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Stimulation of muscle cell glucose uptake by resveratrol through sirtuins and AMPK

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ABSTRACT

Although recent studies *in vitro* and *in vivo* indicate that the polyphenol resveratrol (RSV) has anti-diabetic properties, the exact mechanisms involved are not known. In the present study, we examined the effects of RSV and the mechanism of regulation of glucose uptake in skeletal muscle cells.

In L6 myotubes RSV (100 μM) induced maximum stimulation of glucose (2DG) uptake (201 ± 8.90% of control, $p < 0.001$), an effect that was similar to insulin action. RSV-stimulated glucose uptake was abolished by AMPK inhibition. In the presence of the sirtuin inhibitor nicotinamide, RSV-stimulated 2DG uptake and AMPK phosphorylation were abolished. RSV did not stimulate significant translocation of GLUT4 or GLUT1 transporters. However, treatment with indinavir, a GLUT4 specific inhibitor, blocked RSV-stimulated glucose uptake. We propose that RSV elevates glucose uptake in muscle cells through a mechanism that involves sirtuins and AMPK and possibly stimulation of GLUT4 transporter intrinsic activity.

Skeletal muscle is responsible for more than 75% of glucose disposal in response to insulin in the post-prandial state and is therefore quantitatively the most important glucose-utilizing tissue. Defects in the muscle glucose transport system can lead to insulin resistance [1].

The insulin signalling pathway leading to increased muscle glucose uptake involves binding of insulin to its receptor, phosphorylation of downstream insulin receptor substrates (IRS) and activation of phosphatidylinositol-3 kinase (PI3-K) and Akt which promotes GLUT4 glucose transporter translocation from an intracellular pool to the plasma membrane [2,3].

AMP-activated protein kinase (AMPK) is a heterotrimeric ser/thr kinase that functions as a cellular energy sensor that becomes activated when the cellular AMP/ATP ratio is increased [4] and in recent years has become an attractive pharmacological target for the treatment of insulin resistance and type 2 diabetes. In skeletal muscle, AMPK is activated by exercise/contraction (reviewed in [5]), metformin [6,7], and thiazolidinediones [7] resulting in an increase in glucose uptake. An in depth understanding of the glucose transport mechanism in skeletal muscle cells and the finding of new compounds that stimulate glucose uptake could provide new options for the treatment of insulin resistance and type 2 diabetes.

The polyphenol resveratrol (RSV) (*trans*-3, 4′, 5-trihydroxystilbene) is found in the skin of grapes and in highest concentration in red wine. The many health benefits of RSV have been the focus of many papers and reviews [8,9]. RSV has been shown to have anti-oxidant, anti-cancer, and anti-cardiovascular disease properties and to prolong life span in mice [8–10].

Recent *in vitro* and *in vivo* studies have shown RSV to have anti-diabetic properties [10–14]. RSV has been reported to stimulate glucose uptake [12] and abolish the palmitate-induced impairment in insulin signaling in C_2C_{12} muscle cells [11]. *In vivo*, RSV was shown to protect mice against high-fat diet-induced insulin resistance [10,14] and to attenuate the increase in blood glucose, lipid levels and diabetic symptoms in streptozotocin-induced diabetic rats [13].

The mechanism(s) by which RSV exerts its anti-diabetic properties are not clear. A potentiation of the insulin signaling cascade and/or activation of AMPK have been proposed [8,10,11]. RSV has been reported to be an activator of sirtuins, a family of histone/protein deacetylases (HDACs) named after their homology to the *Saccharomyces cervisiae* gene silent information regulator 2 (Sir2) [15]. Seven mammalian sirtuins (SIRT1-7), family of class III NAD^+-dependent HDACs, exist and evidence indicate that they regulate many important biological processes ranging from life span extension, to apoptosis, energy metabolism, muscle differentiation, and gluconeogenesis in mammals [14,15]. SIRT1 has been suggested to play a role in regulating glucose homeostasis [14,16] and may be involved in the insulin signalling cascade [17].

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The role of sirtuins on muscle glucose transport has not been examined. In addition, although stimulation of glucose transport by RSV has been reported previously, its mechanism of action and the effect on glucose transporters is not known.

