Stilbene Analogs of Resveratrol Improve Insulin Resistance through Activation of AMPK

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Resveratrol (RSV), 3,5,4'-trihydroxy-trans-stilbene, is known to have many beneficial physiological activities. We have synthesized several stilbene analogues and have reported that the hydroxyl group in the 4' position of RSV exhibited strong radical scavenging action. Using stilbene analogues, we investigated the structure of RSV to explain its protective effect against obesity and type 2 diabetes. All six analogs used in this study inhibited the differentiation of 3T3-L1 adipocytes. 3-Hydroxy-trans stilbene (3(OH)ST), and 3,4'-dihydroxy-trans stilbene (3,4'(OH)2ST) increased glucose uptake and induced adenosine monophosphate kinase (AMPK) phosphorylation in C2C12 myotubes independently of insulin. An in vitro study using mice fed high-fat diets indicated that 3(OH)ST was more effective than RSV in improving insulin resistance. In conclusion, RSV and its derivatives, particularly 3(OH)ST, inhibited adipocyte differentiation and enhanced glucose uptake in the myotubes, resulting in a reduction of obesity and an improvement in glucose tolerance in vivo.

Key words: resveratrol; stilbene analog; adenosine monophosphate kinase (AMPK); insulin resistance; myotube

Resveratrol (RSV) (3,5,4’-trihydroxy-trans-stilbene), a polyphenol abundant in grapes and red wine, possesses a range of biological activities including anti-aging,1–5) anti-cancer,5) repression of fat accumulation,6,7) anti-oxidative activity,8,9) and improvement of insulin sensitivity.10) Especially, it is accepted as an activator of Sirtuin 1 (Sirt1), NAD-dependent deacetylase, which is related to longevity and calorie restriction, and has drawn the attention of many researchers. Some RSV derivatives with higher bioavailability are being tested in clinical trials for type 2 diabetes treatment,11) but, information on the correlation between the physiological activities and the structural characteristics of RSV analogs is sparse.

We have investigated the physiological activities of RSV analogs using synthesized RSV derivative analogs showing various numbers and positions of the hydroxyl group on the stilbene backbone, and have reported that the 4'-hydroxy group on RSV is essential for strong anti-oxidative activity. On the other hand, RSV has been reported to induce chromosomal aberrations, micro-nuclei, and sister chromatid exchanges in mammalian cells in a bacterial reverse mutation assay12) due to its strong radical scavenging action.12–15) We found that the 4-hydroxy group on the RSV structure was responsible for in vitro clastogenic activity.13) RSV and its analog 4-hydroxy-trans-stilbene (4(OH)ST), but not 3-hydroxy-trans-stilbene (3(OH)ST), scavenge the tyrosyl free radical of the R2 subunit of mammalian ribonucleotide reductase.14) This observation should prove useful for safe drug design.

While most antioxidants are useful for human health, it is not known whether the many biological benefits of RSV are explained solely by its anti-oxidative function. In this study, we compared the effects of several stilbene analogs to clarify the structural importance of its hydroxyl groups in the prevention of obesity-induced disorders.

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Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; AMPK, adenosine monophosphate kinase; BAT, brown adipose tissue; cAMP, cyclic AMP; CamKKβ, calcmodulin kinase kinase β; DMEM, Dulbcco’s Modified Eagle Medium; FBS, fetal bovine serum; GLUT4, glucose transporter 4; GST, glutathione S-transferase; 3,4'(OH)2ST, 3,4'-dihydroxy-trans-stilbene; 3,3'(OH)2ST, 3,3'-dihydroxy-trans-stilbene; 3,5(OH)2ST; 3,5-dihydroxy-trans-stilbene; 4(OH)ST, 4-hydroxy-trans-stilbene; 3(OH)ST, 3-hydroxy-trans stilbene; HF, high fat; OGTT, oral glucose tolerance test; isoRSV, iso-resveratrol; ITT, insulin tolerance test; KRBB, Krebs-Ringer bicarbonate buffer; LF, low fat; NA, nicotine amide; NAD, nicotine amide dinucleotide; PPAR, peroxisome proliferator-activated receptor; RSV, resveratrol; Sirt1, sirtuin 1; WAT, white adipose tissue
Material and Methods

