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 (E_2) is known to modulate insulin sensitivity and, consequently, glucose homeostasis. Resveratrol (RSV), an agonist of estrogen receptor (ER), has exerted antihyperglycemic effects in streptozotocin-induced type 1 diabetic rats in our previous study and was 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 AND METHODS—Male Sprague-Dawley rats were given a high cholesterol–fructose (HCF) diet for 15 weeks and were treated with RSV for either 15 days or 15 weeks.

RESULTS—Here, we show that RSV shifts the metabolic characteristics of rats on an HCF diet toward those of rats on a 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 GLUT4 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 an 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 dependent on extracellular signal–related kinase/p38 (early phase, 1 h) and p38/phosphoinositide 3-kinase (late phase, 14 h) activation. 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. *Diabetes* **57:1814–1823, 2008**

Recent investigations have revealed a pivotal role for estradiol (E_2) in regulating energy metabolism and have opened new insights into the physiological role of 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 E_2–ER action for the maintenance of glucose homeostasis (3). Phytoalexin resveratrol (RSV) (3,4′,5-trihydroxy-trans-stilbene) has structural similarities to 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). A previous study (8) has demonstrated that RSV binds ER-β with lower affinity than ER-α. 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 (14) showed that RSV exerted an antihyperglycemic effect in streptozotocin-induced type 1 diabetic rats. Taken together, it is suspected that the activation of the ER signaling pathway might also play a crucial role in the beneficial effects of RSV on insulin resistance 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 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 an obesity-induced insulin-resistant animal model (12,13), HCF diet–fed rats instead of obese rats were chosen in the present study to test whether ER signaling is directly

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TABLE 1
Metabolic characteristics in rats fed with standard diet (control) or with HCF diet for 15 weeks

	Control group	HCF-diet group	HCF + RSV 15D group	HCF + RSV 15W group
n	30	28	15	15
Body weight (g)	536 ± 9	470 ± 11 (<0.001)*	470 ± 10	484 ± 13
Caloric intake (kcal · rat^{-1} · day^{-1})	138 ± 7	135 ± 6	125 ± 10	148 ± 8
Glucose (mg/dl)	89 ± 5	93 ± 5	95 ± 4	92 ± 3
Insulin (mg/l)	0.45 ± 0.03	2.84 ± 0.46 (<0.001)*	1.53 ± 0.18 (<0.05)†	0.62 ± 0.05 (<0.001)†
Cholesterol (mg/dl)	73 ± 5	369 ± 21 (<0.001)*	236 ± 14 (<0.001)†	159 ± 10 (<0.001)†
Triglycerides (mg/dl)	95 ± 4	127 ± 4 (<0.001)*	112 ± 3 (<0.001)†	73 ± 3 (<0.001)†
HDL (mg/dl)	28 ± 1	25 ± 1	27 ± 2	30 ± 1 (<0.05)†
Uric acid (mg/dl)	0.71 ± 0.01	1.08 ± 0.02 (<0.01)*	1.05 ± 0.02	0.83 ± 0.01 (<0.01)†
Mean arterial pressure (mmHg)	106 ± 1	123 ± 2 (<0.01)*	123 ± 1	119 ± 1

Data are means ± SE or means ± SE (*P*). The HCF-diet rats were either treated with RSV for 15 days (HCF + RSV 15D) or for 15 weeks (HCF + RSV 15W). *Versus control; †versus HCF.

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 the 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 diet–induced insulin resistance syndrome. It implicates a therapeutic potential of RSV in the treatment of patients, in particular menopausal women with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Animals and diets. Male Sprague-Dawley 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-h 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 g were randomly assigned to four groups fed regular standard diet (5.1% fat, 23.5% protein, and 50.3% carbohydrate, LabDiet 5010) (control, $n = 30$) or a high-cholesterol diet (4% cholesterol, 10.1% fat, 17% protein, and 51.6% carbohydrate, TD03468; Harlan Teklad, Indianapolis, IN) with 10% fructose in drinking water ($n = 58$) for 15 weeks. Then, the HCF diet–fed rats were further divided into three subgroups concomitantly treated with vehicle (HCF diet group, $n = 28$), RSV for 15 days (HCF + RSV 15D group, $n = 15$), or RSV for 15 weeks (HCF + RSV 15W group, $n = 15$). Resveratrol (1 mg · kg^{-1} · day^{-1}) was suspended in a 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. A separate set of rats ($n = 8$ per group) were killed with an overdose of pentobarbital (100 mg/kg i.p.) at the end of basal and euglycemic-hyperinsulinemic clamp periods to measure the GLUT4 translocation and phosphorylation of insulin resistance and ERs 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 detailed procedures are described in the online appendix (available at http://dx.doi.org/10.2337/db07-1750).

