OBESITY has become an ongoing health concern not only in industrialized but also in some developing countries in these past few decades. Many clinical studies have disclosed that obesity dramatically contributes to various chronic diseases including atherosclerosis, hypertension, metabolic syndrome, stroke, and specific forms of cancer [1-4]. Obesity is characterized by adipogenesis and lipogenesis. Adipogenesis is known as the hyperplastic transformation from undifferentiated pre-adipocytes to mature adipocytes and, lipogenesis, the conversion and accumulation of lipid droplets in adipocytes from free fatty acids in bloodstream. Emerging evidence has indicated that uncontrolled adipogenesis and lipogenesis lead to obesity and may provide a potential target in prevention and treatment of obesity-related disorders [5]. Adipogenesis and lipogenesis are complex interconnections taking place among the differentiation and proliferation of pre-adipocytes and synthesis of fatty acids [6]. The adipogenic and lipogenic capacities of adipose tissue depend on a group of transcription factors [7, 8]. Peroxisome proliferator-activated receptor gamma (PPARγ), one of the regulators in lipid and glucose homeostasis, is also involved in regulating adipocyte-specific protein expressions. Previous investigations have demonstrated both expression and transactivation of PPARγ were associated with development of insulin resistance and ultimately obesity [9-11]. In addition, obesity was closely associated with inflammatory stress. It was reported that obesity increased...
infiltration of macrophages in adipose tissue, releasing numerous pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), and interleukins, which consequently contributed to progressive exacerbation of insulin resistance and eventually, metabolic syndrome [12, 13].

Resveratrol (3,4,5-trihydroxystilbene), a natural bioactive phytochemical mainly found in grapes and berries, has been previously identified as a potent anti-oxidation [14-16] and anti-inflammation [17, 18] agent. It has been demonstrated that resveratrol possesses broad bioactivities in prevention of cardiovascular disease [19-22], cancer progression [23-25], neurodegenerative disorders [26, 27], and metabolic syndrome [28-30]. Recently, growing interest in resveratrol has been targeted on its potential effects against obesity and other complicated disorders [31]. It was also indicated that resveratrol down-regulated PPARγ expression in attenuation of obesity [32, 33]. The detailed mechanism of resveratrol, nevertheless, needs to be further elucidated in terms of the composition of various cellular activities and effects exerted against obesity. To clarify the anti-obesity effects of resveratrol, we evaluated its morphological and biochemical profiles using high fat diet-fed mice with different dosage of resveratrol supplement. In vitro study was also conducted to evaluate the dosage effects of resveratrol on cytotoxicity, proliferation, differentiation, and lipolysis in 3T3-L1 cells. The present study was aimed to provide a more generalized and deepening understanding with respect to the attenuation of obesity by resveratrol treatment.

Materials and Methods

Animal experiments

Male C57BL/6C mice (10 wk old, 23-25 g in body weight) were purchased from BioLASCO Taiwan Co., Ltd (Taiwan, ROC). All animals were housed individually in an environmentally-controlled room of 12h/12h light-dark cycle and provided with ad libitum access to food and water. All experimental procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Chang Gung University at Taiwan. The mice were randomly divided into five groups: mice on chow diet (Laboratory Autoclavable Rodent Diet 5010; 13.5% of calories from fat) served as normal control group (CON), mice fed with high-fat diet (Research Diet 12451; 45.3% of calories from fat) treated with vehicle (0.9% NaCl) for 10 weeks (HFD), mice on HFD treated with resveratrol 1 mg/kg (HFR1), 10 mg/kg (HFR10), and 30 mg/kg (HFR30) for 10 wk. Resveratrol was dissolved in DMSO and diluted by 0.9% NaCl then was administered as oral gavage daily. All mice were fasted overnight (8 hr) and were sacrificed at chow or HFD feeding for 10 wk after being anesthetized by chloral hydrate at a dosage of 448 mg/kg, and blood plasma, adipose tissues, heart, and liver were collected and stored at -80°C for further examination.

