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Resveratrol stimulates glucose transport in C2C12 myotubes by activating AMP-activated protein kinase

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Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; PI-3 kinase, phosphatidylinositol-3 kinase; t-RVT, *trans*-resveratrol

Abstract

***trans*-Resveratrol (t-RVT), a naturally occurring polyphenol found in *Polygonum cuspidatum*, grape, and red wine, has been reported to have anti-inflammatory, cardioprotective, and cancer chemo-preventive properties. However antidiabetic effect of t-RVT has not yet been reported. In this study, we show that t-RVT increases glucose uptake in C2C12 myotubes by activating AMP-activated protein kinase (AMPK), uncovering an antidiabetic potential of t-RVT for the first time. AMPK plays a central role in the regulation of glucose and lipid metabolism, and hence it is considered a novel therapeutic target for metabolic syndrome such as type 2 diabetes. t-RVT significantly induced glucose uptake in C2C12 cells, via AMPK activation, but not a phosphatidylinositol-3 kinase (PI-3 kinase) signal pathway. The induced glucose uptake was attenuated by pretreatment with a pharmacological inhibitor for AMPK, indicating that the effect of t-RVT primarily depends on AMPK activation. However, in the presence of insulin, t-RVT also potentiated the effect of insulin on glucose uptake via AMPK activation, which led to further activation of PI-3 kinase/Akt signal pathway.**

Keywords: 1-phosphatidylinositol 3-kinase; 5'-AMP-activated protein kinase; acetyl-CoA carboxylase; glucose; proto-oncogene proteins c-akt; resveratrol

Introduction

trans-Resveratrol (t-RVT), a naturally occurring stilbene (3,4',5-trihydroxystilbene), is a phytoalexin found in *Polygonum cuspidatum*, mulberry (*Morus* species), grape, and red wine (Chen *et al*., 2001; Signorelli and Ghidoni, 2005). t-RVT has been proposed to contribute to the “French paradox” (the epidemiological evidence that links the low incidence of cardiovascular events in France with red wine consumption) (Renaud and de Lorgeril, 1992). t-RVT has been reported to have a variety of cardioprotective, cancer chemopreventive, and anti-inflammatory properties (Fremont, 2000; Das *et al*., 2005; Signorelli and Ghidoni, 2005). However antidiabetic effect of t-RVT has not yet been reported.

AMP-activated protein kinase (AMPK) is a phylogenetically conserved intracellular energy sensor that plays a central role in the regulation of glucose and lipid metabolism. AMPK, a heterotrimeric complex comprising a catalytic α-subunit and regulatory β- and γ-subunits, is activated when the cellular energy is depleted (Hardie and Carling, 1997). When activated by allosteric binding of AMP or phosphorylation at Thr 172 of catalytic subunit by AMPK kinase, AMPK accelerates ATP-generating catabolic pathways, including glucose and fatty acid oxidation (Makinde *et al*., 1997; Ai *et al*., 2002; Zong *et al*., 2002) while reducing ATP-consuming anabolic pathways including cholesterol, fatty acid, and triacylglycerol synthesis (Henin *et al*., 1995).

Metformin (dimethylbiguanide), the most commonly used glucose-lowering reagent, leads to AMPK activation which is thought to be mandatory for the increase in glucose uptake in skeletal muscle (Musi *et al*., 2002). Muscle contraction (exercise) and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside(AICAR), an AMPK activator, activate glucose transport by a mechanism dependent upon AMPK, independent of phosphatidylinositol-3 kinase (PI-3 kinase)

(Hayashi *et al*., 1998). Furthermore overexpression of a dominant negative mutant of AMPK in skeletal muscle blocked AICAR-induced glucose uptake (Mu *et al*., 2001). Thiazolindinediones (TZDs), another antidiabetic drugs which are specific ligands for peroxisome proliferator-activated receptor-γ, has also been shown to improve insulin sensitivity by activating AMPK (Fryer *et al*., 2002). In addition, antiobesitic effect of leptin, adiponectin, and α-lipoic acid is mediated by AMPK (Minokoshi *et al*., 2002; Yamauchi *et al*., 2002; Lee *et al*., 2005). Therefore, AMPK is thought to be a novel therapeutic target to reduce blood glucose levels in type 2 diabetes mellitus.