Immunoblotting. To assess GLUT4 and GLUT1 distribution between the intracellular membranes and the plasma membranes, rat soleus muscle and C_2C_{12} 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 mmol/l Tris, pH 7.4; 50 mmol/l glycerophosphate; 20 mmol/l NaF; 2 mmol/l EDTA; 2 mmol/l Na_3VO_4; 250 mmol/l sucrose; and 1% β-mercaptoethanol) with 1 mmol/l phenylmethylsulfonylfluoride as a protease inhibitor. A sample of the crude homogenate was saved for protein determination, and the remainder was centrifuged (15,000*g*). The supernatant was then further centrifuged at 44,000*g*, the pellet discarded, and the liquid phase pelleted at 200,000*g*, resulting in an intracellular membrane–enriched fraction. For plasma membrane enrichment, the 15,000*g* pellet was dissolved in a buffer containing 1% Triton 100 and centrifuged at 200*g*. Fractions enriched in intracellular membrane and plasma membrane were dissolved in sucrose buffer and stored at −70°C. Protein samples of intracellular membranes, plasma membranes, and total lysates were subjected to 10% SDS-PAGE and electrophoretically transferred to polyvinylidene fluoride membrane for 2 h. The membrane was blocked in 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20. It was then washed and blotted with anti-GLUT1, GLUT4 (Chemicon), ER-α, *p*-ER-α-ser 118, insulin receptor (InsR), *p*-InsR-β-tyr 1146 (Cell Signaling), Akt, *p*-Akt-thr308, *p*-Akt-ser 473 (Santa Cruz), p38, *p*-p38-thr 180/tyr 1146, extracellular signal–related kinase (Erk), and *p*-Erk-thr 202/tyr 204 (Chemicon) antibodies. The membrane was then incubated with horseradish peroxidase–conjugated secondary antibody before chemiluminescence detection (Pierce).

2-Deoxy-[^{3}H] glucose uptake in C_2C_{12} myotubes. Mouse C_2C_{12} myoblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C with 95% air and 5% CO_2. To induce differentiation, cells were switched to Dulbecco's modified Eagle's medium containing 1% fetal bovine serum for 3 days. For the glucose uptake assay, cells were cultured on 12-well cluster dishes, washed twice with PBS and incubated in serum-free media for 16 h and then treated with or without insulin (0.792 μmol/l) or RSV (0.1 μmol/l) in Dulbecco's modified Eagle's medium for 1 or 14 h at 37°C. After treating with the agonist, uptake of 2-deoxy-[^{3}H] glucose (0.24 μmol/l) was measured over 10 min. Reactions were terminated by the removal of the permanent solution and rapid washing with ice-cold 20 mmol/l 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 Ly294002 (100 nmol/l, inhibitors of phosphatidylinositol 3-kinase [PI3K]), PD98059 (10 μmol/l, an inhibitor of mitogen-activated protein kinase kinase), SB203580 (10 μmol/l, an inhibitor of p38), or ICI 182780 (1 μmol/l, an inhibitor of ER) for 30 min at 37°C before treatment with RSV (0.1 μmol/l).

Biochemical analysis. Blood was collected from the tail vein for biochemical measurements in experimental rats after overnight fasting. Plasma was used for the measurements of total cholesterol, HDL, triglycerides, and uric acid (Randox reagent kits; Randox Laboratories, Antrim, U.K.). Insulin was measured using a commercially available kit provided by Mercodia (rat insulin enzyme-linked immunosorbent assay 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 the 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 ± SE. The difference in RSV-stimulated 2-deoxy-[^{14}C]glucose (2-DG) uptake was analyzed by one-way ANOVA and followed by Bonferroni's post hoc test. Others were analyzed by Student's *t* test, with the significant difference set at $P < 0.05$.

RESULTS

RSV protects against HCF diet–induced insulin resistance syndrome in rats. As shown in Table 1, the average body weight of HCF diet–fed rats was slightly lower than that of control rats and failed to change after RSV (1 mg · kg^{-1} · day^{-1}) treatment for 15 days or 15 weeks. There was no difference in caloric intake between control rats and

HCF diet–fed rats with or without RSV administration throughout the study. HCF diet feeding for 15 weeks progressively increased the severity of insulin resistance syndrome characterized by elevated blood pressure, increased fasting plasma cholesterol, triglycerides, insulin, and uric acid in a time-dependent manner. However, HCF diet feeding did not alter fasting plasma glucose and HDL levels. RSV treatment for 15 days and 15 weeks time-dependently attenuated the elevated insulin, cholesterol, and triglyceride levels. In addition, RSV treatment for 15 weeks significantly reduced the increased uric acid levels and conversely increased plasma HDL levels in HCF diet–fed rats. The RSV treatment for 15 weeks also slightly lowered mean arterial pressure in HCF diet–fed rats (difference is not significant). These results suggest that RSV shifts the metabolic characteristics of rats on the HCF diet toward 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 diet–induced whole-body and muscular insulin resistance in a euglycemic-hyperinsulinemic clamp study. As shown in Fig. 1, under similar euglycemic and hyperinsulinemic conditions, RSV treatment significantly alleviated HCF diet–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 diet–fed rats in a time-dependent manner (Fig. 1 and online appendix Fig. 1*S*). The improving effect of RSV on insulin-stimulating muscular glucose uptake (soleus muscle) (Fig. 1) was concomitant with increases in GLUT4 membrane translocation and InsR phosphorylation (Fig. 2). Notably, RSV also significantly reversed HCF diet–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 pathways in HCF diet–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 diet–induced insulin-resistant soleus muscles. Taken together with Fig. 2, these results suggest that both RSV treatments for 15 days and 15 weeks could significantly enhance ER and InsR activities as well as GLUT4 translocation to the plasma membrane in HCF diet–induced insulin-resistant soleus muscles (Fig. 3*A*). On the other hand, RSV (0.1 μmol/l) treatment also significantly increased phosphorylation of ER-α at ser118 during the 2-h observation period in C2C12 myotubes (Fig. 3*B*). 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 pads, and hepatic tissue in a dose-dependent manner in normal rats, as shown in online appendix Fig. 2*S*. 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 and 0.3 μmol/l, which is close to those in isolated rat soleus muscle. RSV-stimulated glucose uptake reached a peak level at the 1-h mark (first wave), and then the second phase of activation was observed in 14 h. RSV also directly induced glucose uptake and stimulated GLUT4 translocation to the plasma membrane in a dose-dependent manner in C2C12 myotubes (online appendix Fig. 3*S*).