Serum biochemical analysis

Blood samples were obtained by retro-orbital bleeding under anesthesia. Glucose levels were determined upon blood collection using SureStep Glucometer Kit (LifeScan Inc.). Separation of serum was performed by centrifugation at 9,300 × g for 10 min at 4°C and stored at -80°C before analysis. Serum insulin concentration was measured using an enzyme-linked immunosorbent assay (ELISA) commercial kit (Mercodia, Uppsala, Sweden). Serum cholesterol, triglyceride, and non-esterified fatty acid levels were determined by colorimetric assay commercial kits (Randox, Crumlin, UK), respectively. All examinations were performed in accordance with the manufacturer’s protocols.

Histological examinations

Subcutaneous, epididymal and brown adipose tissues were dissected and fixed in 4% paraformaldehyde and subsequently embedded in paraffin. Paraffin sections were trimmed at a thickness of 10 μm and stained with hematoxylin and eosin (H&E) for morphologic evaluation. The 3T3-L1 adipocyte was fixed in 4% paraformaldehyde and subjected to the Oil-Red-O staining for lipid content assessment. Images were observed and photographed in randomly chosen microscopic fields.

Culture and differentiation of 3T3-L1 cells

3T3-L1 pre-adipocytes were incubated in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% of fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO₂. While 3T3-L1 cells were grown to confluence, cells were stimulated by differentiation medium consisting of 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 1 μM dexamethasone (DEX) and incubation for 8 days and then insulin (10 μg/mL) was added at the last 2 days, at which time
more than 90% of cells were mature adipocytes with accumulated fat droplets (confirmed by Oil-Red-O staining). For evaluation of anti-proliferative and anti-differentiated effects of resveratrol, 3T3-L1 cells were cultured in 96-well plates and treated with varied concentrations of resveratrol (0.03 to 100 µM) for 7 and 8 days, respectively.

**Cell viability assay**

Viability of 3T3-L1 cells with or without resveratrol treatment was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO) method. Briefly, each cell culture was added with 50 µL of 5 mg/mL MTT in phosphate-buffered saline (PBS) solution and maintained for 2 hr at 37°C in a humidified atmosphere with 5% CO₂. After incubation, dimethyl sulfoxide (DMSO) was added to each well. Absorbance was detected by chemical well spectrophotometry at 570 nm (Epoch, Bio-Tek Co., Winooski, VT) for evaluation of cell viability.

**Lipolysis assay**

3T3-L1 pre-adipocytes were seeded in 24-well plates and incubated in the differentiation medium for 14 days to develop into mature adipocytes as described above. Mature adipocytes treated with varied concentrations of resveratrol (0.03 to 100 µM) in combination with or without recombinant mouse TNFα (R&D Systems, Inc., Minneapolis, MN) and incubated for 24 hr. The incubation medium was collected and the contents of glycerol release from 3T3-L1 cells were determined using Free Glycerol Reagent kit (Sigma-Aldrich Chemical Co) based on the manufacturer’s instructions.

**Western blot analysis**

Cells were collected and extracted in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 50 mM glycero-phosphate, 2 mM ethylenediaminetetraacetic acid (EDTA), 20 mM sodium fluoride (NaF), 2 mM sodium orthovanadate (Na₃VO₄) and 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton-100, and 1% β-mercaptoethanol. Cell protein extracts (25 µg) were loaded on 10% SDS-polyacrylamide gels for electrophoresis, followed by transfer onto PVDF membranes and then blotted with PPARγ and perilipin antibodies. Protein levels were determined by chemiluminescent detection and analyzed by densitometric method.

**Statistical analysis**

Results are presented as the mean ± S.E. One-way ANOVA coupled with Student’s t-test was performed in comparison of group means. P < 0.05 was considered significant.

**Results**

**Resveratrol attenuated body weight gain in HFD-induced obese mice in a dose-dependent manner**

Mice on HFD feeding for 10 wk showed significant body weight gain with lower calorie intake compared to chow diet control mice (Table 1). Treatment with resveratrol at concentrations of 1 to 30 mg/kg for 10 wk significantly attenuated HFD-induced obesity in a dose-dependent manner compared to non-treated HFD mice (Table 1). We further evaluated the tissue weight of a variety of vital organs. There was a noticeable weight gain in all kinds of adipose tissue (including subcutaneous, epididymal, and brown adipose tissues) in HFD mice compared to chow diet control mice (Table 2). Resveratrol (30 mg/kg) treatment significantly mitigated the weight augmentation of subcutaneous and epididymal adipose tissues in HFD mice, but not that of brown adipose tissue (Table 2). Notably, resveratrol also reduced liver weight at dosage of 30 mg/kg compared to non-treated HFD mice (Table 2).