In the present study, we examined the effects of RSV on glucose uptake and glucose transporter translocation and attempted to elucidate the RSV mechanism of action in L6 skeletal muscle cells. Our data show that RSV increases glucose uptake without stimulating translocation of glucose transporters. RSV-induced AMPK phosphorylation and glucose uptake were abolished by the sirtuin inhibitor nicotinamide indicating that sirtuins play a role in muscle glucose transport.

Materials and methods

Materials. All tissue culture materials were purchased from GIBCO Life Technologies (Burlington, ON). Antibodies against phosphorylated and total Akt, AMPK, mTOR, p70S6 K, HRP-conjugated anti-rabbit secondary antibody, and ChemiGLOW were from New England Biolabs (Mississauga, ON, Canada). 9E10 anti-myc monoclonal antibody was from Santa Cruz (Santa Cruz, CA) and HRP-conjugated donkey anti-mouse IgG from Jackson ImmunoResearch Labs (West Grove, PA). Compound C was from Calbiochem (New Jersey) and [^{3}H] 2-deoxy-D-glucose from Perkin-Elmer (Boston, MA). All other chemicals including RSV, nicotinamide and cytochalasin B (CB) were from Sigma (St. Louis, MO).

Cell culturing and 2-deoxy-D-glucose uptake. L6 rat skeletal muscle cells (wild-type, GLUT4*myc* and GLUT1*myc* overexpressing) were grown in α-MEM as previously described [18]. The final concentration and the time of incubation for each compound are indicated in each figure (a vehicle control group was always included). [^{3}H] 2-deoxy-D-glucose uptake measurements performed as described previously [18]. Cellular protein content was measured by the Bio-Rad Protein Assay method.

Measurement of GLUT4myc and GLUT1myc translocation in L6 myotubes. After treatment myotubes were fixed with 3% paraformaldehyde for 3 min at RT, incubated with 1% glycine, blocked with 10% goat serum and 3% BSA in PBS, and then exposed to anti-myc antibody followed by incubation with peroxidase-conjugated donkey anti-mouse IgG. Cells were washed, and the OPD reagent was added for 30 min at RT. The reaction was stopped with 3 N HCl. The supernatant was collected, and the absorbance was measured.

Western blotting. Equal amounts of protein samples (12 μg) were separated by SDS–PAGE, transferred to PVDF membrane, blocked with 5% (w/v) dry milk in TBS and incubated with primary antibody overnight at 4 °C. The primary antibody was detected with HRP-conjugated anti-rabbit secondary and ChemiGLOW reagent and visualized by autoradiography using Kodak XAR-5 film or FluroChem software (Thermo Fischer).

Statistical analysis. The significance of the differences between groups was determined using analysis of variance (ANOVA) followed by Tukey's post-hoc analysis. Differences were considered statistically significant at $P < 0.05$. Calculations were preformed using SPSS v14.0 software.

Results

Myotubes were incubated with various concentrations of RSV as indicated in Fig. 1A, followed by glucose uptake measurements. RSV concentrations of 1, 5, and 10 μM did not affect glucose uptake ($p > 0.05$) while a significant increase was observed at 25 μM (139 ± 5.61% of control, $p < 0.05$) with maximum response reached at 100 μM (201 ± 8.90%, $p < 0.001$). Higher RSV concentrations, 125 μM (200 ± 12.61%, $p < 0.001$) and 150 μM (data not shown), did not result in any greater stimulation of glucose uptake. Cell morphology, observed microscopically, was not affected by treatment. The effect of RSV did not require the presence of insulin and in parallel experiments, maximum insulin-stimulated glucose uptake was 208 ± 10.87% of control ($p < 0.001$). The effect of RSV on glucose uptake was time-dependent with a significant increase seen after 30 min (141 ± 6.50%, $p < 0.05$) and maximum response at 120 min (201 ± 8.90%, $p < 0.001$) (Fig. 1B).