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, E_2), and ER antagonist (ICI 182780) on glucose uptake of C2C12 myotubes. Both RSV treatments for 1 and 14 h exerted synergistic effect with insulin but not with E_2 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 h) RSV treatment. RSV-induced glucose uptake and GLUT4 membrane translocation for 1 and 14 h 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 a two-wave pattern after RSV administration. These results further support that RSV and E_2 are via the same signaling pathway to enhance muscular glucose uptake. Moreover, the result in online appendix Fig. 4*S* 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 (Figs. 3*B* and 5*A* and *B*). Subsequently, InsR, Akt (thr 308), and Akt (ser 473) were phosphorylated at 10, 20, and 30 min, respectively (Fig. 5*A* and *B*).

Because both RSV treatments for 1 and 14 h have shown to enhance glucose uptake and GLUT4 translocation to the plasma membrane in C2C12 myotubes (as shown in Fig. 4 and online appendix Fig. 3*S*), 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. 6*A*, cotreatment with MEK and p38 inhibitors (PD 98059 and SB 203580), but not PI3K inhibitor (wortmannin and Ly 294002), completely blocked the early-phase effect (1 h) of RSV-stimulating glucose uptake and GLUT4 membrane translocation. Inhibition of p38 and PI3K, but not Erk, blunted the late-phase effect (14 h) of RSV-induced glucose uptake and GLUT4 membrane translocation. These results suggest that the signaling

