ethics Committee of Universidade Federal do Rio Grande do Sul (no. 2007/936). All subjects were informed about the aim of the study and signed the informed consent form.

Sample of VAT (10 g) was immediately immersed in Hanks' medium kept at 4°C and brought to the laboratory to start cell preparation within 15 min after tissue sampling. Adipocytes were isolated by collagenase digestion (0.5 mg/ml, type II; Sigma) at 37°C during 55 min, in Hank's medium buffered with 20 mmol/l HEPES (Sigma) and supplemented with 200 nmol/l adenosine (Sigma) and 4% (wt./vol.) fatty acid free bovine serum albumin (Sigma). Cells were filtered through a 500-μm mesh nylon filter and washed three times in Dulbecco's modified Eagle's medium (DMEM) containing 4% fatty acid free bovine serum albumin [17, 18].

Adipocytes Incubation

Isolated adipocytes were incubated at 37°C under 5% CO_2 in DMEM containing 4% fatty acid free bovine serum albumin in a final volume of 1 ml with resveratrol (Sigma) at 1 μM (resveratrol group, $n=10$) or without resveratrol (control group, $n=13$) for 4 h [17, 18]. To determine the culture time and concentration, we first treated adipocytes for different times (4, 5, and 6 h) with resveratrol at different concentrations (1, 10, and 30 μM). We found no difference in SIRT1 mRNA expression between the tested concentrations and times. Thus, 1 μM was used to modulate isolated adipocytes to achieve resveratrol modu-

Table 1 Anthropometrics and biologic parameters of morbidly obese patients (mean±SE)

	Mean (±SE)
BMI (kg/m^2)	46.78 (±2.06)
Waist (cm)	134.75 (±5.08)
Hip (cm)	146.0 (±4.57)
Age (year)	41.75 (±2.80)
ALT (U/l)	24.50 (±3.12)
AST (U/l)	30.25 (±4.46)
Triglycerides (mg/dl)	151.0 (±17.11)
Total cholesterol (mg/dl)	181.0 (±9.61)
HDL-C (mg/dl)	45.0 (±0.89)
LDL-C (mg/dl)	102.75 (±10.52)
HOMA-IR	10.75 (±2.06)

Table 2 Oligonucleotides used in qRT-PCR

B2M	Forward 5′-TGCTGTCTCCATGTTTGATGTATCT-3
	Reverse 5′-TCTCTGCTCCCCACCTCTAAGT-3′
SIRT1	Forward 5′-GAGTGGCAAAGGAGCAGA-3′
	Reverse 5′-TCTGGCATGTCCCACTATC-3′
FOXO1	Forward 5′-TGGACATGCTCAGCAGACATC-3′
	Reverse 5′-TTGGGTCAGGCGGTTCA-3′
Adiponectin	Forward 5′-TGGTGAGAAGGGTGAGAA-3′
	Reverse 5′-AGATCTTGGTAAAGCGAATG-3′
PPARγ1–3	Forward 5′-AGGCCATTTTCTCAAAC-3′
	Reverse 5′-AGAAATGCTGGAGAAGTCAACA-3′
PPAR β/δ	Forward 5′-AATGCCTACCTGAAAAACTTCAAC-3′
	Reverse 5′-GTGCACGCTGATTCCTTGT-3′

lation with minimal concentration, because of its low systemic bioavailability in humans [19]. Resveratrol stock solutions (1 mM) were prepared in absolute ethanol and stored at −20°C in sterilized eppendorfs, away from light. Dilutions were held at 37°C for at least 1 h before addition to the cells. The average cell number in the incubation medium varied from 50,000–150,000 cells/ml. At the end of the incubation periods, the infranatant was removed by aspiration, and cells were immediately immersed in Trizol reagent for total RNA extraction.