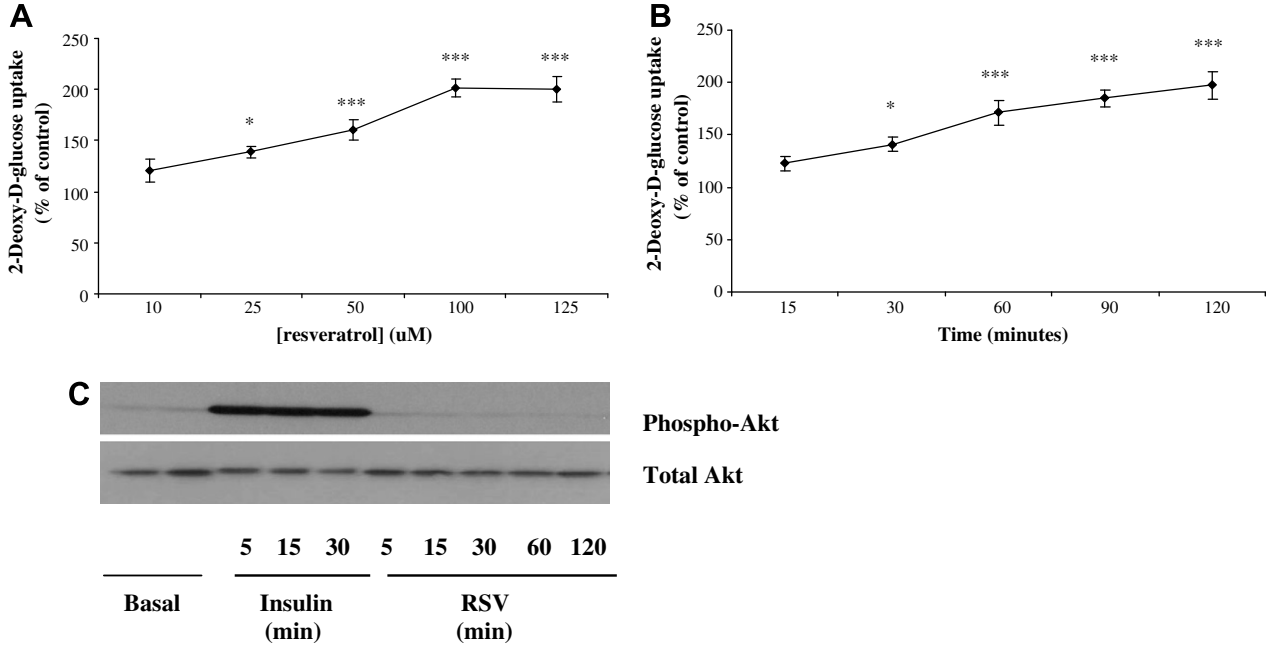

Fig. 1. Effects of RSV on 2DG uptake and Akt phosphorylation. L6 myotubes were incubated with the indicated concentrations of RSV for 120 min (A) or with 100 μM RSV for the indicated time (B) followed by 2DG uptake measurement. The results are the mean ± SE of 4–8 independent experiments, each performed in triplicate. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control. (C) Cell lysates were prepared from cells treated with 10^{-7}M insulin or 100 μM RSV and immunoblotted with specific antibodies that recognize total or phosphorylated Akt. A representative immunoblot of three experiments is shown.

In view of these data, we examined the effect of RSV on the phosphorylation of Akt, a key player of insulin-stimulated glucose uptake. Insulin caused a robust phosphorylation of Akt/PKB while RSV treatment had no significant effect (Fig. 1C) implying that Akt is not involved in the action of RSV.

Hypoxia, metformin, and thiazolidinediones have been shown to increase glucose uptake in skeletal muscle [5] through AMPK phosphorylation. We therefore examined the effect of RSV on AMPK phosphorylation. Our data indicate that RSV significantly stimulated AMPK phosphorylation (15 min = 4.10 ± 0.75 fold of control, $p < 0.05$; Fig. 2A and B). The total levels of AMPK were not changed by any treatment (Fig. 2C). Insulin did not significantly increase AMPK phosphorylation (1.32 ± 0.04, $p > 0.05$) (Fig. 2A and B). Phosphorylation of the catalytic α-subunit on Thr 172 results in activation of AMPK. To assess AMPK activity we examined mTOR phosphorylation the downstream target of AMPK as well as phosphorylation of p70 S6K. Activated AMPK results in inhibition of mTOR and p70 S6K phosphorylation [19]. RSV inhibited both the basal and insulin-stimulated mTOR and p70 S6K phosphorylation (Fig. 2D) indicating that the activity of AMPK was increased by RSV.

