Activation of Estrogen Receptor is Crucial for Resveratrol-stimulating Muscular Glucose Uptake via Both Insulin-dependent and Independent Pathways

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OBJECTIVE
Estradiol (E2) is known to modulate insulin sensitivity and consequently, glucose homeostasis. Resveratrol (RSV), an agonist of estrogen receptor (ER), has exerted antihyperglycemic effect in streptozotocin-induced type 1 diabetic rats in our previous study and also shown to improve insulin resistance in other reports. However, it remains unknown whether activation of ER is involved in the metabolic effects of RSV via insulin-dependent and independent mechanisms.

RESEARCH DESIGN/METHODS AND RESULTS
Here we show that RSV shifts the metabolic characteristics of rats on high cholesterol-fructose (HCF) diet towards those of rats on standard diet. RSV treatment increased insulin-stimulated whole body glucose uptake and steady-state glucose uptake of soleus muscle and liver in HCF-fed rats as well as enhanced membrane trafficking activity of glucose transporter 4 (GLUT 4) and increased phosphorylation of insulin receptor in insulin resistant soleus muscles. Interestingly, the phosphorylated ER level in insulin-resistant soleus muscle was significantly elevated in rats with RSV treatment in both basal and euglycemic hyperinsulinemic conditions. RSV exerted insulin-like stimulatory effect on isolated soleus muscle, epididymal fat and hepatic tissue and C2C12 myotubes. The RSV-stimulated glucose uptake in C2C12 myotubes was depended on Erk/p38 (early phase, 1 hr) and p38/PI3k (late phase, 14 hrs) activation respectively. Inhibition of ER abrogated RSV-induced glucose uptake in both early and late phases.

CONCLUSIONS
Collectively, these results indicate that ER is a key regulator in RSV-stimulating insulin-dependent and independent glucose uptake which might account for the protective effects of RSV on diet-induced insulin resistance syndrome.
Recent investigations have revealed a pivotal role for estradiol (E2) in regulating energy metabolism and have opened new insights into the physiological role of the estrogen receptors (ERs) (1, 2). Mice that lack ER-α resulted in insulin resistance, impaired glucose tolerance, adipocyte hyperplasia and hypertrophy. Both males and females exhibited these features, highlighting the importance of the E2-ER action for the maintenance of glucose homeostasis (3). Phytoalexin RSV (3, 4', 5-trihydroxy-trans-stilbene) has structural similarities of the synthetic estrogen diethylstilbestrol; it has been shown to bind to and activate gene transcription via the ER in estrogen-sensitive tissues and cell lines (4, 5). In addition, it has been revealed that ER-α is a positive regulator of GLUT4 expressions, whereas ER-β has a suppressive role (6, 7). Previous study has demonstrated that RSV binds ER-β with lower affinity than ER-α (8). Therefore, the present study was focused on the involvement of ER-α instead of ER-β activation in RSV action.

Furthermore, the potential therapeutic effects of RSV have been revised intensely in recent years. RSV has been documented to possess a cardioprotective effect, a chemoprotective agent in cancer therapy, and an activator of sirtuin deacetylases which is related to the extension of lifespan (9-11). More recently RSV treatment has been reported to protect mice against diet-induced obesity and insulin resistance (12, 13). On the other hand, our previous study showed that RSV exerted antihyperglycemic effect in streptozotocin-induced type I diabetic rats (14). Taken together, it is suspected that the activation of ER-signaling pathway might also play a crucial role in the beneficial effects of RSV on insulin resistant syndrome.

Components of the diet related to the changes in eating habits that characterize the modern Western world are important factors in the increasingly high prevalence of chronic diseases, including obesity, diabetes and hypertension. To mimic Western eating habits characterized by consuming a lot of high cholesterol diet combined with sugar sweetened beverages, we fed rats with a high cholesterol diet combination with 10% fructose in drinking water (HCF diet) for 15 weeks and found that these rats could develop a phenotype of insulin resistance syndrome characterized by an increase in blood pressure, hyperlipidemia, hyperinsulinemia, and impaired glucose tolerance with relative normal body weight gain as previously described (15). Since the beneficial effects of RSV on obesity and insulin resistance were functionally intertwined in obese-induced insulin resistant animal model (12, 13), HCF-fed rats instead of obese rats were chosen in the present study to test whether ER signaling is directly involved in the effects of RSV on diet-induced whole body and muscular insulin resistance without the potential disturbance of RSV-mediated anti-obese effect to data interpretation. Our findings suggest that RSV-activated ER-signaling pathway is crucial for its stimulating effects on muscular glucose uptake which might account for at least in part the beneficial effect of RSV on HCF-induced insulin resistant syndrome. It implicates a therapeutic potential of RSV in treatment of patients, in particular menopause women with type 2 diabetes.