In the present study we have shown that t-RVT increases glucose uptake in skeletal muscle by activating AMPK. By checking a cross-talk between AMPK and PI-3 kinase/Akt pathway, we further investigated the underlying mechanism.

Materials and Methods

Materials

DMEM and FBS were purchased from Invitrogen. 2-deoxy-D-[^{3}H]glucose (6.0 Ci/mmol) was from PerkinElmer Life Sciences. LY294002 was purchased from TOCRIS. Compound C was a generous gift from Merck. Resveratrol and insulin were from Sigma. Antibodies that recognize the phosphorylated ACC-Ser^{79}, AMPKα-Thr^{172}, Akt-Ser^{473}, Akt and Erk antibodies were from Cell signaling technology. AMPKα antibody was from Upstate Biotechnology. ACC antibody was purchased from Santa Cruz.

Cell culture

The skeletal muscle cell line C2C12 myoblasts were maintained in DMEM supplemented with 10% heat-inactivated FBS at 37°C with 95% air and 5% CO_2. Differentiation into myotubes was performed as previously described (Epting *et al*., 2004). To induce differentiation, media was replaced with DMEM containing 1% FBS when they were confluent. Experiments were performed in differentiated C2C12 myotubes after 7 days in 1% FBS/DMEM.

MTT assay

Cytotoxicity was determined by the MTT assay. C2C12 myoblasts or myotubes were incubated with or without resveratrol for 24 h. 10 μl of MTT solution (5 mg/ml, pH 7.5) was added. After 1 h, blue formazan crystals were resolved with 100 μl of dimetyl sulfoxide. Absorbance was measured at 595 nm. Cell viability (percent of control) was calculated relative to untreated control.

Glucose uptake

Cells were cultured on 12-well cluster dishes, washed with Krebs-Ringer phophate buffer (KRB) (25 mM HEPES, pH 7.4, 118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 5 mM $NaHCO_3$, 0.07% BSA, and 5.5 mM glucose) and incubated in serum-free media for 3 h. After t-RVT treatment, cells were incubated in KRB containing 0.5 μCi of 2-deoxy-D-[^{3}H] glucose for 20 min. The reaction was terminated by placing the plates on ice