To examine whether the RSV stimulation of glucose uptake was mediated by AMPK activation we pre-incubated the cells with compound C (50 μM), an inhibitor of AMPK [6], for 30 min before stimulation with RSV. Compound C abolished the RSV-induced increase in AMPK phosphorylation (Fig. 3A) and the increase in glucose uptake seen with RSV treatment (86 ± 6.31%) (Fig. 3B), indicating that AMPK activation plays an important role in the RSV mechanism of stimulation of glucose uptake.

RSV is an activator of sirtuins [20] and therefore it is possible that sirtuins are involved in the stimulation of glucose uptake seen with RSV. To investigate the possible involvement of sirtuins and whether they are upstream of AMPK, we incubated the cells with the sirtuin inhibitor nicotinamide [21] followed by stimulation with RSV. Our data show that nicotinamide abolished the RSV-induced increase in AMPK phosphorylation (Fig. 3C) suggesting that sirtuins mediate the increase in AMPK phosphorylation seen with RSV. We also used splitomicin, a cell permeable SIRT1 selective inhibitor [22,23] and found that RSV-stimulated AMPK phosphorylation was abolished (data not shown) suggesting that the effect of RSV may be mediated by SIRT-1. Importantly, nicotinamide abolished the RSV-induced increase in glucose uptake (Fig. 3D) indicating an important role of sirtuins in RSV-stimulated glucose uptake.

Insulin, through activation of the PI3K-Akt signalling pathway, and stimuli such as hypoxia, metformin and thiazolidinediones though activation of AMPK, stimulate glucose uptake in muscle cells via a well documented recruitment of glucose transporters from an intracellular storage compartment to the plasma membrane [5,7,24]. To investigate whether RSV also stimulates glucose uptake by increasing glucose transporter translocation we examined the effect of RSV on plasma membrane GLUT4 and GLUT1 glucose transporters in L6 cells overexpressing GLUT4*myc* and GLUT1*myc*, respectively. In GLUT4*myc* cells insulin increased plasma membrane GLUT4 by 194 ± 11.66% of control cells ($p < 0.001$)