**RESEARCH DESIGN AND METHODS**

**Animals and diets** Male Sprague-Dawley (SD) rats were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The rats were housed in an animal room with a constant temperature of 22±1°C and a fixed 12 hrs light-dark cycle. All animals were handled and housed according to the guidelines and manual of the
Committee of the Care of Laboratory Animals of Chang Gung University.

Rats weighing 150-170 gm were randomly assigned into four groups fed regular chow diet (5.1% fat, 23.5% protein, 50.3% carbohydrate, LabDiet® 5010) (control, n=30) or a high cholesterol diet (4% cholesterol, 10.1% fat, 17% protein, 51.6% carbohydrate, Harlan Teklad TD03468, Indianapolis, Ind, USA) with 10% fructose-contained drinking water (n=58) for 15 weeks. Then, the HCF rats were further divided into three subgroups concomitantly treated with vehicle (HCF, n=28), RSV for 15 days (HCF + RSV 15D, n=15) or RSV for 15 weeks (HCF + RSV 15W, n=15). Resveratrol (1 mg/kg/day) was suspended in 0.9% saline solution and administered by oral gavage once a day for 15 days or 15 weeks. Body weight, water, and food intake were recorded weekly. In a separate set of rats (n=8 per group) were sacrificed with overdose pentobarbital (100 mg/kg, ip) at the end of basal and euglycemic hyperinsulinemic clamp (EHC) periods respectively to measure the GLUT4 translocation and phosphorylation of IR and ER in soleus muscle.

**Euglycemic hyperinsulinemic clamp experiment with tracer dilution method**

A euglycemic hyperinsulinemic clamp technique with tracer dilution method was performed as previously described (16-20). The detail procedures are described in the online supplementary information.

**Immunoblotting**

To assess GLUT4 and GLUT1 distribution between the intracellular membranes (IM) and the plasma membrane (PM), rat soleus muscle and C₂C₁₂ skeletal muscle cells (ATCC, Manassas, VA) were subjected to subcellular membrane fractionation as recently described (21). In brief, tissues were first homogenized in a lysis buffer [50 mM Tris (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 2 mM EDTA, 2 mM Na₃VO₄, 250 mM sucrose, 1% β-mercaptoethanol] with 1 mM phenylmethylsulfonylfluoride (PMSF) as a protease inhibitor. A sample of the crude homogenate (CH) was saved for protein determination, and the remainder was centrifuged (15,000 g). The supernatant was then further centrifuged at 44,000g, the pellet discarded, and the liquid phase pelleted at 200,000g, resulting in an IM-enriched fraction. For PM enrichment, the 15000g pellet was dissolved in a buffer containing 1% Triton 100 and centrifuged at 200g. Fractions enriched in IM and PM were dissolved in sucrose buffer and stored at -70°C. Protein samples of IM, PM, and total lysates were subjected to 10% SDS-PAGE and electrophoretically transferred to PVDF membrane for 2 hrs. The membrane was blocked in 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20. It was then washed and blotted with anti-GLUT1, GLUT4 (Chemicon, USA), ER-α, p-ER-α-ser118, InsR, p-InsR-β-tyr1146 (Cell signaling, USA), Akt, p-Akt-thr308, p-Akt-ser473 (Santa Cruz), p38, p-p38-thr180/tyr1146, Erk, and p-Erk-thr202/tyr204 (Chemicon, USA) antibodies. The membrane was then incubated with HRP-conjugated secondary antibody prior to chemiluminescence detection (Pierce, USA).

**2-Deoxy-[³H] glucose uptake (2-[³H] DG) in C₂C₁₂ myotubes**

Mouse C₂C₁₂ myoblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C with 95% air and 5% CO₂. To induce differentiation, cells were switched to DMEM containing 1% FBS for 3 days. For glucose uptake assay, cells were cultured on 12-well cluster dishes, washed twice with PBS and incubated in serum-free media for 16 hrs, and then treated with or without insulin (0.792 µM) or RSV (0.1 µM) in DMEM for 1 or 14 hrs at 37°C. After treating with the agonist, uptake of 2-[³H]DG
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(0.24 μM) was measured over 10 min. Reactions were terminated by the removal of the permanent solution and rapid washing with ice-cold 20 mM phloretin, a specific inhibitor of equilibrative glucose transport. Cells were then dissolved in 0.1 N KOH, and aliquots were counted by liquid scintillation counting or were used to determine protein concentration. In some experiments, cells were preincubated with wortmannin or Ly 294002 (100 nM, inhibitors of phosphatidylinositol 3-kinase), PD98059 (10 μM, an inhibitor of mitogen-activated protein kinase kinase), SB203580 (10 μM, an inhibitor of p38), or ICI 182780 (1 μM, an inhibitor of ER) for 30 min at 37°C before treatment with RSV (0.1 μM).