To evaluate the effect of resveratrol on morphology of adipose tissue in high fat diet-fed mice, biopsy

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Dosage effects of resveratrol on body weight and caloric intake in mice fed with high-fat diet for 10 weeks</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CON</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>29.36 ± 0.70</td>
</tr>
<tr>
<td>% of initial body weight</td>
<td>115.98 ± 1.42</td>
</tr>
<tr>
<td>Caloric intake (cal/g/day)</td>
<td>471.41 ± 21.69</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE for n=10-11 in each group. * p < 0.05, vs. CON. † p < 0.05, vs. HFD. CON, control; HFD, high fat diet; HFR1, HFD mice treated with resveratrol 1 mg/kg; HFR10, HFD mice treated with resveratrol 10 mg/kg; HFR30, HFD mice treated with resveratrol 30 mg/kg. Resveratrol was dissolved in DMSO and diluted by 0.9% NaCl then was administered as oral gavage daily.
of subcutaneous (Fig. 1A), epididymal (Fig. 1B), and brown (Fig. 1C) adipose tissue was performed and observed in a microscopic view with H&E staining. Enlargement of lipid droplets was observed in every kind of adipose tissue mentioned above in HFD mice compared with mice on chow diet feeding. Resveratrol treatment at concentrations of 10 and 30 mg/kg in the HFD group showed remarkable reduction in fat drop-let accumulations of every kind of adipose tissue when compared with HFD non-treated group (Fig. 1).

**Resveratrol induced cytotoxicity and suppressed adipogenesis in 3T3-L1 cells**

To determine whether the anti-obese effects of resveratrol produce cytotoxicity in adipose tissues, the pre-adipocyte and maturing pre-adipocyte were treated with wide range of resveratrol (0.03-100 μM) for 2 and 8 days, respectively, and survival rate was examined by MTT assay. The results showed that resveratrol treatment significantly decreased the survival rate of 3T3-L1 pre-adipocytes to 70.13±1.10%, 35.67±6.29%, and 23.63±5.04% in concentrations of 10, 30, and 100 μM compared with control, respectively (Fig. 2). Also, the survival rate of mature adipocytes treated with 10, 30, and 100 μM resveratrol for 8 days was decreased to 90.53±1.99%, 3.23±1.02%, and 1.51±0.38% with statistical significance in comparison with the control group, respectively (Fig. 2B).

Table 2 Dosage effects of resveratrol on tissue weight in mice fed with high-fat diet for 10 weeks

<table>
<thead>
<tr>
<th>Tissue weight (g)</th>
<th>CON</th>
<th>HFD</th>
<th>HFR1</th>
<th>HFR10</th>
<th>HFR30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>0.12±0.01</td>
<td>0.14±0.01</td>
<td>0.12±0.00†</td>
<td>0.12±0.01</td>
<td>0.12±0.01</td>
</tr>
<tr>
<td>Liver</td>
<td>1.08±0.08</td>
<td>1.19±0.10</td>
<td>1.06±0.04</td>
<td>0.90±0.08</td>
<td>0.93±0.04†</td>
</tr>
<tr>
<td>Adipose tissues (AT)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcutaneous AT</td>
<td>0.35±0.08</td>
<td>2.16±0.36*</td>
<td>1.33±0.12</td>
<td>1.03±0.22†</td>
<td>1.21±0.26†</td>
</tr>
<tr>
<td>Epididymal AT</td>
<td>0.38±0.05</td>
<td>2.67±0.13†</td>
<td>1.90±0.13†</td>
<td>1.75±0.43</td>
<td>1.58±0.30†</td>
</tr>
<tr>
<td>Brown AT</td>
<td>0.06±0.01</td>
<td>0.16±0.02†</td>
<td>0.13±0.02</td>
<td>0.11±0.02</td>
<td>0.13±0.03</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.29±0.02</td>
<td>0.38±0.02</td>
<td>0.35±0.02</td>
<td>0.31±0.01†</td>
<td>0.35±0.01</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.38±0.01</td>
<td>0.43±0.04</td>
<td>0.40±0.02</td>
<td>0.33±0.02</td>
<td>0.37±0.03</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE for n=5-6 in each group. *p<0.05, vs. CON. †p<0.05, vs. HFD. CON, control; HFD, high fat diet; HFR1, HFD mice treated with resveratrol 1 mg/kg; HFR10, HFD mice treated with resveratrol 10 mg/kg; HFR30, HFD mice treated with resveratrol 30 mg/kg.