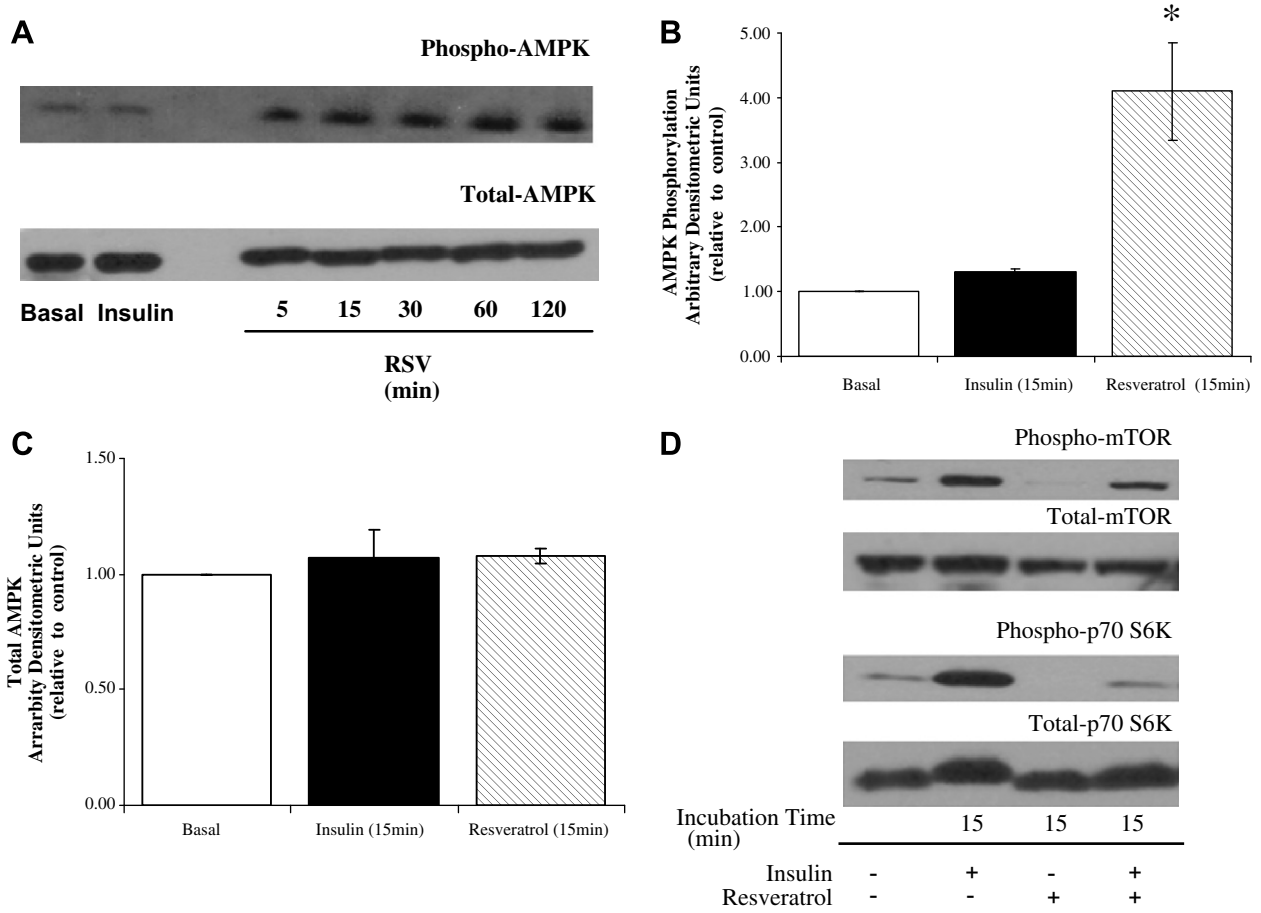

Fig. 2. (A) AMPK phosphorylation by RSV. Cells were treated with 10^{-7}M insulin or 100 μM RSV. Lysates were immunoblotted with specific antibodies against total or phosphorylated AMPK. (B and C) Immunoblots were scanned to quantitate the density of the bands. Results are the mean ± SE of four experiments. Values are arbitrary densitometric units expressed relative to control. $^{*}p < 0.05$ (D) Total and phosphorylated mTOR and p70 S6 K. The cells were incubated in the absence or the presence of 100 μM RSV and /or 100 nM insulin for 15 min.