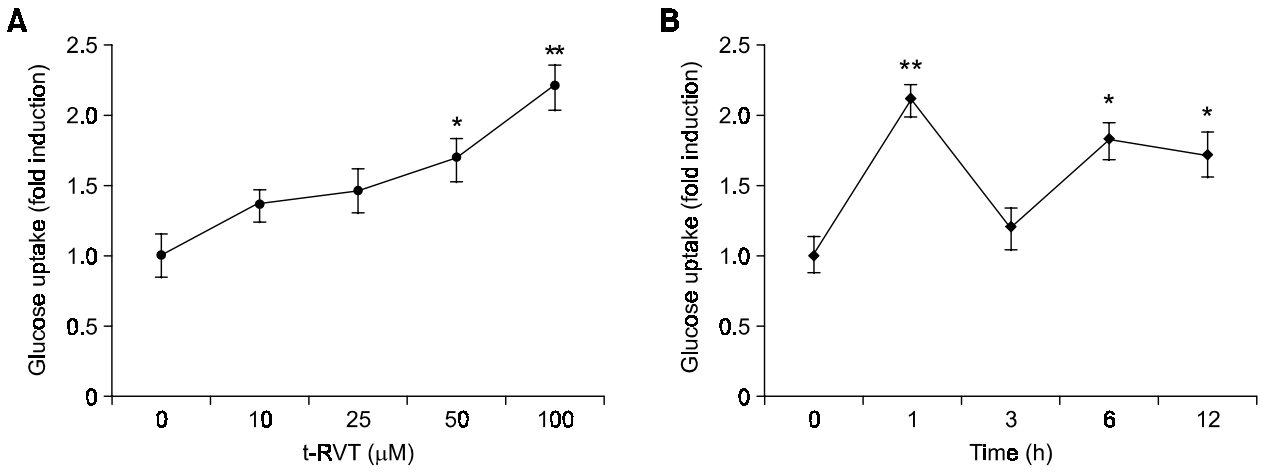

Figure 1. Effect of t-RVT on glucose uptake of C2C12 myotubes. C2C12 myotubes were incubated with the indicated concentrations of t-RVT in serum-free media for 1 h (A) or with t-RVT (100 μM) in serum-free media for the indicated time period (B). Glucose uptake was determined as described in Materials and Methods. The results represent the means ± S.E. for two independent assays in triplicate (*$P < 0.05$, **$P < 0.01$; compared with control).

and adding ice-cold PBS. After washing three times with PBS, the cells were dissolved in 0.1% SDS. Trace activities were determined by liquid scintillation counter.

Protein extract and western blot analysis

C2C12 cells grown in 6 well culture plate were washed with cold PBS, and 150 μl of extraction buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.25% sucrose, 0.4 mg/ml digitonin, and 1.5 mM PMSF) was added to a culture dish. The plate was rocked on ice for 3 min, and the buffer was collected for western blot. The protein in the cytosolic fractions were separated on a 7% SDS-PAGE, transferred to a nitrocellulose membrane, and probed using horseradish-peroxidase conjugates. ERK protein levels were used as a control for equal protein loading.

Statistical analysis

Data were analyzed using a one way ANOVA, followed by Tukey's post hoc test for multiple comparison. A P value of < 0.05 was considered significant.