Materials. RSV and iso-resveratrol (3,5,3’-trihydroxy-trans-stilbene) were purchased from Sigma (St. Louis, MO), 3(OH)ST, 4(OH)ST, 3,4’dihydroxy-trans-stilbene (3,4’(OH)2ST), 3,3’dihydroxy-trans-stilbene (3,3’(OH)2ST), and 3,5-dihydroxy-trans-stilbene (3,5(OH)2ST) were synthesized as previously reported.15,16) The chemical structures are shown in Fig. 1.

Cell culture. 3T3-L1 pre-adipocytes were purchased from ATCC (Rockville, MD) and were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Nissui, Tokyo) containing 10% fetal bovine serum (FBS, Bio West, Inc., Nuaille, France) at 37 °C in a humidified atmosphere with 5% CO2. Cells were plated at a density of 4 × 10^4 cells in a 75 cm2 flask and passed at 80% confluence. In each experiment, 15 × 10^5 cells were plated in 60 mm dishes, and the DMEM (low glucose) was changed every 2 d. At confluence, the medium was removed and replaced (day 0) with a differentiation medium containing 10% FBS at 37 °C, and were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Nissui, Tokyo) containing 10% fetal bovine serum (FBS, Bio West, Inc., Nuaille, France) at 37 °C in a humidified atmosphere with 5% CO2. Cells were plated at a density of 4 × 10^4 cells in a 75 cm2 flask and passed at 80% confluence. In each experiment, 15 × 10^5 cells were plated in 60 mm dishes, and the DMEM (low glucose) was changed every 2 d. At confluence, the medium was removed and replaced (day 0) with a differentiation medium containing 5% FBS in the insulin until day 8. The cells were treated with RSV or stilbene analogs from day 0 to day 2.

The skeletal muscle cell line of C2C12 myoblasts was purchased from ATCC (Rockville, MD), and was maintained in DMEM containing 10% FBS at 37 °C in a humidified atmosphere with 5% CO2. Cells were plated at a density of 7.5 × 10^4 cells in a 75 cm2 flask and passed when it reached 80% confluence. In the glucose uptake experiments, 1.5 × 10^5 cells were plated in 6-well plates, and for Western blotting, 4.5 × 10^5 cells were plated in 60 mm dishes. At confluence (day 0), the medium was exchanged for differentiation medium, DMEM containing 2% horse serum (Invitrogen, San Diego, CA). Since myogenin mRNA expression levels increased (data not shown), it was thought that the C2C12 cells were differentiated into myotube cells at day 3 or 4. Hence, the cells were treated with RSV or stilbene analogs in differentiated myotube cells at day 4–6 in each experiment.

Oil Red O staining. Adipocytes were fixed using 10% formalin/PBS (−) for 10 min at day 3. Then they were stained with Oil Red O (Sigma, St. Louis, MO) and lysed with 60% isopropanol for 20 min.

Real-time RT-PCR. Total RNA was extracted from the mature adipocytes (day 8) with Isogen (Nippon Gene, Tokyo) following the manufacturer’s instructions. PCRs of the peroxisome proliferator-activated receptor γ (PPARγ), resistin, and adiponectin were performed for 40 cycles under the following conditions: denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min by the 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The primers used had the following base sequences: for peroxisome proliferator-activated receptor γ (PPARγ), 5’-GCACTGCTATGAG-CACTTCAC-3’, for resistin, 5’-AGAGGTCCACAGAGCTGTACCC-3’, 5’-TGGAACATGATGGAAAGGA-3’ and 5’-TGTAATCAAGTGCCCTAGTG-3’; and for adiponectin, 5’-GTGGATCTGACGACACAAAAAG-3’ and 5’-AGCTGATCTCAGCCGACTG-3’. Glucose uptake into C2C12 cells. Differentiated C2C12 myotube cells at days 4–6 were incubated in differentiation medium containing 50 μM RSV or stilbene analogs for 60 min. After they were washed twice with PBS (−), the cells were preincubated with KRBB (1 mL/well) for 40 min. Then 10 μL of 20 mM [3H]-deoxyglucose (0.68 μCi/mmol) was added (final concentration, 200 μg) and this was incubated for 5 min. After being washed 3 times with ice-cold PBS (−), the cells were dissolved in 0.1% SDS. The radioactivity of the cell lysate was measured by liquid scintillation counting.