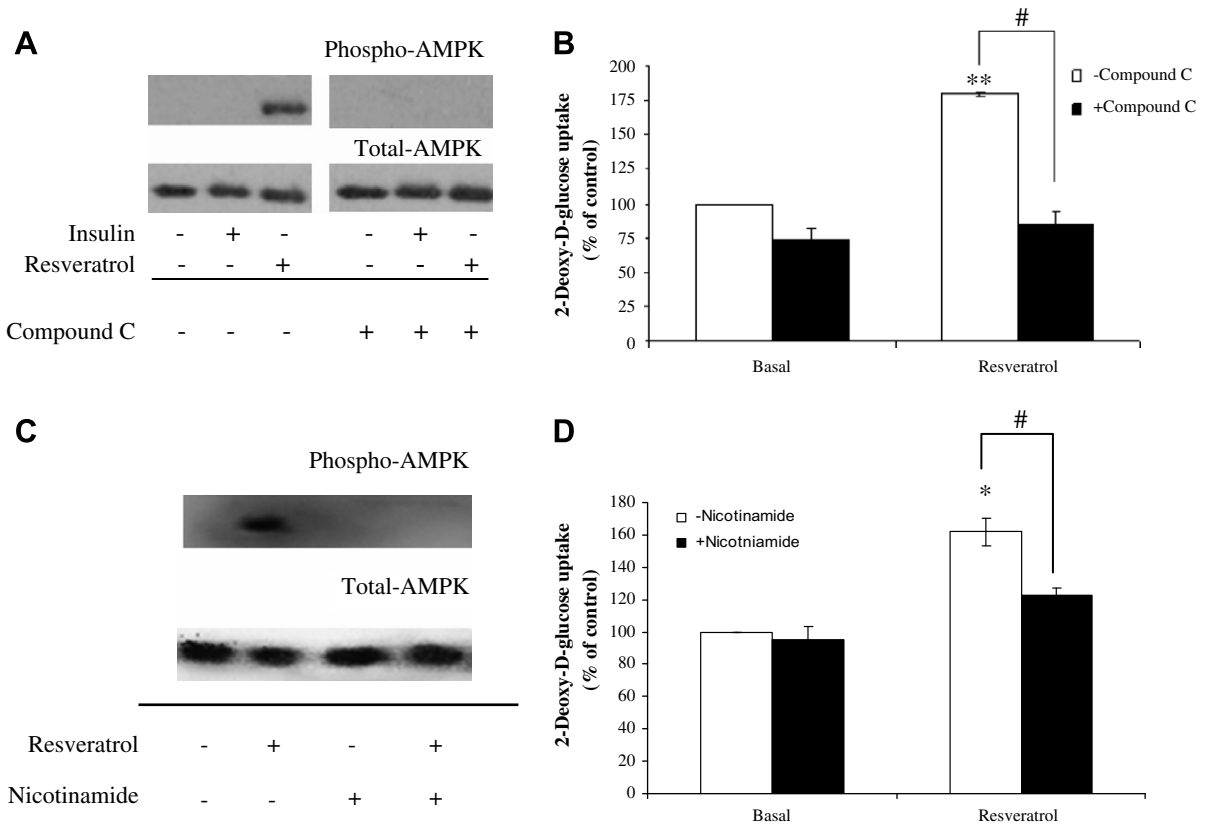

Fig. 3. Effect of Compound C on resveratrol-stimulated AMPK phosphorylation (A) or glucose transport (B). Cells were incubated in the absence (□) or presence of 50 μM Compound C (■) for 30 min, followed by the addition of 100 μM RSV or 100 nM insulin, cell lysis and immunoblotting for total and phosphorylated AMPK (A) or 2DG uptake measurement (B). Effect of nicotinamide on RSV-stimulated AMPK phosphorylation (C) or glucose uptake (D). Cells were treated with or without 6 mM nicotinamide for 6 h followed by stimulation with or without 100 μM RSV, cell lysis and immunoblotting for total or phosphorylated AMPK (C) or 2DG uptake measurement (D). Mean ± SE of four experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, vs. control, $^{\#}p < 0.05$ vs. RSV alone.

but interestingly no significant changes were seen on plasma membrane GLUT4 with RSV (78 ± 9.81%, $p > 0.05$) (Fig. 4A), despite a significant stimulation of glucose uptake (Fig. 4C). The GLUT1 glucose transporter is also expressed in the L6 cell line and although it is primarily responsible for basal glucose uptake, it is also responsive to insulin but to a much smaller degree than GLUT4 [25]. To explore the possibility that RSV may elevate the plasma membrane GLUT1 levels, we examined the effect of RSV in GLUT1-*myc* cells. However, no significant changes were seen with RSV (98 ± 3.6%, $p > 0.05$) while insulin increased plasma membrane GLUT1 by 145 ± 2.8%, $p < 0.001$, (Fig. 4B). These results indicate that GLUT4 and GLUT1 translocation to the plasma membrane is not likely the mechanism contributing to the RSV stimulation of glucose uptake. It is possible that RSV increases glucose transport by modulating the activity of glucose transporters. Since RSV did not significantly increase glucose uptake in parental myoblasts expressing mainly GLUT1 (Fig. 4C), its effect may be due to alteration in GLUT4 glucose transporter activity and not GLUT1.

Indinavir is a protease inhibitor reported to specifically inhibit GLUT4 glucose transporter-mediated transport [26,27] by direct binding to and blocking the GLUT4 transporter protein [28]. To examine the involvement of GLUT4 activity in RSV-stimulated glucose uptake we measured glucose uptake in the presence of 50 μM indinavir. Both insulin (196 ± 6.3%, $p < 0.001$)- and RSV (179 ± 19%, $p < 0.05$)-stimulated glucose uptake were abolished by indinavir (111 ± 19.0% and 95 ± 13%, respectively, both $p < 0.05$), indicating that an increase in GLUT4 intrinsic activity may mediate the majority of the RSV-stimulated glucose uptake in muscle cells (Fig. 4D).