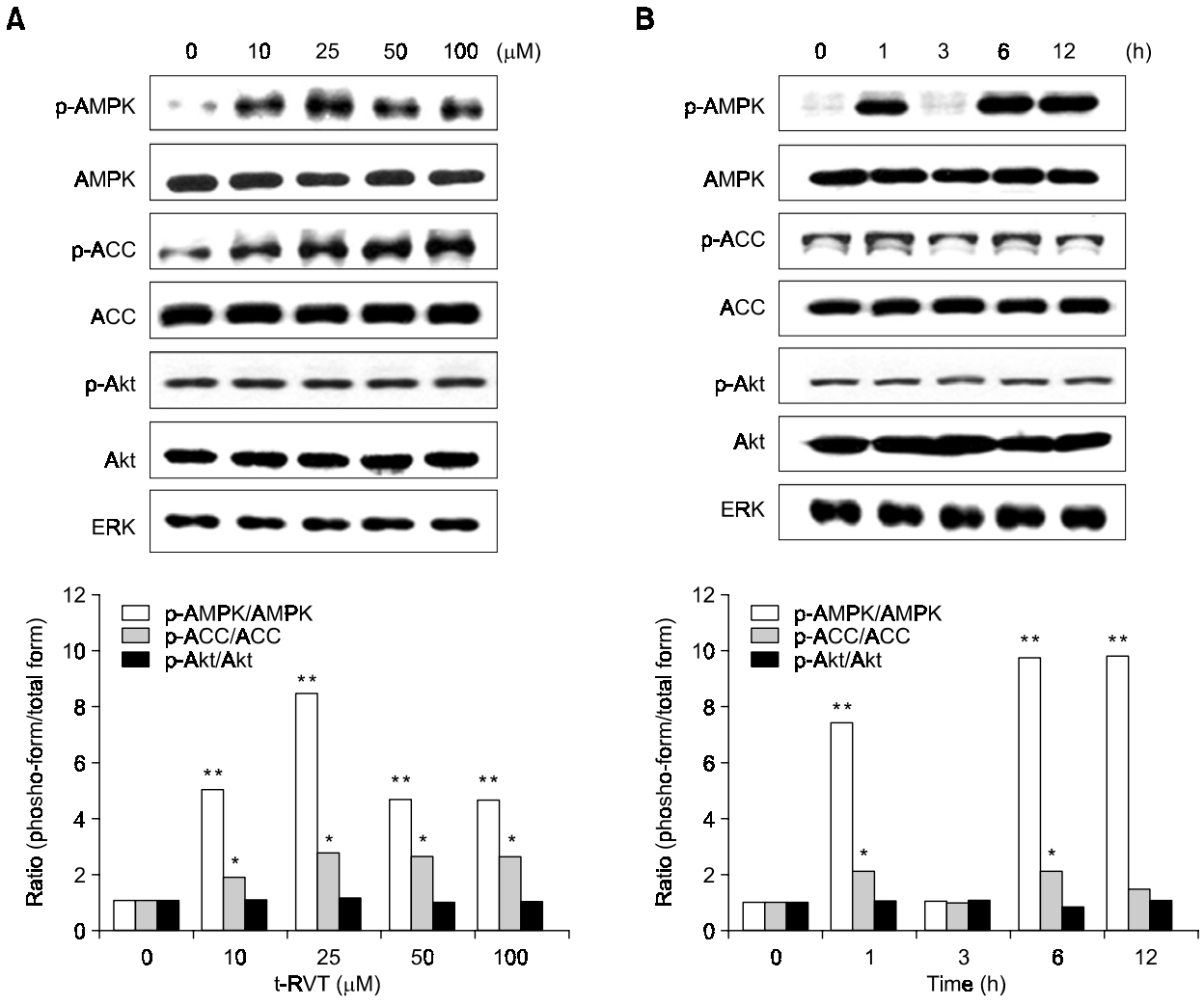

Figure 2. Effects of t-RVT on the activity of AMPK and Akt. C2C12 cells were incubated in 1% FBS/DMEM with the indicated concentrations of t-RVT for 1 h (A) or with 100 μM for the indicated time period (B). Protein extracts were prepared and subject to western blot assay using anti-phosphospecific ACC-Ser^{79} (pACC), anti-ACC (ACC), anti-phosphospecific AMPK-Thr^{172} (pAMPK), anti-AMPK (AMPK), anti-phosphospecific Akt-Ser^{473} (pAkt), anti-Akt (Akt), and anti-ERK antibodies. ERK protein levels were used as a control for equal protein loading. A ratio between phosphorylated form and total form of AMPK, ACC, and Akt was presented in a bar graph. *$P < 0.05$, **$P < 0.01$; compared with none (A), or time = 0 (B).

Results

t-RVT increases glucose uptake in C2C12 myotubes by activating AMPK

We first examined the dose- and time-dependent effect of t-RVT on glucose uptake in C2C12 myotubes. For short treatment of t-RVT, cells were incubated in serum-free media for 3 h, and then with the indicated concentrations of t-RVT for 1 h (Figure 1A). Glucose uptake was induced in a concentration-dependent manner, showing a maximum ~2-fold induction at 100 μM. t-RVT did not influence the viability of C2C12 cells in the dose range between 0 and 100 μM, as assessed by MTT assay, but a severe toxicity was observed at high concentrations (data not shown). Thus, the concentrations of up to 100 μM were used in subsequent experiments. We next determined a time-dependent effect, and cells were maintained in serum-free media with t-RVT (100 μM) for the indicated time (Figure 1B). t-RVT-induced activation reached a peak level at 1 h mark, and then gradually decreased until 3 h. Then, second phase of activation was observed in 6-12 h.