Biochemical analysis

Blood were collected from the tail vein for biochemical measurements in experimental rats after overnight fasting. Plasma was used for the measurements of total cholesterol, high-density lipoprotein, triglyceride, and uric acid (Randox reagent kits, Randox Laboratories LTD. Antrim, United Kingdom). Insulin was measured using a commercial available kit provided by Mercodia (rat insulin ELISA kit, Uppsala, Sweden). Blood glucose samples were determined by the glucose oxidase method (Chemistry Analyzer; Auto analyzer Quik-Lab., Ames, Spain).

Direct blood pressure measurement

Direct blood pressure measurement was performed in the experimental rats by connecting the femoral artery catheter to Maclab data acquisition system (AD Instruments, Castle Hill, NSW, Australia) through a pressure transducer for arterial pressure and heart rate monitoring.

Statistical analysis

Data were expressed as means ± SEM. The difference in RSV-stimulated 2-DG uptake was analyzed by one-way analysis of variance (ANOVA) and followed by Bonferroni’s post hoc test. Others were analyzed by student t-test, with the significant difference was set at P< 0.05.

RESULTS

RSV protects against HCF-induced insulin resistance syndrome in rats

As shown in table 1, the average body weight of HCF-fed rats was slightly lower than that of control rats and failed to change after RSV (1 mg/kg/day) treatment for 15 days or 15 wks. There was no difference in caloric intake between control rats and HCF-fed rats with or without RSV administration throughout the study. HCF-diet feeding for 15 wks progressively increased the severity of insulin resistance syndrome characterized by elevated blood pressure, increased fasting plasma cholesterol, triglyceride, insulin, and uric acid in a time-dependent manner. However, HCF feeding did not alter fasting plasma glucose and high-density lipoprotein (HDL) levels. RSV treatment for 15 days and 15 wks time-dependently attenuated the elevated insulin, cholesterol and triglyceride levels. In addition, RSV treatment for 15 wks significantly reduced the increased uric acid levels and conversely increased plasma HDL levels in HCF-fed rats. The RSV treatment for 15 wks also slightly lowered MAP in HCF-fed rats (difference is not significant). These results suggest that RSV shifts the metabolic characteristics of rats on HCF diet towards those of rats on standard diet, significantly alleviating their insulin resistance syndrome. Notably, all these changes occurred without the significant changes in body weight and caloric intake among the groups.

RSV attenuates HCF-induced whole body and muscular insulin resistance in euglycemic hyperinsulinemic clamp study

As shown in Fig. 1, under similar euglycemic and hyperinsulinemic conditions, RSV treatment significantly alleviated HCF-induced insulin resistance as indicated by improving the diminished insulin-mediated
stimulatory effect on whole body glucose uptake as well as tissue-specific (soleus muscle, vastus lateralis red muscle, and liver but not epididymal fat, extensor digitorum longus muscle, and vastus lateralis white muscle) glucose uptake in HCF-fed rats in a time-dependent manner (Fig. 1 and supplemental Fig. 1S of the online appendix). The improving effect of RSV on insulin-stimulating muscular glucose uptake (soleus muscle, Fig. 1) was concomitant with increases in GLUT4 membrane translocation and insulin receptor (InsR) phosphorylation (Fig. 2). Notably, RSV also significantly reversed HCF-induced decreases in basal membranous GLUT4 and phosphorylated InsR protein levels in soleus muscles. These data implicate that RSV could improve whole body and muscular glucose uptake via insulin-dependent and independent pathway in HCF-induced insulin resistant rats.

**RSV increases ER phosphorylation in vivo and in vitro**

As shown in Fig. 3, under both basal and euglycemic hyperinsulinemic conditions, RSV treatment markedly increased ER-α protein phosphorylation in HCF-induced insulin resistant soleus muscles. Taken together with fig. 2, these results suggest that both RSV treatments for 15 days and 15 wks could significantly enhance ER and InsR activities as well as GLUT4 translocation to the plasma membrane in HCF-induced insulin resistant soleus muscles (Fig. 3A). On the other hand, RSV (0.1 μM) treatment also significantly increased phosphorylation of ER-α at ser118 during 2 hrs observation period in C2C12 myotubes (Fig. 3B). These in vivo and in vitro results suggest that RSV might directly elicit ER activation in skeletal muscles.