Analysis of mRNA Expression

Approximately 2 μg of total RNA obtained from Trizol were added to each cDNA synthesis reaction using the M-MLV reverse transcriptase (Invitrogen). Reactions were performed at 42°C for 1 h using the primer T23V (5′ TTT TTT TTT TTT TTT TTT TTT TTV 3′). Quantitative real-time polymerase chain reaction (qRT-PCRs) amplification was carried out using specific primer pairs designed with Primer 3 calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html) and synthesized by Prodimol (São Paulo, Brazil). The sequences of the primers used are listed in Table 2. Applied Biosystem 7500 real-time cycler was used to carry out the qPCRs. Reaction settings comprised of an initial denaturation step of 5 min at 95°C, followed by 40 cycles of 15 s at 95°C, 10 s at 60°C, 15 s at 72°C, and 35 s at 60°C; samples were held for 15 s at 95°C for annealing and then heated for 1 min at 60°C with a ramp of 0.1°C/s to acquire data to produce the denaturing curve of the amplified products. qRT-PCRs were done on a final volume of 20 μL, composed of 10 μL of each reverse transcription sample diluted 40–100 times, 2 μL 10 times PCR buffer, 1.2 μL of 50 mM $MgCl_2$, 0.1 μL of 5 mM dNTPs, 0.4 μL of 10 μM primer pairs, 4.25 μL of water, 2.0 μL of SYBR green (1:10.000 Molecular Probe), and 0.05 μL of Platinum Taq DNA polymerase (5 U/μL; Invitrogen).

Data Analyses

The gene expression was quantified using the $2^{-\Delta\Delta Ct}$ (threshold cycle) method [20]. For each well, analyzed in quadruplicate, a ΔC_T value was obtained by subtracting the beta-2-microglobulin (B2M) C_T value from the C_T value of the interest gene. The ΔC_T mean value obtained from the control group of each gene was used to calculate the $\Delta\Delta Ct$ of the respective gene ($2^{-\Delta\Delta Ct}$).

Statistics

Results were analyzed with the parametric independent sample *T* test. Data are shown as mean±standard error and they were calculated using the SPSS program (version 15.0). Differences were considered statistically significant at $p \leq 0.05$.

Results

The adipocytes were isolated from obese patients with similar biochemical and anthropometric parameters. The ability of 1 μM resveratrol to modulate SIRT1, FOXO1, adiponectin, PPARγ1–3, and PPARβ/δ mRNA expression in isolated visceral adipocytes was investigated. The expression of mRNA genes in visceral adipocytes of morbidly obese subjects was assessed by qRT-PCR (Fig. 1). Resveratrol significantly modulated the SIRT1 mRNA pattern in isolated visceral adipocytes ($p=0.021$). The relative amounts of SIRT1 mRNA were higher in

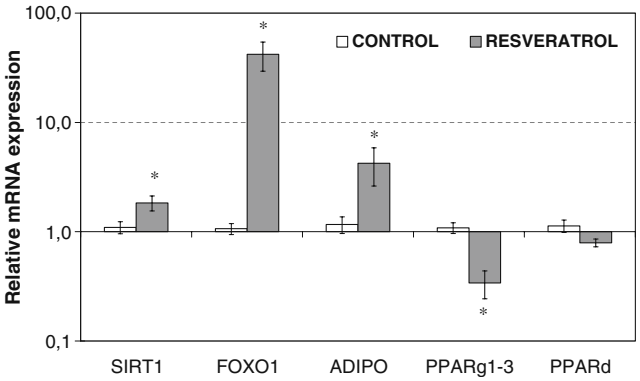

Fig. 1 Effects of 1 μM resveratrol on SIRT1, FOXO1, adiponectin, PPARγ1–3, and PPARβ/δ mRNA expression on isolated human visceral adipocytes. The parametric independent sample *T* test was used to compare the expression pattern of SIRT1, FOXO1, adiponectin, PPARγ1–3, and PPARβ/δ mRNA in treated isolated human visceral adipocytes after 4 h of incubation with 1 μM of resveratrol. *Open boxes* indicate the control values. The values are presented as logarithmic mean±standard error. Relative expressions were normalized against B2M (ΔCt) and calibrated to the mean value of control cells in each gene ($2^{-\Delta\Delta Ct}$). * represents a statistically significant difference between treated and control cells ($p \leq 0.05$)