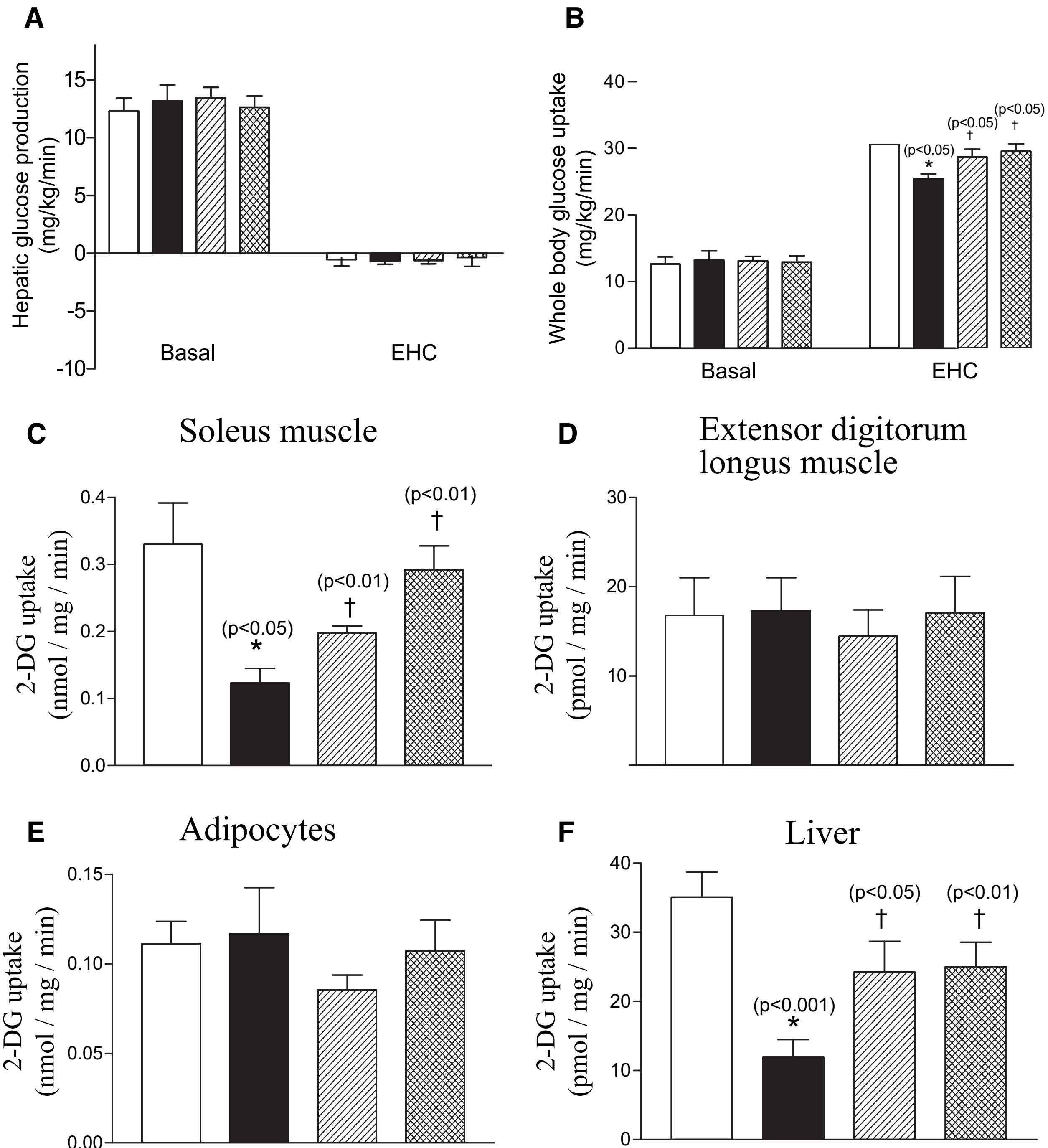

FIG. 1. RSV improved whole-body glucose uptake and insulin-stimulated steady-state glucose uptake in HCF diet–induced insulin-resistant rats. Hepatic glucose production (*A*), whole-body glucose uptake (*B*), and tissue-specific glucose uptake (*C–F*) under hyperinsulinemic-euglycemic conditions were performed in control, HCF diet–fed, 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 min of intravenous injection of 2-DG. Values of 2-DG uptake were adjusted by the tissues protein content (g). Graph shows the means ± SE of eight independent experiments (*Versus control; †versus HCF group; *n* = 8 per group). □, Control (rats fed with standard diet for 15 weeks); ■, HCF (rats fed with HCF for 15 weeks); ▨, HCF + RSV 15D (HCF-fed rats treated with RSV for 15 days); ▩, HCF + RSV 15W (HCF-fed rats treated with RSV for 15 weeks). Resveratrol (1 mg · kg^{-1} · day^{-1}) was suspended in 0.9% saline solution and administered by oral gavage once a day for 15 days or 15 weeks.

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. 6*B*–*D*), indicating that the phosphorylation of p38, Erk, InsR, and Akt is downstream of RSV-activating ER-α–mediated signaling pathway.

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazdium bromide assay was used to evaluate mitochondria activity and cell survival rate in C2C12 myotubes. The results show that mitochondria activity and cell survival

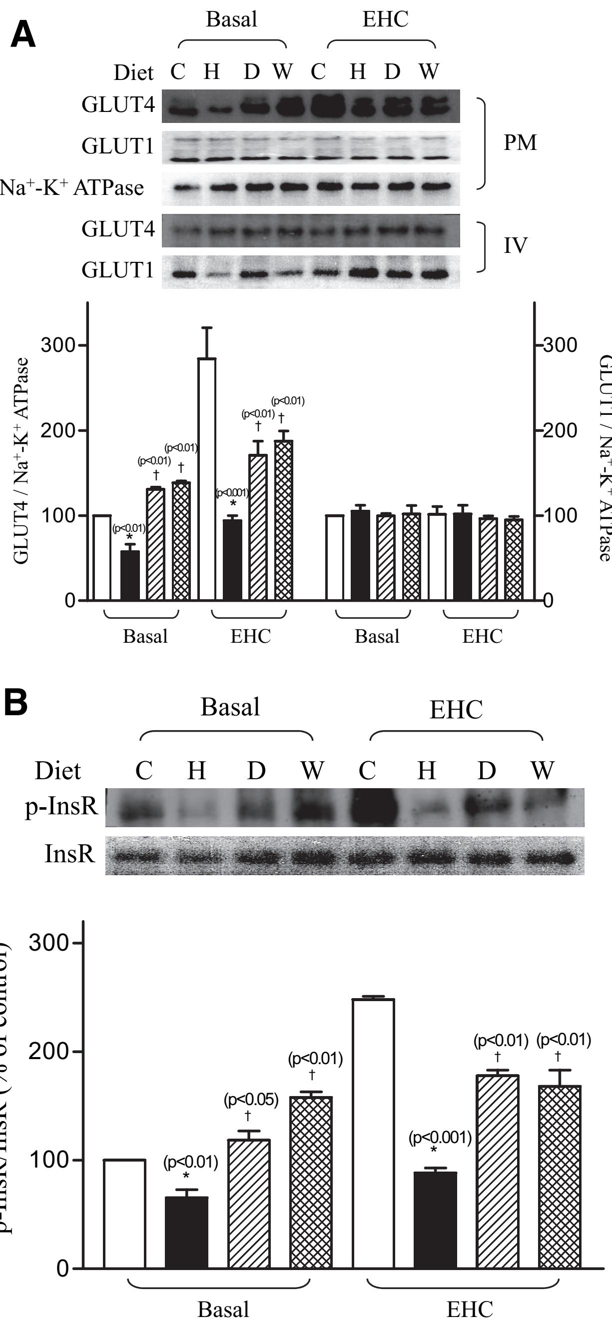

FIG. 2. RSV enhanced GLUT4 translocation to the plasma membrane and elevated insulin receptor (InsR) phosphorylation in insulin-resistant soleus muscles under conditions with and without euglycemic-hyperinsulinemic clamp (EHC). *A*: The intracellular vesicles (IV) and plasma membrane (PM) GLUT1 and GLUT4 protein levels were examined in control (□), HCF (■), HCF + RSV 15D (▨), and HCF + RSV 15W (▩) rats. *B*: In RSV-treated HCF diet–fed rats, phosphorylated InsR protein levels were significantly increased in conditions with and without EHC as compared with the HCF diet–fed rats. 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 reprobed with an antibody for Na^+-K^+ ATPase. All experiments were performed in triplicates from three animals (*Versus control; †versus HCF group; $n = 3$ per group). *Upper panels* show blots and quantified ratio is shown in the lower panels. Resveratrol ($1\ mg \cdot kg^{-1} \cdot day^{-1}$) was suspended in 0.9% saline solution and administered by oral gavage once a day for 15 days or 15 weeks. □, control; ■, HCF; ▨, HCF + RSV 15D; ▩, HCF + RSV 15W.