**RSV directly stimulates glucose uptake in isolated soleus muscle and C2C12 myotubes**

Acute RSV treatment for 30 min could directly stimulate glucose uptake in isolated rat soleus muscle, epididymal fat pad, and hepatic tissue in a dose-dependent manner in normal rats shown in supplemental Fig. 2S. To investigate the underlying mechanism of RSV-stimulating muscular glucose uptake in the model of muscle cell line, the 2-DG uptake and expression of membranous GLUT4 were measured in C2C12 myotubes with RSV treatment. We first examined the dose- and time-dependent effects of RSV on glucose uptake in C2C12 myotubes. The maximal effective dose of RSV-stimulated glucose uptake was between 0.1-0.3 μM close to those in isolated rat soleus muscle. RSV-stimulated glucose uptake reached a peak level at 1 hr mark (first wave), and then second phase of activation was observed in 14 hrs. RSV also directly induced glucose uptake and stimulated GLUT4 translocation to the plasma membrane in a dose-dependent manner in C2C12 myotubes (supplemental Fig. 3S of the online appendix).

**RSV stimulates glucose uptake in C2C12 myotubes in an ER-dependent manner**

To further elucidate the role and characteristic of ER-mediated signaling pathway in RSV-induced glucose uptake and GLUT4 membrane translocation, we compared the effect of RSV with or without insulin, ER agonist (estradiol, E2), and ER antagonist (ICI 182780) on glucose uptake of C2C12 myotubes. Both RSV treatments for 1 hr and 14 hrs exerted synergistic effect with insulin but not with E2 on glucose uptake and GLUT4 membrane translocation (Fig. 4 A and B). Notably, the synergistic effect of RSV with insulin was blunted after longer (14 hrs) RSV treatment. RSV-induced glucose uptake and GLUT4 membrane translocation for 1 hr and 14 hrs were both suppressed by ER blocker (Fig. 4 C and D). As shown in fig. 3 B, the level of ER phosphorylation but not total ER protein expression was increased in two-wave pattern after RSV administration. These results further supports that RSV and E2 are via the same signaling pathway to enhance muscular glucose uptake. Moreover, the result in supplemental Fig. 4S of the online appendix.
showed that tamoxifen, another ER blocker significantly antagonized RSV-induced glucose uptake (from 129.1±9.53 % to 107.2±3.94 %, p<0.05) consistent with those with ICI 182780 cotreatment. Collectively, these results indicate that ER is a key mediator in acute and slow actions of RSV-stimulated GLUT4 membrane translocation and glucose uptake in C2C12 myotubes.

The signaling pathways involved in RSV-induced muscular glucose uptake

To probe the potential ER and InsR-mediated signaling pathways involved in RSV-induced muscular glucose uptake, C2C12 myotubes were treated with RSV and probed with phosphorylation-specific antibodies. As shown in Fig. 5, RSV treatment significantly increased phosphorylation of p38 at thr180 and tyr1146, Erk at thr202 and tyr204, Akt at thr308 and ser473, and InsR at tyr1146 in a time-dependent manner. The ER-α, p38, and Erk phosphorylation was started at 5 min in response to RSV (Fig. 3B, 5A, and 5B). Subsequently, InsR, Akt (thr 308), and Akt (ser 473) was phosphorylated at 10, 20, and 30 min respectively (Fig. 5A and 5B).

Because both RSV treatments for 1hr and 14 hrs have shown to enhance glucose uptake and GLUT4 translocation to the plasma membrane in C2C12 myotubes as shown in Fig. 4 and supplemental Fig. 3S, we were interested to further test whether there are different underlying mechanisms beneath the early and late-phase actions of RSV in this part of study. As shown in Fig. 6A, co-treatment with Erk and p38 inhibitors (PD 98059 and SB 203580) but not PI3k inhibitor (wortmannin and Ly 294002) completely blocked the early-phase effect (1 hr) of RSV-stimulating glucose uptake and GLUT4 membrane translocation. Inhibition of p38 and PI3k but not Erk blunted the late-phase effect (14 hrs) of RSV-induced glucose uptake and GLUT4 membrane translocation. These results suggest that the signaling cascades of RSV-stimulating muscular glucose uptake shifted Erk/p38 activation in the early phase to p38/PI3k activation in the late phase. Moreover, RSV-evoked phosphorylation of p38, Erk, and InsR protein was markedly suppressed by adding ICI 182780 (ER blocker, Fig. 6B-D), indicating that the phosphorylation of p38, Erk, InsR, and Akt is downstream of RSV-activating ER-α-mediated signaling pathway.