resveratrol-treated cells (1.84±0.29) compared to the control (1.10±0.14). In addition, results showed that FOXO1 mRNA expression was statistically increased in adipocytes treated with 1 μM resveratrol (p=0.001). Amounts of FOXO1 were higher in treated (41.9±12.5) compared to control (1.07±0.12) cells. Moreover, resveratrol-treated cells showed significantly increased adiponectin mRNA expression (4.24±1.62) compared to control (1.17±0.20, p=0.025). Considering PPARγ1–3, resveratrol significantly downmodulates the mRNA expression (p=0.003). Resveratrol decreased PPARγ1–3 quantities in treated visceral adipocyte cells (0.34±0.01) compared with the control (1.08±0.12). Finally, resveratrol has no effect on adipocytes PPARβ/δ mRNA expression.

Discussion

Adipose tissue accumulation, especially VAT, is closely associated with metabolic syndrome [16]. Adipocytes can be controlled under the influence of various nutrients [2]. Resveratrol has been identified as a potent modulator of adipocytes metabolism [8]. There is little information related to resveratrol modulation on human visceral adipocytes. This study aimed at verifying the ability of resveratrol to modulate SIRT1, FOXO1, adiponectin, PPARγ1–3, and PPARβ/δ mRNA expression on human isolated visceral adipocyte cells. Although resveratrol is absorbed efficiently by humans, it has a very low systemic bioavailability [19]. We found that resveratrol, in vitro and in low concentrations (1 μM), could be sufficient to alter genes expression in human visceral adipocytes culture, increasing SIRT1, FOXO1, and adiponectin mRNA quantities and decreasing PPARγ1–3 mRNA amounts. Resveratrol did not modulate PPARβ/δ mRNA in human adipocytes at this concentration.

SIRT1 is a NAD^+-dependent enzyme that is involved in a variety of biological processes such as fatty acids mobilization in AT, stimulus of mitochondrial biogenesis, and prevention of metabolic syndrome [21, 22]. Recently, the authors of the present study found that decreased SIRT1 mRNA expression in VAT of morbidly obese patients could be involved with the worsening of nonalcoholic fat liver disease [23]. Resveratrol is one of the natural compounds that are able to activate SIRT1 [16]. Shan et al. [21] found that when porcine adipocyte cells are exposed to resveratrol, SIRT1 mRNA levels were increased. In agreement, another study found that resveratrol significantly increased SIRT1 mRNA expression in pig adipocytes [2]. The present study is the first done on resveratrol modulation on SIRT1 mRNA expression in human adipocytes. The present results of qRT-PRC show that 1 μM resveratrol increased SIRT1 mRNA expression in human visceral adipocyte cells. Picard et al. [8] have shown that resveratrol reduces blood TG content, stimulates free fatty acid release, and inhibits adipocyte differentiation and fat accumulation by activation of SIRT1 in 3T3-L1 adipocytes. Moreover, Lagouge et al. [7] found that treating mice with resveratrol, through increasing SIRT1 activity, significantly increases mitochondrial activity in brown AT. Mitochondria malfunction, especially in VAT, is central to the development of metabolic syndrome [24]. We suggest that resveratrol SIRT1 stimulation can possibly act on human visceral lipid metabolism, improving adipocytes activity.