Discussion

Our data in L6 cells show a significant increase in glucose uptake by RSV in the absence of insulin in agreement with studies using isolated soleus muscle strips [13] and C_2C_{12} cells [12]. The concentration of RSV (micromolar range) required to achieve significant stimulation in our system is in agreement with other *in vitro* studies [12,23] indicating that RSV, at pharmacological concentrations, may have a significant role in increasing glucose uptake in normal and insulin resistant muscle [11,12].

AMPK is an important energy sensor in mammalian cells [4]. In skeletal muscle cells AMPK may be activated by contraction or by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) leading to increase in glucose uptake [5]. Metformin [6] and thiazolidinediones [7] which are used in the treatment of type 2 diabetes, have been shown to increase glucose uptake via AMPK. Our studies showed a rapid and significant increase in AMPK phosphorylation by RSV in agreement with other recent studies in hepatocytes [23] and C_2C_{12} mouse muscle cells [12]. Using compound C, an inhibitor of AMPK, we demonstrated a significant reduction in RSV-stimulated glucose uptake providing support of the notion that AMPK is a mediator of the RSV effects on glucose uptake. We measured phosphorylation levels of mTOR, the downstream target of AMPK, and also p70 S6K, which is downstream of mTOR [29]. Activated AMPK results in inhibition of mTOR [19]. Our data show that RSV inhibited the phosphorylation of both mTOR and its downstream target p70 S6K. mTOR is also downstream of Akt [19]. However we did not see any changes in Akt phosphorylation with RSV.