The MTT assay was used to evaluate mitochondria activity and cell survival rate in C2C12 myotubes. The results show that mitochondria activity and cell survival rate were not affected by inhibitors (SB 203580, PD 98059, Ly 294002, wortmannin, ICI 182780) treated alone or combination treated with RSV in C2C12 myotubes (data not shown). In addition, the glucose uptake activity and GLUT4 translocation to the plasma membrane were not significantly changed in those treated with inhibitors alone (Fig. 6A).

Taken together, these results suggest that activation of estrogen receptor is essential for RSV-evoked p38, Erk, Akt, and InsR phosphorylation and that inhibition of ER is sufficient to abrogate RSV-induced GLUT4 membrane translocation and consequently, glucose uptake in skeletal muscles via insulin dependent and independent signaling pathways (Fig. 7).

DISCUSSION

Recent studies have indicated that E2 via ER-α and ER-β-mediated signaling pathway participates in glucose homeostasis by modulating the expression of genes involved in insulin sensitivity and glucose uptake (22, 23). RSV has higher affinity to ER-α, a positive regulator of GLUT4 expression, than ER-β, a suppressive role in skeletal muscle (6-8). Here, we showed that RSV stimulated muscular glucose uptake in HCF-stimulated rats (soleus muscle, vastus lateralis red muscle),
isolated soleus muscle, and C2C12 myotubes. Acute RSV administration directly increased glucose uptake not only in isolated soleus muscle but also in isolated epididymal fat and hepatic tissue. RSV could go through eliciting ER-α and InsR phosphorylation, stimulating GLUT4 translocation to plasma membrane, and consequently increasing muscular glucose uptake in HCF-fed rats and C2C12 myotubes. Inhibition of ER activation could markedly suppress RSV-stimulated glucose uptake via p38/Erk (early phase) and p38/Akt (late phase) dependent pathways. Our result has demonstrated that activation of ER is crucial for the RSV-evoked insulin-dependent and independent signaling pathway, i.e. p38, Erk, Akt and InsR phosphorylation in a time-dependent manner.

The present study shows that RSV treatment significantly attenuated HCF-induced insulin resistance indicated by reducing fasting hyperinsulinemia and also whole body glucose uptake during euglycemic hyperinsulinemic clamp. In addition, RSV administration can induce InsR phosphorylation and GLUT4 translocation to the plasma membrane under both basal and euglycemic hyperinsulinemic conditions, indicating that RSV may possess both insulin-like action and the improving effect on HCF-induced insulin resistance in rats. Furthermore, RSV has showed to directly stimulate tissue glucose uptake in major insulin sensitive tissues such as soleus muscle, epididymal fat and hepatic tissues. This could support that RSV possesses insulin-like effect on glucose uptake of insulin-sensitive tissues. In support to the present observation, our previous study also demonstrated that RSV exerted antihyperglycemic effect without altering the plasma insulin levels in streptozotocin-induced type 1 diabetic rats, suggesting metabolic effect of RSV was not solely due to improve the insulin action on insulin-dependent tissues (14). On the other hand, the present result from C2C12 myotubes has shown that RSV could stimulate ER-α, p38 and Erk phosphorylation at first and follow by InsR and Akt phosphorylation. Moreover, the synergistic effect of RSV and insulin was diminished after RSV treatment for longer period (14 hrs). This diminished synergistic effect in parallel with the shift of RSV-mediated p38/Erk activation to p38/PI3K/Akt activation also supports that RSV could stimulate muscular glucose uptake via insulin independent and dependent pathways in a time-dependent manner.

On the other hand, the present result showed that RSV has dominant stimulatory effects on glucose uptake in slow-twitch muscle fibers (soleus muscle, vastus lateralis red muscle) than fast-twitch muscle fibers (EDL, vastus lateralis white muscle). Soleus and vastus lateralis (red) muscles are slow-twitch muscle fibers. The slow-twitch muscle fibers usually account for ~50% or more of total muscle mass in humans and are more insulin responsive than fast-twitch fibers, implicating the potential improving effect of RSV on muscular insulin resistance in humans. In addition, it has been reported that calcineurin up-regulates slow muscle fiber genes in C2C12 cells (24). Therefore, we think that the mouse myoblast cell line C2C12 could be an appropriate model for studying the underlying mechanism of RSV action.

Furthermore, E2 has been considered as an important regulator of glucose homeostasis. ER-α and ER-β have been proposed to be the important modulators of GLUT4 expression in skeletal muscle (6, 7). The present study shows that RSV (a phytoestrogen) could directly induce ER-α phosphorylation but not ER protein expression in skeletal muscle both in vivo and in vitro. Inhibition of ER markedly suppressed RSV-stimulated muscular glucose uptake, suggesting that the ER activation is crucial for the RSV-mediated metabolic actions. RSV has been documented to serve as a SIRT1 activator and could affect the regulation of energy homeostasis via
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modulation of PGC-1α functions (12, 13, and 25). Furthermore, RSV has also been reported to have a close connection with pathways of insulin signaling and lifespan regulation (26-30). Our observations raise the possibility that RSV induced ER activation might be another key regulator to its stimulating effect on muscular glucose uptake, independent of its activation of SIRT1 histone deacetylase.