Forkhead proteins and FOXO1, in particular, play a significant role in regulating whole body energy metabolism [25]. FOXO1, depending on tissue localization, may have protective or negative effects on insulin resistance, diabetes, and vascular function [26]. In adipose tissue, it seems that FOXO1 improves glucose tolerance and insulin sensitivity [26]. The present results show that resveratrol upregulated FOXO1 mRNA expression in mature adipocytes. Bai et al. [2] found that resveratrol significantly decreased the expression of FOXO1 mRNA in pig preadipocytes. However, Subauste and Burant [11] found that treatment with resveratrol was able to increase FOXO1 protein levels, decreasing the generation of reactive oxygen species (ROS) and reversing the changes associated with fatty acid overloading, in 3T3-L1 adipocytes. ROS production has been established as an essential contributor in the pathogenesis of obesity-associated insulin resistance [11]. Furthermore, Qiao and Shao [27] recently suggest that SIRT1 deacetylates FOXO1, promoting its transcriptional activity. Then, it is possible that resveratrol can be a health nutrient for human visceral adipocytes, since it induces SIRT1 and FOXO1 activation.

Adiponectin is an adipocyte-derived protein that has antiobesity, antidiabetic, and anti-inflammatory properties [12]. Resveratrol treatment increased adiponectin serum concentration, improved dyslipidemia, hyperinsulinemia, and hypertension, and produced anti-inflammatory effects in VAT of obese Zucker rats [4]. Rogers et al. [13] found that resveratrol treatment of ethanol-fed mice markedly increased serum adiponectin concentrations with prevention of hepatic fat accumulation. The authors observed that the adiponectin upregulation was associated with increased mRNA levels of SIRT1 and FOXO1 in mice adipose tissue. Furthermore, Qiao and Shao [27] suggest that SIRT1 increases adiponectin transcription by activating FOXO1 in 3T3-L1 cells. Once adiponectin is considered to be a protective cytokine, one can conclude that resveratrol, through adiponectin upregulation, possibly has a protective role in human visceral adipocytes metabolism.

Wang et al. [28] described that overexpression of PPARβ/δ in AT displays an upregulation of genes involved in fatty acid oxidation and energy dissipation. A study

published by our group suggests that a probable imbalance between PPARβ/δ (involved in fatty acid oxidation) and PPARγ1–3 (related to adipogenesis) expression can regulate adipocytes' development in obesity [29]. In the present study, results show that resveratrol significantly decreases PPARγ1–3 but does not modulate PPARβ/δ mRNA expression in human visceral adipocytes. Nothing is known about resveratrol modulation of PPARγ1–3 and PPARβ/δ mRNA expression in human VAT. In murine 3T3-L1 preadipocytes, resveratrol functions as a nutrition inhibitor of PPARγ activity expression [5]. Rayalam et al. [30] found that resveratrol downregulated PPARγ in mice 3T3-L1 cell. Resveratrol was shown to decrease adipogenesis in pig primary preadipocytes [31]. Moreover, Picard et al. [8] had shown that SIRT1 promotes fat mobilization by repressing PPARγ1–3 in mice adipocytes. Also, FOXO1 represses PPARγ gene expression in primary adipocytes and increases insulin sensitivity [25]. The results of the present study suggest that resveratrol is probably an important nutrient related to the control of obesity in visceral human AT, since it can modulate SIRT1, FOXO1, and PPARγ1–3.

In the present study, we evaluated the modulation of gene expression by resveratrol, but message levels does not necessarily equate to protein synthesize. Therefore, further studies are necessary to reveal the post-transcriptional control.

In summary, the present results show that resveratrol positively modulates SIRT1, adiponectin, and FOXO1 and decreases PPARγ1–3 mRNA expression in isolated visceral adipocytes. It is possible that resveratrol modulation genes' pathways are interconnecting. More researches are necessary to better understand this relationship and to provide data for using this nutrient as future treatment in human obesity and metabolic syndrome.

Acknowledgments This study was supported by research grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Fundação de Apoio à Pesquisa do Rio Grande do Sul (FAPERGS). Dr. R. Margis is a recipient of a research fellowship from the CNPq.

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