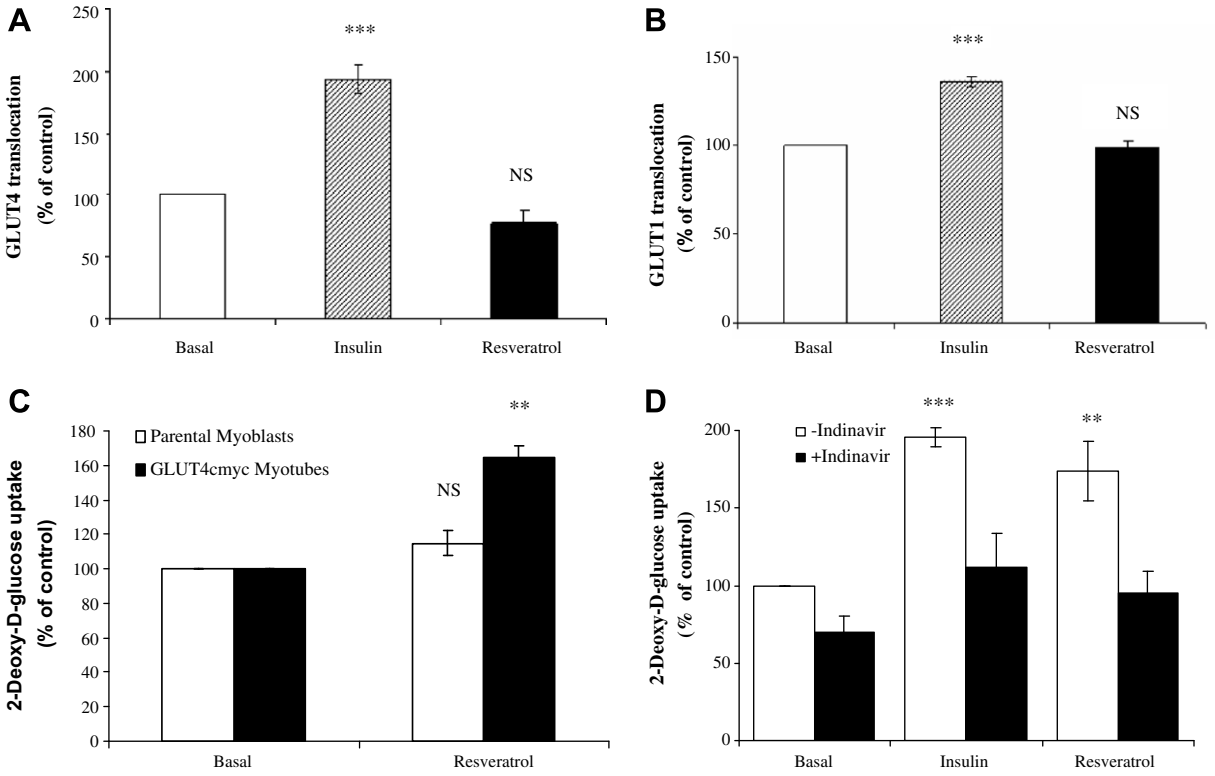

Fig. 4. Effect of resveratrol or insulin on plasma membrane GLUT4 (A) and GLUT1 levels (B). GLUT4*myc* (A) or GLUT1*myc* (B) overexpressing myotubes were treated with RSV (100 μM, 120 min) (■) or insulin (10^{-7}M, 30 min) (▒) followed by GLUT4 or GLUT1 transporter translocation measurements. (C) Effects of RSV on 2DG uptake in L6 parental myoblasts and GLUT4*myc* tagged myotubes. Cells were incubated with RSV (100 μM, 120 min) followed by 2DG uptake measurements. (D) Effect of indinavir on RSV- and insulin- stimulated glucose transport. L6 parental cells were incubated in the absence (□) or presence of indinavir (■), followed by the addition of RSV (100 μM, 120 min) or insulin (10^{-7} M, 30 min). Mean ± SE of 4–5 experiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

The cellular mechanism through which AMPK activation leads to increased glucose uptake in response to various stimuli is unclear. Hypoxia stimulates glucose uptake through AMPK-dependent GLUT4 translocation [30]. However, AICAR, an AMPK activator, increased glucose uptake but not GLUT4 translocation to transverse tubules representing 60–80% of the total surface area of skeletal muscle [31] raising the possibility that AMPK activation may also regulate glucose transporter activity. Interestingly, we observed that RSV stimulated glucose uptake without a parallel increase in GLUT4*myc* or GLUT1*myc* translocation to the plasma membrane. This was in contrast to the well documented insulin-induced GLUT4 and GLUT1 translocation. RSV may stimulate the activity of the GLUT4 or GLUT1 transporters already present at the plasma membrane. Activation of AMPK using AICAR has been suggested to enhance the intrinsic activity of GLUT4 protein in 3T3-L1 adipocytes [32] and GLUT1 in clone 9 cells [33,34] and C_2C_{12} myoblasts [35]. To examine whether RSV may modulate GLUT4 intrinsic activity to induce the enhancement of glucose uptake, we used indinavir to selectively block the GLUT4 transporter in wild-type L6 cells as shown previously [26]. Indinavir treatment was successful in blocking both insulin- and RSV-stimulated glucose uptake. In addition, no significant stimulation of glucose uptake was seen with RSV treatment in parental myoblasts expressing mainly GLUT1. In contrast, RSV induced significant stimulation of glucose uptake in GLUT4*myc* cells. Taken together, these data suggest that RSV may stimulate glucose uptake through an increase in the intrinsic activity of GLUT4 glucose transporters. Alternatively, RSV may stimulate glucose transport indirectly by increasing the glucose gradient because of increased intracellular glucose metabolism.

RSV is an activator of SIRT-1 [20]. Although SIRT-1 has been reported very recently to mediate the anti-diabetic effects of RSV in hepatocytes [23] and to be involved in glucose-stimulated insulin secretion in beta cells [36], its role in regulating muscle glucose transport has not been previously examined. The blockage in RSV-stimulated glucose uptake seen with nicotinamide indicates that sirtuins may play an important role. Furthermore, the observation that RSV-stimulated AMPK phosphorylation is abolished by nicotinamide and splitomicin suggests that sirtuins are upstream of AMPK. This is in agreement with a recent study by Hou et al. [23] showing that in hepatocytes RSV stimulates SIRT-1 leading to downstream AMPK activation.

Although nicotinamide and splitomicin are used widely to inhibit SIRT-1 [21,22] it is possible that they inhibit other members of the sirtuin family. Therefore whether RSV's action in muscle cells involves SIRT-1 specifically remains to be examined.

In summary, this is the first study to show that RSV stimulates glucose uptake in L6 skeletal muscle cells in a dose- and time-dependent fashion, independent of insulin. Akt/PKB, and GLUT4 or GLUT1 translocation do not appear to be involved in RSV's action but the mechanism appears to involve SIRT-dependent AMPK activation that may lead to stimulation of the intrinsic activity of the GLUT4 glucose transporters.

Acknowledgments

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