The male SD rats were chosen in the present study to test whether ER signaling is directly involved in the effects of RSV without the potential disturbance of estrogen to data interpretation. In addition, estradiol via estrogen receptor has been reported to play a pivotal role in regulation of insulin-mediated glucose metabolism in both male and female (31-33). Therefore, this study did provide persuasive evidence to implicate the potential therapeutic effect of RSV on systemic and muscular insulin resistance both in male and female.

Recently, oral RSV (22.4 mg/kg/day) administration was shown to improve insulin sensitivity and slightly reduce the body weight in high-calorie-diet-fed mice (12). In addition, RSV (400 mg/kg/day) has been demonstrated to diminish high-fat diet-induced obesity and related insulin resistance via SIRT1-mediated deacetylation of PGC-1α (13). In a more recent report, RSV (0.01-1 μM) enhances insulin sensitivity in C2C12 myotubes by repressing PTP1B in a SIRT1-dependent manner and RSV (2.5 mg/kg/day) is sufficient to attenuate high-fat-diet-induced insulin resistance. The dosages of RSV used in these in vivo and in vitro studies are much lower than those in the above-mentioned reports (25). The wide range of concentrations and doses used to achieve the various effects reported for RSV (~32 nM-100 μM in vitro and ~100 ng-1,500 mg/kg in animals) raises many questions about the concentrations that are achieved or achievable in vivo (34). The dose of RSV applied in our study was 1 mg/kg/day (in vivo) and 0.1-0.3 μM (in vitro), which can possibly be achieved by oral red wine consumption. Recently, it has been demonstrated that a 2-week consumption of red wine (360 ml/day) substantially attenuates insulin resistance in type 2 diabetic patients (35). The calculated maximal concentration of RSV (red wind contained trans-RSV 0.1-14.3 mg/L) in the above case is approximately 73 μg/kg. It is of therapeutic importance to choose a relatively low-dose of RSV for clinical application since lower concentrations mean greater biological safety and lower pharmaceutical cost.

It has been reported that RSV treatment could reduce many of the negative consequences under excess caloric intake, especially its protective effect against high-caloric-diet-induced obesity (12, 13, and 25). Nevertheless, our results demonstrated that RSV diminished HCF diet-induced insulin resistance in the absence of the changes in body weight and caloric intake. Accordingly, under euglycemic hyperinsulinemic condition, insulin-stimulated glucose uptake on epididymal fat pad exhibited no significant difference among the groups; indicating that visceral fat tissue may not be the major target for RSV action on insulin resistance in our model.

The present study was focused on the RSV effect on HCF-fed insulin resistant rats but not on healthy rats. However, our previous study did show that normal SD rats treated with RSV acutely (0.5 mg/kg, 90 minutes after RSV feeding) alone significantly decreased blood glucose as well as improved insulin sensitivity during glucose tolerance test (14). It has been reported that RSV, 400 mg/kg/day for 15 weeks significantly increases the animal’s capability to resist muscle fatigue and concomitantly increased mitochondria activity in absence of body weight change (13).

In conclusion, RSV, an ER agonist, via ER-α activation is critical for its effects on enhancing muscular glucose uptake via both insulin-dependent and independent pathways. The dual effects of RSV on glucose
metabolism are of clinical importance to treat the subjects with type 2 diabetes, especially in those with end-stage diabetes. The metabolic effects of RSV-enhanced ER activation might also provide a valuable new strategy for treating menopause women with type 2 diabetes.

Acknowledgments
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References


Table 1  Metabolic characteristics in rats fed with chow (control), or with high cholesterol-fructose diet for 15 weeks (HCF), the HCF rats were either treated with RSV for 15 days (HCF + RSV 15D) or for 15 weeks (HCF + RSV 15W)