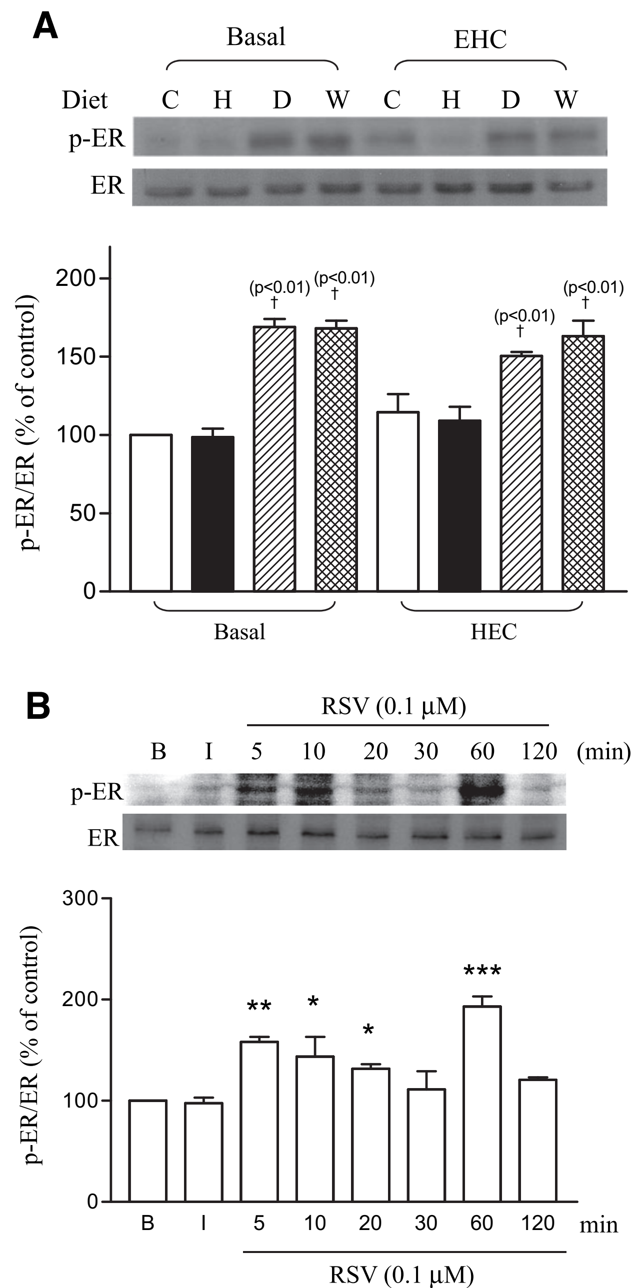

FIG. 3. RSV directly increased ER phosphorylation in vivo (soleus muscles, $n = 3$ per group) and in vitro (C2C12 myotubes, $n = 3$). *A*: In RSV (1 mg/kg)-treated HCF diet–fed rats, phosphorylated ER, but not protein expression, was significantly increased in the conditions with and without euglycemic-hyperinsulinemic clamp (EHC) as compared with HCF diet–fed rats. However, the ER phosphorylation was not enhanced by insulin challenge among the groups (□, control; ■, HCF; ▨, HCF + RSV 15D; ▩, HCF + RSV 15W). *B*: RSV (0.1 μmol/l) evoked ER protein phosphorylation in C2C12 myotubes in a time-dependent manner. Equal amounts of proteins were resolved on 10% SDS-PAGE and blotted with respective *p*-ER-α (ser118) and ER-α antibodies. In vivo experiments were performed in triplicates from three animals (*Versus control; †versus HCF group). In vitro studies, representative images, and densitometries from three independent experiments are shown (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. 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 μmol/l, positive control).

rate were not affected by inhibitors (SB 203580, PD 98059, Ly 294002, wortmannin, and ICI 182780) treated alone or in 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. 6*A*).