Western-blot analysis. The differentiated myotube cells (at day 5) were incubated in serum-free DMEM for 3 h, and then 50 μM RSV and the stilbene analogs were added and this was incubated for a further 30 min. The cells, in 60 mm dishes, were washed with cold PBS (−) and harvested by cell scraping in 200 μL of lysis buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 10% glycerol, and 1 mM EDTA-2NA) supplemented with protease and phosphatase inhibitors (20 mM NaF, 30 mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM aprotinin, 10 mM leupeptin, 200 mM PMSF, and 200 mM DTT). The cell lysates were centrifuged at 15,000 rpm for 10 min to obtain the supernatants, and were stored at −80 °C until use. For Western-blot analysis, cell lysates containing 10 μg proteins were resuspended in sample buffer (500 mM Tris–HCl pH 6.8, 2% glycerol, 8% SDS, and 0.004% bromophenol blue supplemented with 20% 2-mercaptoethanol), and heated for 2 min at 95 °C. Samples were separated by 9% SDS–PAGE and transferred to a PVDF membrane, which was blocked with 2% non-fat skim milk in PBS-Tween 20 (0.1% Tween20 in PBS) and phosphatase blocking buffer (Phospho BLOCKER™ Blocking Reagent, Cell Biolabs, San Diego, CA) in PBS-Tween 20 and incubated with the primary antibody in PBS-Tween 20. The bound primary antibody was detected with a goat anti-rabbit IgG HRP-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit polyclonal antibody against AMPK alpha and phospho-AMPK alpha (Thr 172) was purchased from Cell Signaling Technology (Beverly, MA). Target protein bands were detected by the ECL plus Western Blotting Detection System (GE Healthcare UK, Buckinghamshire, UK).
Animals and diets. Six-week-old female C57BL/6J mice were purchased from Tokyo Laboratory Animals Science (Tokyo). They were housed in rooms at a constant temperature of 22 ± 1 °C under a fixed 12-h light-dark cycle. After feeding of a CLEA Rodent Diet CE-2 (CLEA Japan, Tokyo) for 1 week, the mice were randomly divided into four groups (n = 6 per group) and fed the following diets: a low-fat (LF) diet (20% fat, 20% protein, and 60% carbohydrate); high-fat (HF) diet (50% fat, 15% protein, and 35% carbohydrate); or HF with 0.4% 3(OH)ST. The compositions of the diets are shown in Table 1.

After 6 weeks of feeding, blood and tissues of the mice were collected. The weights of the liver, gastrocnemius, white adipose tissue (WAT) around the testis, and brown adipose tissue (BAT) were measured. All procedures were approved by the Animal Ethics Committee of Ochanomizu University.

Oral glucose tolerance test (OGTT). At the 5th week of the experiment, d-glucose (1.5 mg/g of body weight) was administrated to mice orally after overnight fasting. Blood samples were obtained from the tail at 0, 15, 30, 60, and 90 min after glucose administration. Blood glucose levels were measured with glucose analyzer Dexter Z II (Bayer, Tokyo).

Insulin tolerance test (ITT). At the 2d after the OGTT, human insulin (0.40 mU/g the body weight) (Eli Lilly, Kobe, Japan) was injected into the mice intraperitoneally. Blood samples were obtained at 0, 15, 30, 60, and 90 min after insulin injection, and blood glucose levels were measured.