<table>
<thead>
<tr>
<th></th>
<th>Control (n=30)</th>
<th>HCF (n=28)</th>
<th>HCF+RSV 15D (n=15)</th>
<th>HCF+RSV 15W (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>536±9</td>
<td>470±11* (p&lt;0.001)</td>
<td>470±10</td>
<td>484±13</td>
</tr>
<tr>
<td>Caloric intake (kcal/rat/day)</td>
<td>138±7</td>
<td>135±6</td>
<td>125±10</td>
<td>148±8</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>89±5</td>
<td>93±5</td>
<td>95±4</td>
<td>92±3</td>
</tr>
<tr>
<td>Insulin (mg/l)</td>
<td>0.45±0.03</td>
<td>2.84±0.46* (p&lt;0.001)</td>
<td>1.53±0.18†(p&lt;0.05)</td>
<td>0.62±0.05†(p&lt;0.001)</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>73±5</td>
<td>369±21* (p&lt;0.001)</td>
<td>236±14†(p&lt;0.001)</td>
<td>159±10†(p&lt;0.001)</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>95±4</td>
<td>127±4* (p&lt;0.001)</td>
<td>112±3†(p&lt;0.001)</td>
<td>73±3†(p&lt;0.001)</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>28±1</td>
<td>25±1</td>
<td>27±2</td>
<td>30±1†(p&lt;0.05)</td>
</tr>
<tr>
<td>Uric acid (mg/dl)</td>
<td>0.71±0.01</td>
<td>1.08±0.02* (p&lt;0.01)</td>
<td>1.05±0.02</td>
<td>0.83±0.01†(p&lt;0.01)</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>106±1</td>
<td>123±2* (p&lt;0.01)</td>
<td>123±1</td>
<td>119±1</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM (*, versus control; †, versus HCF). HDL, high density lipoprotein; MAP, mean arterial pressure.
FIG. 1.
RSV improved whole body glucose uptake and insulin-stimulated steady-state glucose uptake in HCF-induced insulin resistant rats. Hepatic glucose production (A), whole body glucose uptake (B), and tissue-specific glucose uptake.
FIG. 1. (C-F) under hyperinsulinemic euglycemic condition were performed in control, HCF, HCF + RSV 15D, and HCF + RSV 15W rats. Soleus muscle (C), extensor digitorum longus muscle (D), epididymal fat pad (E), and liver (F) were harvested after 30 minutes of intravenous injection of 2-[\textsuperscript{14}C]deoxyglucose (2-DG). Values of 2-DG uptake were adjusted by the tissues protein content (gm). Graph shows the means ± SE of eight independent experiments (*, versus control; †, versus HCF; n=8 per group). Control, rats fed with chow diet for 15 weeks; HCF, rats fed with high cholesterol-fructose diet for 15 weeks; HCF + RSV 15D, HCF rat treated with RSV for 15 days; HCF + RSV 15W, HCF rat treated with RSV for 15 weeks. Resveratrol (1 mg/kg/day) was suspended in 0.9% saline solution and administered by oral gavage once a day for 15 days or 15 weeks.
FIG. 2.
RSV enhanced GLUT4 translocation to the plasma membrane and elevated insulin receptor (InsR) phosphorylation in insulin resistant soleus muscles under conditions with and without euglycemic hyperinsulinemic clamp (EHC). The intracellular vesicles (IV) and plasma membrane (PM) GLUT1 and GLUT4 protein levels were examined in control, HCF, HCF + RSV 15D, and HCF + RSV 15W rats (A). In RSV-treated HCF rats, phosphorylated InsR protein levels were significantly increased in conditions with and without EHC as compared to the HCF-fed rats.
RSV-stimulating ER activation and glucose uptake

**FIG. 2.**

(B). Equal amounts of proteins were resolved on 10% SDS-PAGE and blotted with respective GLUT1, GLUT4, p-InsRβ (tyr 1146), and InsR antibodies. All blots from PM were stripped and re-probed with an antibody for Na\(^+\)-\(K\)^+ ATPase. All experiments were performed in triplicates from three animals (*, versus control; †, versus HCF; n=3 per group). Upper panels show blots and quantified ratio is shown in the lower panels. Resveratrol (1 mg/kg/day) was suspended in 0.9% saline solution and administered by oral gavage once a day for 15 days or 15 weeks.
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**FIG. 3.**

RSV directly increased estrogen receptor (ER) phosphorylation in vivo (soleus muscles, n=3 per group) and in vitro (C2C12 myotubes, n=3). (A) In RSV (1 mg/kg)-treated HCF rats, phosphorylated ER, but not protein expression, was significantly increased in the conditions with and without euglycemic hyperinsulinemic clamp (EHC) as compared to HCF-fed rats. However, the ER phosphorylation wasn’t enhanced by insulin challenge among the groups (control, HCF, HCF + RSV 15D, and HCF + RSV 15W).
FIG. 3. (B) RSV (0.1 μM) evoked ER protein phosphorylation in C2C12 myotubes with a time-dependent manner. Equal amounts of proteins were resolved on 10% SDS-PAGE and blotted with respective p-ER-α (ser 118) and ER-α antibodies. In vivo experiments were performed in triplicates from three animals (*, versus control; †, versus HCF). In vitro studies, representative images and densitometries from three independent experiments are shown (*P<0.05, **P<0.01, ***P<0.001; compared with basal control). Upper panels show blots for ER-α and p-ER-α. The quantified ratio of ER-α to p-ER-α is shown in the lower panels. B, basal control; I, insulin (0.792 μM, positive control).
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Figure 4

A.