Resveratrol inhibited lipolysis in mature 3T3-L1 adipocytes

Lipolysis implies the breakdown of triacylglycerols in adipocytes and subsequently release of glycerol and fatty acids. To evaluate whether resveratrol affects lipolysis in maturing 3T3-L1 adipocytes, the amount of glycerol release was examined in cultured medium. Isoproterenol (10 μM) treatment for 24 hr led to a remarkably higher released of glycerol in mature 3T3-L1 adipocytes. In contrast, resveratrol treatment at concentrations of 0.03 to 10 μM for 24 hr triggered a significant inhibition in adipose lipolysis in a dose-dependent manner (Fig. 4).

It was known that both PPARγ and perilipin played essential roles in regulation of adipogenesis and lipogenesis. Thus, western blot analysis was performed to estimate the effect of resveratrol on protein expressions of PPARγ and perilipin in differentiated 3T3-L1 cells. Our results showed that PPARγ and perilipin protein expressions were significantly down-regulated in resveratrol-treated mature adipocytes with dosage of 3 to 10 μM in comparison with the control group (Fig. 3A and 3B).
Fig. 1  Dosage effect of resveratrol on cellular morphology of adipose tissues in high fat diet-fed mice. Subcutaneous (A), epididymal (B), and brown (C) adipose tissues were sectioned and observed with H&E staining in HFD obese mice with or without resveratrol treatment for 10 wk. CON, age-matched chow diet feeding for 10 wk; HFD, high-fat diet feeding for 10 wk; HFR1, HFD mice treated with resveratrol 1 mg/kg; HFR10, HFD mice treated with resveratrol 10 mg/kg; HFR30, HFD mice treated with resveratrol 30 mg/kg for 10 wk.
Fig. 2 Dosage effect of resveratrol (RSV) on cytotoxicity, proliferation, and adipogenesis in 3T3-L1 cells. Resveratrol decreased survival rate was examined by MTT assay in pre-adipocyte (A) and mature adipocyte (B). Anti-proliferative effect of resveratrol was examined by cell growth curve (C). Dosage effect of resveratrol on adipogenic differentiation was evaluated with Oil-Red-O staining (D) and the quantified ratios are shown with normalization by control group (E). Data are expressed as the mean ± SE, n=5 in each group. *p < 0.05, **p < 0.01, ***p < 0.001 vs. control.
Discussion

Resveratrol has recently become available in tablet form and is recommended as a dietary supplement, with commercial resveratrol available at between 50 μg and 300 mg per dosage. A 25 mg oral dose results in peak plasma levels of resveratrol at about 2 μM and a plasma half-life of 9.2±0.6 hr in human subjects [34]. This pharmacokinetic study shows that the bioavailability of resveratrol is very low and only a small proportion reaches plasma and tissues. Interestingly, despite its low bioavailability, resveratrol exerts a wide range of biological effects that could benefit human health.
[19-31]. The functional discrepancies may result from different concentrations of resveratrol and treatment period lengths that different cells are exposed to in different experimental settings. Therefore it is necessary to have a comprehensive grasp of the diverse and contradictory effects of resveratrol. Additionally, studies of resveratrol in a wide range of concentrations on adipose cells can provide a more generalized and deepening understanding with respect to the attenuation of obesity by resveratrol treatment. In the present study, we found that resveratrol exhibited anti-obesity effects on high-fat diet-induced obese mice in a dose-dependent manner. The anti-obesity effect of resveratrol was most likely through attenuation of lipid deposition in adipose tissues (including subcutaneous and epididymal fat) but not via influence of calorie intake. High concentration of resveratrol treatment (10 to 100 μM) significantly induced cytotoxicity in both 3T3-L1 pre-adipocytes and mature adipocytes. Resveratrol also inhibited preadipocyte proliferation at concentrations of 10 μM. These results indicate that the anti-proliferative effects of resveratrol may result from exertion of cytotoxicity but not through direct growth inhibition. At sub-toxic concentrations (1 to 10 μM) of resveratrol treatment significantly suppressed adipogenic differentiation and lipolysis in preadipocytes and mature adipocytes. Accordingly, protein expression of PPARγ, a main adipogenesis-inducing regulator, was down-regulated by 0.3 to 10 μM resveratrol treatment, suggesting that resveratrol inhibited adipocyte differentiation and lipolysis by modulating of PPARγ protein expressions. Taken together, these results indicate that the anti-obesity mechanisms of resveratrol could be involved with cell viability, cell proliferation, intracellular lipid accumulation, and lipolysis in adipocytes and pre-adipocytes, depending on the treatment dosage.