Measurement of glucose uptake into skeletal muscle. The soleus muscles of the mice were excised from both legs, and the tendons were tied by stainless-steel clips. These muscles were used for measurement of basal (without insulin) and insulin-stimulated glucose uptake respectively. To stabilize cell metabolism, the muscles were preincubated with continuous shaking at 37 °C in 4 mL of KRBB (Krebs-Ringer bicarbonate buffer at pH 7.4 containing 0.1%w/v BSA and 2 mm sodium pyruvate oxygenated with 95% O2 and 5% CO2) supplemented with 8 mm glucose and 32 nm mannitol for 30 min, and then incubated with or without 100 nm human insulin for 20 min. After washing with glucose free KRBB supplemented with 40 nm mannitol with or without insulin for 10 min, the muscles were incubated with KRBB supplemented with 1 mm [3H]-2-deoxyglucose and 39 mm[14C]-mannitol with or without insulin for 15 min. Then they were washed in ice-cold PBS containing 0.1% BSA for 30 min, boiled in 1 mL of 1 N NaOH for 5 min, and neutralized with 60 mL of 5 N HCl. Radioactivity levels were measured by liquid scintillation counting. The amount of glucose uptake was determined by calculating specific glucose uptake by the ratio of [3H]-2-deoxyglucose to [14C]-mannitol.

Statistical analysis. Results were expressed as mean ± SD. Data were analyzed by a one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. Significance was set at p < 0.05.

Results

Effects of RSV and stilbene analogs on the differentiation of adipocytes

RSV and its simple analogs, 3(OH)ST, and 4(OH)ST, were examined for their effects on fat accumulation during the differentiation of 3T3 pre-adipocytes by Oil Red O staining. Fat accumulation decreased markedly in the presence of RSV, 3(OH)ST, and 4(OH)ST as compared with control (Fig. 2A–D). GPDH activities, the differentiation marker of adipocytes, were decreased by RSV, 4(OH)ST, and 3(OH)ST compared with control in a dose-dependent manner (data not shown). Furthermore, the mRNA expression levels of adiponectin in the small adipocytes were significantly increased by RSV, 3(OH)ST, and 4(OH)ST compared with the control. The effect of 3(OH)ST was the strongest (Fig. 2E). Hence, we compared the effects of other stilbene analogs with different numbers and positions of the hydroxyl group, as shown in Fig. 1. As Fig. 3 shows the mRNA expression levels of PPARγ, the master regulator of the differentiation of pre-adipocytes, were decreased by RSV (79%), 3(OH)ST (60%), 4(OH)ST (71%), 3,4′(OH)2ST (41%), 3′,3′(OH)2ST (57%), 3,5(OH)2ST (44%), and iso-RSV (63%) as compared to control. All the stilbene analogs, but not RSV, decreased the mRNA expression levels of resistin, the initiation factor for insulin resistance (Fig. 3B). This showed a significant decrease in 3,4′(OH)2ST (25%), 3′,3′(OH)2ST (48%), and 3,5(OH)2ST (51%) as compared with control.

Effects of RSV and stilbene analogs on glucose uptake in C2C12 myotube cells

RSV and the stilbene analogs did not influence the viability of C2C12 cells in the dose range between 1 and 100 μM, as assessed by MTT assay (data not shown). Since it has been reported that glucose uptake was increased by 50 μM RSV,17) 50 μM RSV and stilbene analogs were added to the cells after differentiation for 1 h, and then glucose uptake into the C2C12 myotubes was measured. 3(OH)ST, 3′,4′(OH)2ST, and isoRSV significantly increased the amount of glucose uptake into the C2C12 myotubes, to 153%, 160%, and 147% respectively (Fig. 4A). Although neither RSV nor any of the stilbene analogs changed the ratio of the phosphorylated form of Akt (data not shown), they all