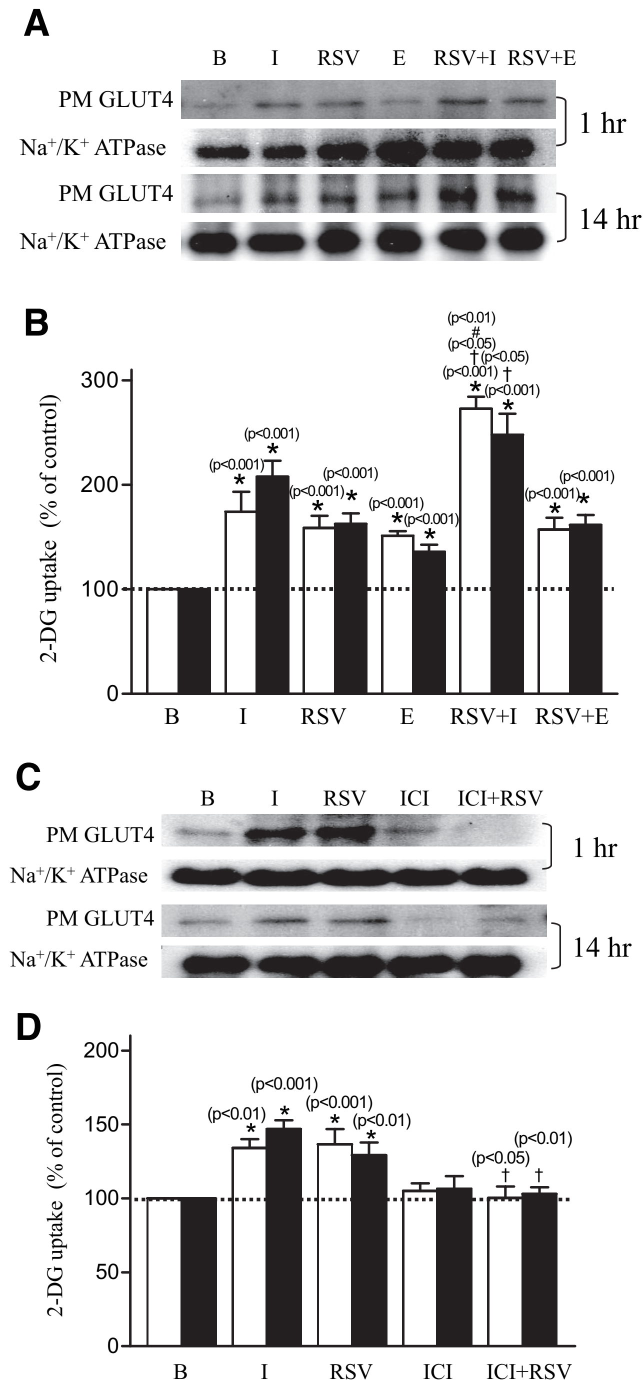

FIG. 4. ER was involved in RSV-stimulated membrane GLUT4 recruitment and glucose uptake in C2C12 myotubes. Plasma membrane (PM) GLUT4 protein levels (*A* and *C*, *upper panels*) and 2-DG uptake (*B* and *D*, *lower panels*) were determined after insulin, RSV, estradiol, ICI, insulin and RSV, estradiol and RSV, or ICI and RSV treatment for 1 h (□) and 14 h (■). 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 μmol/l, positive control); RSV, RSV (0.1 μmol/l); E, estradiol (0.1 μmol/l, ER agonist); ICI, ICI182780 (1 μmol/l, ER inhibitor).

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 (22,23) have indicated that E_2 via ER-α– and ER-β–mediated signaling pathways participate in glucose homeostasis by modulating the expression of genes involved in insulin sensitivity and glucose uptake. 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 diet–fed 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 diet–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 results have 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 diet–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 diet–induced insulin resistance in rats. Furthermore, RSV has been shown 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 of the present observation, our previous study (14) also demonstrated that RSV exerted antihyperglycemic effects without altering the plasma insulin levels in streptozotocin-induced type 1 diabetic rats, suggesting that the metabolic effect of RSV was not solely due to improved insulin action on insulin-dependent tissues. On the other hand, the present results from C2C12 myotubes show that RSV could stimulate ER-α, p38, and Erk phosphorylation at first, followed by InsR and Akt phosphorylation. Moreover, the synergistic effect of RSV and insulin was diminished after RSV treatment for a longer period (14 h). 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 results show 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 (extensor digitorum longus, vastus lateralis white muscle). Soleus and vastus lateralis (red) muscles are slow-twitch muscle fibers. The slow-twitch muscle fibers usually account for