With regard to cytotoxicity, resveratrol significantly reduced cell survival rate at concentrations of 10 to 100 μM in both 3T3-L1 pre- and mature adipocytes. Consistent with our findings, it has been reported that resveratrol-stimulated cell apoptosis at concentrations of 50 and 100 μM [35]. Our observations also found mature adipocytes to be more sensitive to resveratrol treatment than pre-adipocytes. Adipogenesis is known as a hyperplastic transformation from undifferentiated pre-adipocytes to mature adipocytes and, lipogenesis, the convert and accumulation of lipid droplets in adipocyte from free fatty acids. Regarding the anti-adipogenic effects, several studies have demonstrated that resveratrol inhibited adipogenesis at concentrations of 20 to 100 μM and in the treatment period length (24 hr to 8 days) [36-39]. Our results show that resveratrol attenuated adipogenesis and lipogenesis at concentrations of 1 to 10 μM which is much less than the above published studies. Our results suggest that resveratrol directly suppressed adipogenic differentiation and lipid droplets accumulation at concentrations below 10 μM. In contrast, at high concentrations (10 to 100 μM) resveratrol treatment seemed to lead to cell death instead of induction of anti-differentiated effects.

Lipolysis implies the breakdown of triacylglycerols in adipocytes, and subsequently leads to release of glycerol and fatty acids. The effects of resveratrol on lipolysis have been studied in vitro, ex vivo, and in vivo [40, 41]. One study demonstrated that resveratrol (100 μM) failed to promote lipolysis in epididymal adipocytes of rat [40]. Another study suggested that treatment of resveratrol (100 μM) for 12, 24, or 48 hr significantly promoted lipolysis in 3T3-L1 adipocytes and also exerted synergistic action with epinephrine [41]. It is not clear whether this discrepancy is due to differential response of rat epididymal adipocytes versus 3T3-L1 cells. Our results show that resveratrol treatment at concentrations of 0.03 to 10 μM for 24 hr not only inhibited lipolysis in basal conditions but also when lipolysis was stimulated with TNFα in 3T3-L1 mature adipocytes. Inhibition of adipose lipolysis may prevent ectopic lipid accumulation in non-adipose tissues such as liver. Our in vivo study also showed that resveratrol significantly decreased liver weight at concentrations of 30 mg/kg in high-fat diet obese mice, suggesting that resveratrol could attenuate obesity-associated liver steatosis. It is noteworthy that while 3T3-L1 mature adipocytes exposed to high concentrations of resveratrol (above 10 μM) exhibited a dramatic reduction in cell survival rate which may have confounded its anti-lipolysis action. Corresponding to the opposing effects of resveratrol at low (from our study) versus high (from a study by others) concentrations on lipolysis of 3T3-L1 cells is unclear and would be of great interest for future studies.

Our study revealed distinct and much more dynamic actions of resveratrol on both high-fat diet obese mice and 3T3-L1 cells conferred by a wide range of concentrations, as compared to previous studies [35-41]. At low concentrations (below 10 μM), resveratrol appears to inhibit adipogenic differentiation and lipolysis. At high concentrations (above 10 μM), however, resveratrol decreased cell survival rate in pre- and mature
anti-obesity effects of resveratrol

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Disclosure

The authors declare that they have no conflict of interests.

References