Table 1. Compositions of the Diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>LF</th>
<th>HF</th>
<th>HF+RSV</th>
<th>HF+3(OH)ST</th>
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<td>20.00</td>
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<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
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<tr>
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<td>0.0050</td>
<td>0.0050</td>
<td>0.0050</td>
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<tr>
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<td>25.00</td>
<td>25.00</td>
<td>25.00</td>
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<td>Resveratrol</td>
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<td></td>
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<td></td>
<td></td>
<td>0.40</td>
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</table>
induced AMPK phospholylation significantly (Fig. 4C). Especially, the extent of AMPK activation by 3(OH)ST (196%), 3,4(OH)2ST (233%), and 3,3(OH)2ST (226%) was greater than AICAR (192%), the activator of AMPK. We also evaluated AMPK activation in the presence and the absence of nicotine amide (NA), an inhibitor of Sirt1 (Fig. 4D). All the stilbene analogs activated the phospholylation of AMPK without NA, but, activation by 3(OH)ST and 3,3(OH)2ST was suppressed in the presence of NA, while that of RSV and 3,4(OH)2ST did not change. These results suggest that both 3(OH)ST and 3,3(OH)2ST activated AMPK via Sirt1 activation, and that the process of activation by RSV and 3,4(OH)2ST, which holds a 4' position of OH, was independent of Sirt1.

Prevention of HF diet-induced insulin resistance by RSV and 3(OH)ST

To investigate the effect of RSV and the involvement of its chemical structure, we compare the effect of 3(OH)ST, the simplest analog RSV, with RSV on HF diet-induced insulin resistance in vivo. We fed C57BL/6J mice, six mice in each group, the diet shown in Table 1. As shown in Fig. 5A, the HF diet mice (HF group) had a higher mean body weight at the end of the experimental period, as indicated by a 20% increase as compared to the LF diet mice (LF group). The body weight of the RSV-fed mice (RSV group) decreased to the same level as the LF group, and the addition of 3OH to the HF diet decreased in 73% from HF. There was no difference in energy intake among experimental groups (data not shown). No abnormalities were observed in the appearances of the organs. The values for aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glutathione S-transferase (GST) were not changed by 3(OH)ST treatment (data not shown). The weight of WAT around the testis in the HF group was 1.7 fold higher than in the LF group, but the addition of RSV to the HF diet decreased the WAT weight to the level for the LF group. Furthermore, 3(OH)ST treatment reduced the WAT weight to half of the LF groups (Fig. 5B).

We tried OGTT and ITT after 5 weeks of dietary intervention. The addition of 3(OH)ST to the HF diet improved glucose intolerance in the mice fed the HF diet alone to an extent similar to LF diet mice, but RSV failed to reproduce this (Fig. 5C and D). The HF group also showed aggravation of insulin resistance as compared to the LF group, but both RSV and 3-OH improved insulin tolerance to the LF group. We further examined glucose uptake into isolated skeletal muscle with and without insulin induction (Fig. 5E). The basal levels of
Fig. 4. Effects of RSV and the Stilbene Analogs on C2C12 Myotube Cells.
The incorporation of $[^{3}H]2$-deoxyglucose into differentiated C2C12 myotube cell were measured as described in “Materials and Methods.” Values are expressed as uptake ratio relative to control (A). Western-blot analysis was used and measure the phosphorylation of AMPK (B). AMPK activation in the presence and the absence of 10 mM NA. One of the three individual images of Western-blotting is shown (C). All values were expressed as mean ± SD (n = 3). Data were analyzed by a one-way ANOVA. Significant difference was expressed vs. control. $p < 0.05$; $p < 0.01$. Different superscripts by column indicate significant differences ($p < 0.05$).