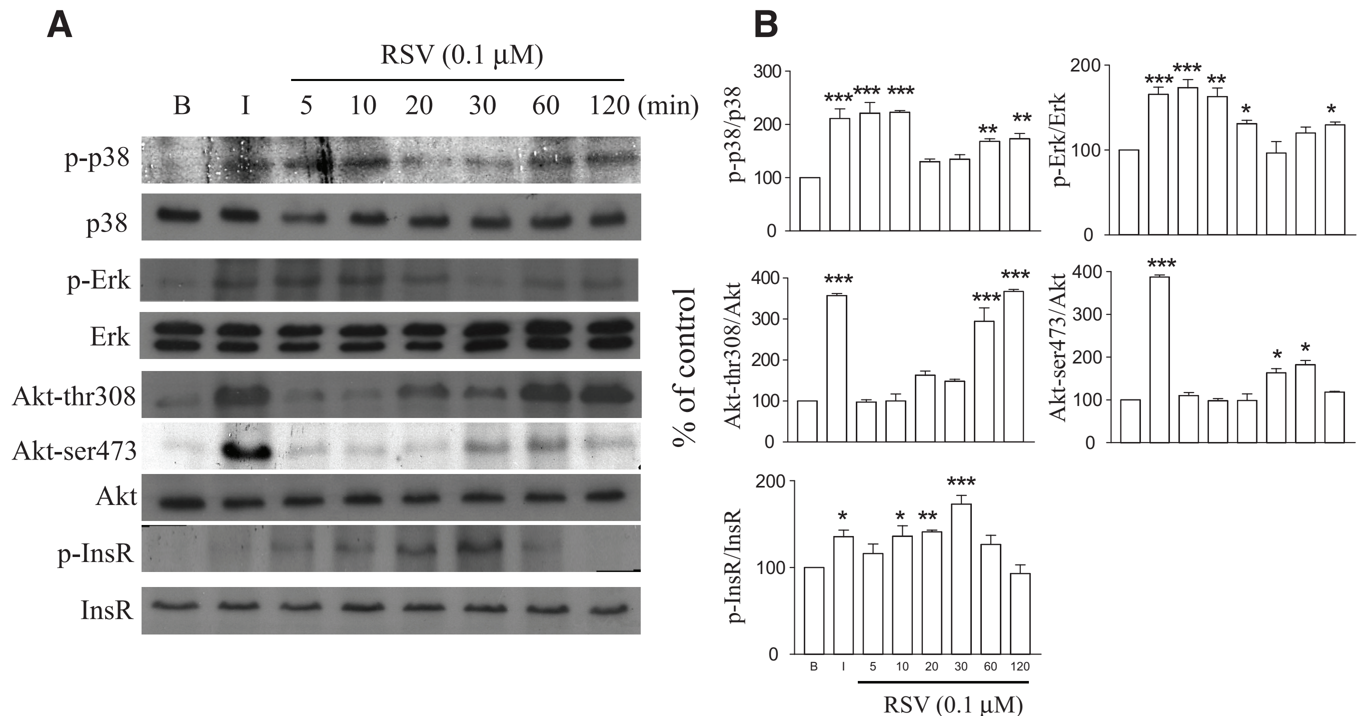

FIG. 5. In C2C12 myotubes, RSV-induced p38, Erk, Akt, and InsR phosphorylation in a time-dependent manner. *A*: Equal amounts of proteins were resolved on 10% SDS-PAGE and blotted with respective *p*-p38 (thr180/tyr182), p38, *p*-Erk (thr202/tyr204), Erk, *p*-Akt (thr308 and ser473), Akt, *p*-InsRβ (tyr1,146), and InsR antibodies. *B*: 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 vs. basal control).**

~50% or more 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 upregulates slow-twitch 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, E_2 has been considered 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 (NAD^+-dependent deacetylase) activator and could affect the regulation of energy homeostasis via modulation of peroxisome proliferator–activated receptor-γ coactivator-1α functions (12,13,25). 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 Sprague-Dawley 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 males and females (31–33). Therefore, this study did provide persuasive evidence to implicate the potential therapeutic effect of RSV on systemic and muscular insulin resistance in both males and females.

Recently, oral RSV (22.4 mg · kg^{-1} · day^{-1}) 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^{-1} · day^{-1}) has been demonstrated to diminish high-fat diet–induced obesity and related insulin resistance via SIRT1-mediated deacetylation of peroxisome proliferator–activated receptor-γ coactivator-1α (13). In a more recent report, RSV (0.01–1 μmol/l) enhances insulin sensitivity in C2C12 myotubes by repressing PTP1B in a SIRT1-dependent manner, and RSV (2.5 mg · kg^{-1} · day^{-1}) 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 (~32–100 μmol/l in vitro and ~100–1,500 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^{-1} · day^{-1} (in vivo) and 0.1–0.3 μmol/l (in vitro), which can possibly be achieved by oral red wine consumption. Recently, it was 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 wine contains 0.1–14.3 mg/l trans-RSV) in the above case is ~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