![Western blot images showing PM GLUT4 and Na+/K+ ATPase levels at 1 hr and 14 hr](image1)

B.

![Bar graph showing 2-DG uptake (% of control) at 1 hr and 14 hr](image2)
FIG. 4.
Estrogen receptor (ER) was involved in RSV-stimulated membrane GLUT4 recruitment and glucose uptake in C2C12 myotubes. Plasma membrane GLUT4 protein levels (upper panels; A and C) and 2-DG uptake (lower panels; B and D) were determined after insulin, RSV, estradiol, ICI, insulin and RSV, estradiol and RSV, or ICI and RSV treatment for 1 hr and 14 hrs. The results represent the means ± SE for five independent assays in triplicates (*, versus B, basal control; †, versus RSV, RSV-treated; #, versus I, insulin-treated; n=5). B, basal control; I, insulin (0.792 μM, positive control); RSV, resveratrol (0.1 μM); E, estradiol (0.1 μM, ER agonist); ICI, ICI 182780 (1 μM, ER inhibitor).
FIG. 5.
In C2C12 myotubes, RSV-induced p38, Erk, Akt, and InsR phosphorylation in a time-dependent manner. (A) Equal amounts of proteins were resolved on 10% SDS-PAGE and blotted with respective p-p38 (thr180/tyr 182), p38, p-Erk (thr 202/tyr 204), Erk, p-Akt (thr 308 and ser 473), Akt, p-InsRβ (tyr 1146), and InsR antibodies. (B) is the densitometric measurements of protein bands in (A). Representative images and densitometries from three independent experiments are shown (*P<0.05, **P<0.01, ***P<0.001; compared with basal control).
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**Figure 6**

Erk/p38 and p38/PI3k signaling pathways were involved in RSV-stimulated first and second phases of membranous GLUT4 recruitment and glucose uptake respectively. C2C12 myotubes were co-treated with Erk, p38, and PI3k inhibitors along with or without RSV (0.1 μM) in serum-free media for 1hr or 14 hrs. The plasma membrane GLUT4 protein levels (A) and 2-DG uptake (B) were determined after RSV treatment of 1 hr and 14 hrs. The results represent the means ± SE for five independent assays in triplicate (*, versus B, basal control; †, versus RSV, RSV-treated; n=5). RSV-induced p38
FIG. 6 (C), Erk (D), and InsR (E) protein phosphorylations were mediated by estrogen receptor. C2C12 cells were incubated with or without the ER antagonist (ICI 182780, 1 μM) followed by incubation with RSV (0.1 μM) for 5 min (p38 and Erk phosphorylation) or 20 min (InsR phosphorylation). Cells were lysed then immunoblotted with antibodies against phosphorylated or total protein as indicated. Representative images and densitometries from three independent experiments are shown in lower panels (*, versus B, basal control; †, versus RSV, RSV-treated; n=3). Upper panels show blots for proteins. The quantified ratio of protein is shown in the lower panels. B, basal control; I, insulin (0.792 μM, positive control); W, wortmannin (100 nM, PI3k inhibitor); L, Ly 294002 (100 nM, PI3k inhibitor); P, PD 98059 (10 μM, MEK inhibitor); S, SB 20358 (10 μM, p38 inhibitor); ICI, ICI 182780 (1 μM, ER inhibitor).
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FIG. 7.
Possible model by which RSV stimulated signaling pathways are involved in the GLUT4 translocation and cellular glucose uptake process. The p38 MAPK (mitogen-activated protein kinase) and Erk 1/2 (extracellular signal-regulated protein kinase) seems to be involved in the first phase of RSV effects on the GLUT4 translocation process. Pharmacological inhibition of p38 (SB 2035810) and PI3k (Ly 294002) blunted RSV-induced glucose uptake and GLUT4 membrane translocation, indicating the involvement of p38 and PI3k (phosphoinositide 3-kinase) in the second phase of RSV effects. Activation of estrogen receptor (ER) is essential for RSV-evoked p38, Erk, Akt, and insulin receptor (InsR) phosphorylation. Inhibition of ER (ICI 182780) is sufficient to abrogate RSV-induced GLUT4 membrane translocation and consequently, glucose uptake. Notably, RSV-induced InsR phosphorylation, also dependent of ER activity, is likewise necessary for a complete and efficient RSV-stimulated GLUT4 translocation.