Fig. 5. Effects of RSV and 3(OH)ST on Glucose Tolerance and Insulin Resistance in HF Induced Obese Mice.
C57BL/6J mice were fed a high-fat diet with 0.4% RSV or 3(OH)ST for 6 weeks. Changes in body weight during the experimental period (A). Weights of WAT around the testis (B). Changes in blood glucose levels during OGTT (C) and ITT (D). Incorporation of $[^{3}H]2$-deoxyglucose into the soleus muscle (E). Levels of adiponectin mRNA in adipose tissue (F). Values were expressed mean ± SD (n = 6). Data were analyzed by one-way ANOVA. Significant difference was expressed vs. control. $p < 0.05$; $p < 0.01$. Different superscripts by column indicate significant differences ($p < 0.05$).
glucose uptake without induction of insulin in all experimental groups were similar. As a result of insulin induction, the HF diet increased glucose uptake by 1.5 fold, while LF increased it twice. Addition of RSV to the HF diet recovered to an extent similar to LF. 3(OH)ST increased glucose uptake by 2.6-fold after insulin induction. These results indicate that 3(OH)ST was effective in improving insulin resistance in the skeletal muscle. As shown in Fig. 5E, adiponectin mRNA levels were suppressed by HF (89%) as compared with LF, and increased by RSV (105%) and 3(OH)ST (127%).

Discussion

To determine how the position of hydroxyl groups on stilbene molecules influences their biochemical role in fat metabolism, we investigated the in vitro and in vivo effects of several stilbene molecules. We found that even the simplest analogs were more effective than RSV itself in preventing obesity and insulin resistance.

RSV is known to suppress body weight gain induced by a high-calorie diet in rats and to reduce the effects of several stilbene molecules. We found that even the simplest analogs were more effective than RSV in increasing glucose uptake by 2.6-fold after insulin induction. These results indicate that 3(OH)ST was more effective than RSV (105%) and 3(OH)ST (127%).

Moreover, in that RSV and 3(OH)ST activated AMPK but not Akt, consistently with the results of et al., who found that activation of Akt did not compete with RSV and the stilbene analogs to inhibit the Akt signal. Thereafter, we confirmed that Akt phosphorylation in phenol red free DMEM was not activated (data not shown). These results suggest that RSV and the stilbene analogs improved insulin sensitivity enhancing glucose uptake in skeletal muscle cells through direct activation of AMPK, but not Akt.

The process of AMPK activation appears different depending on whether the stilbene backbone has the hydroxyl group at the 4th position. As shown in Fig. 4F, NA, an inhibitor of Sirt1, failed to suppress AMPK activation by RSV and 3,4(OH)ST, suggesting that RSV and 3,4(OH)ST did not depend on Sirt1. Since we did not measure the activity of Sirt1 directly, we cannot speculate as to how these analogs act on AMPK.

Adiponectin is secreted specifically from adipose tissue, and it is known that younger, smaller adipocytes secrete more adiponectin than mature ones. Obesity and/or a high-fat diet suppresses the secretion of adiponectin.

Adiponectin increases the expression of PPARγ, a master regulator of the differentiation of adipocytes, and improves insulin resistance through the activation of AMPK. Based on these reports, we suggest that one function of 3(OH)ST in vivo is increased secretion of adiponectin which activates AMPK in the skeletal muscle by suppression of pre-adipocytes differentiation. Lagouge et al. have reported that RSV reduced fat accumulation in the whole body as a result of an increase in the basal metabolic rate and thermogenesis. RSV activity was associated with the induction of genes related to oxidative phosphorylation and mitochondrial biogenesis, and was largely explained by an RSV-mediated decrease in PGC-1α acetylation and an increase in PGC-1α activity. In addition, AMPK directly induced the phosphorylation of PGC-1α.

In a study using AMPK knockout mice, RSV failed to reduce fat mass in mice fed a HF diet, or to increase insulin sensitivity, mitochondrial biogenesis or physical endurance, suggesting that AMPK was the central target of the metabolic effects of RSV. In our study, the weights of BAT were decreased by RSV and 3(OH)ST treatment (data not shown), but we did not examine UCP1 expression in BAT. Mitochondrial function and thermo-
gene expression are the subject of further investigation in our laboratory.

In conclusion, the 3'-hydroxy group of RSV was important to the protective effect of RSV against obesity-induced disorders, such as inhibition of adipocyte differentiation and activation of AMPK, but the 4' hydroxyl group of RSV, which associated with strong antioxidant and genotoxic activity, was not essential. These results provide a basis for a safe drug design without loss of the therapeutic potential of RSV.

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