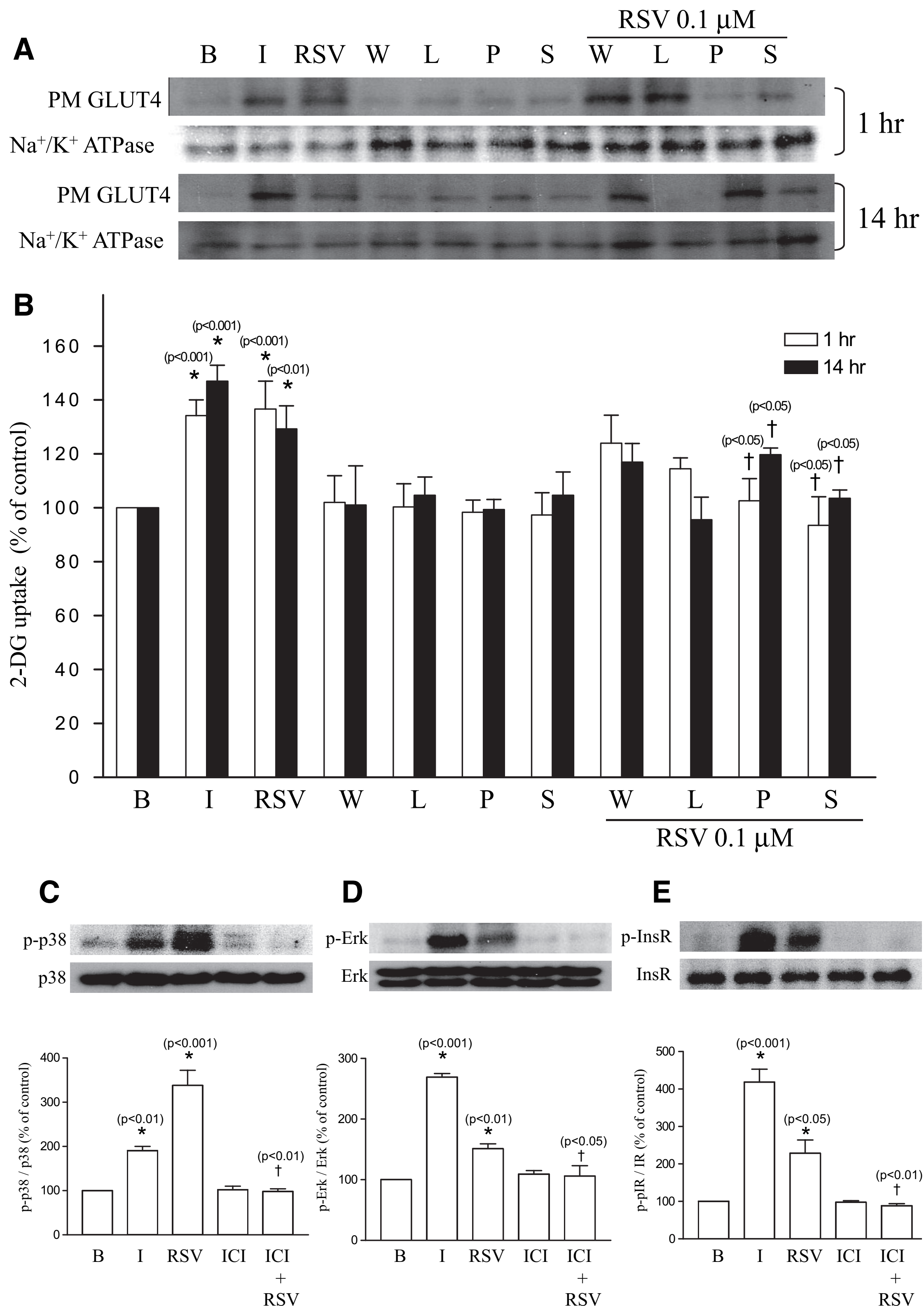

FIG. 6. Erk/p38 and p38/PI3K signaling pathways were involved in RSV-stimulated first and second phases of membranous GLUT4 recruitment and glucose uptake, respectively. C2C12 myotubes were cotreated with Erk, p38, and PI3K inhibitors along with or without RSV (0.1 μmol/l) in serum-free media for 1 h (□) or 14 h (■). The plasma membrane GLUT4 protein levels (*A*) and 2-DG uptake (*B*) were determined after RSV treatment of 1 and 14 h. The results represent the means ± SE for five independent assays in triplicate (*Versus B, basal control; †versus RSV, RSV-treated group; *n* = 5). RSV-induced p38 (*C*), Erk (*D*), and InsR (*E*) protein phosphorylations were mediated by estrogen receptor. C2C12 cells were incubated with or without the ER antagonist (ICI182780, 1 μmol/l) followed by incubation with RSV (0.1 μmol/l) for 5 min (p38 and Erk phosphorylation) or 20 min (InsR phosphorylation). Cells were lysed and then immunoblotted with antibodies against phosphorylated or total protein as indicated. Representative images and densitometries from three independent experiments are shown in the *lower panels* (*Versus B, basal control; †versus RSV, RSV-treated group; *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 μmol/l, positive control); W, wortmannin (100 nmol/l, PI3K inhibitor); L, Ly294002 (100 nmol/l, PI3K inhibitor); P, PD98059 (10 μmol/l, MEK inhibitor); S, SB20358 (10 μmol/l, p38 inhibitor); ICI, ICI182780 (1 μmol/l, ER inhibitor).

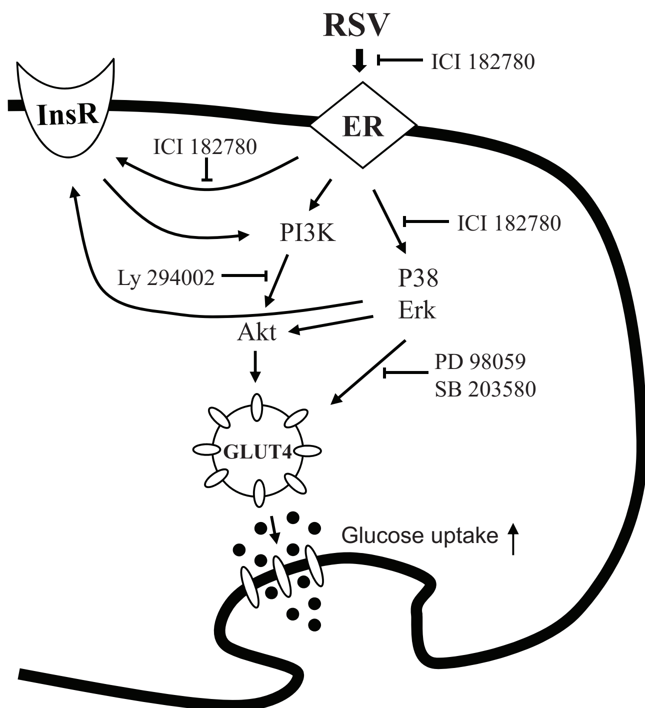

FIG. 7. Possible model by which RSV-stimulated signaling pathways are involved in the GLUT4 translocation and cellular glucose uptake process. The p38 mitogen-activated protein kinase and Erk1/2 seems to be involved in the first phase of RSV effects on the GLUT4 translocation process. Pharmacological inhibition of p38 (SB2035810) and PI3K (Ly294002) blunted RSV-induced glucose uptake and GLUT4 membrane translocation, indicating the involvement of p38 and PI3K in the second phase of RSV effects. Activation of ER is essential for RSV-evoked p38, Erk, Akt, and InsR phosphorylation. Inhibition of ER (ICI182780) 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.

intake, especially its protective effect against high-caloric diet–induced obesity (12,13,25). 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 conditions, 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 diet–fed insulin-resistant rats but not on healthy rats. However, our previous study (14) did show that normal Sprague-Dawley rats treated with RSV acutely (0.5 mg/kg, 90 min after RSV feeding) alone significantly decreased blood glucose as well as improved insulin sensitivity during a glucose tolerance test. It has been reported that 400 mg $\cdot$ kg^{-1} $\cdot$ day^{-1} RSV 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 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 menopausal women with type 2 diabetes.

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