Then we investigated the activities of AMPK, ACC, and Akt in C2C12 myotubes that were exposed to t-RVT at the indicated concentration for 1 h (Figure 2A) or at 100 μM for the indicated time period (Figure 2B). Akt is a serine/threonine protein kinase that leads to a translocation of the insulin-sensitive glucose transporter (GLUT4) to the plasma membrane via activation of a signal transduction cascade involving insulin, insulin receptor substrate (IRS) family, and PI-3 kinase (Alessi and Cohen, 1998). The phosphorylation level of Thr^{172} in the active site of AMPKα catalytic subunit, which is essential for the enzyme activity (Hawley *et al*., 1996), reaches a maximum level at 25 μM (Figure 2A). Furthermore the phosphorylation of ACC-Ser^{79}, which is the best-characterized phosphorylation site by AMPK (Hardie *et al*., 1998), also reached the maximum level at 25 μM. The phosphorylation of ACC-Ser^{79} has been shown to have the tight correlation with an endogenous AMPK activity (Lee *et al*., 2003). Time kinetics shows biphasic activation of AMPK (Figure 2B), which is almost an identical pattern of glucose uptake induction (Figure 1B). Under these conditions, however, the phosphorylation level of Akt- Ser^{473}, which indicates activation of upstream PI-3 kinase, was not altered. The total amount of ACC, AMPKα, and Akt was the same. Therefore, these results indicate that AMPK was activated by t-RVT treatment, whereas the Akt activity was not induced.

AMPK activation is required for the t-RVT-induced increases of glucose uptake, but it is independent of PI-3 kinase/Akt pathway

To determine whether the activated AMPK or the basal activity of Akt is involved in the effect of t-RVT on glucose uptake, we attempted to inhibit the AMPK or Akt activity by pharmacological approach. While pretreatment of C2C12 cell with compound C, an AMPK inhibitor (Zhou *et al*., 2001) significantly attenuated the t-RVT-induced glucose uptake, LY294002, a PI-3 kinase inhibitor, did not prevent it (Figure 3A). Moreover, LY294002 did not inhibit AMPK activation by resveratrol (Figure 3B). These

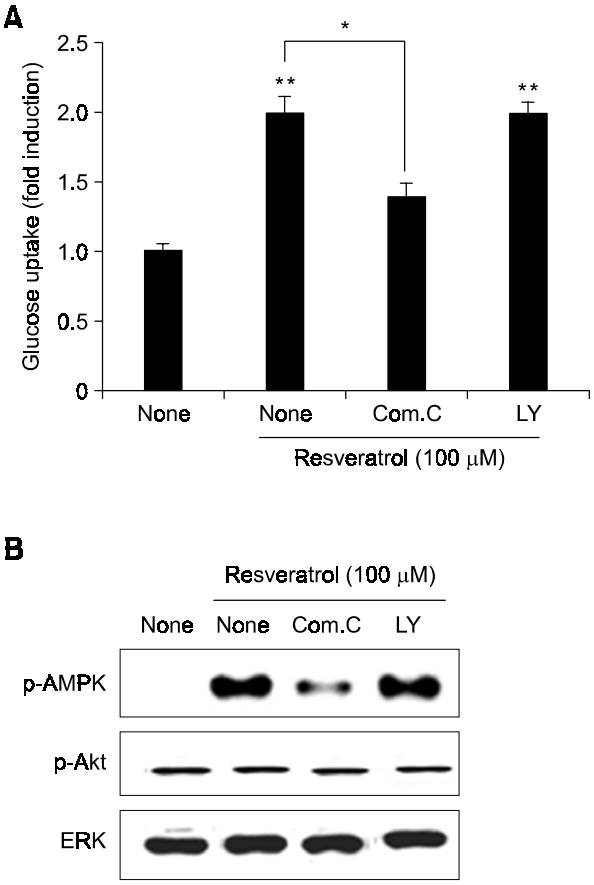

Figure 3. The effect of t-RVT on glucose uptake is dependent upon AMPK, but independent of Akt. (A) C2C12 cells were pretreated with or without 20 μM compound C (Com. C) or 25 μM LY24002 (LY) for 30 min and then treated with 100 μM t-RVT for 1 h. Glucose uptake was measured as described in Materials and Methods. The results represent the mean ± S.E. for two independent assays in triplicate (*$P < 0.05$, **$P < 0.01$; compared with control, or between two groups as indicated). (B) Under the identical conditions, phosphorylation level of AMPK and Akt was measured by western blot. ERK protein levels were used as a control for equal protein loading.

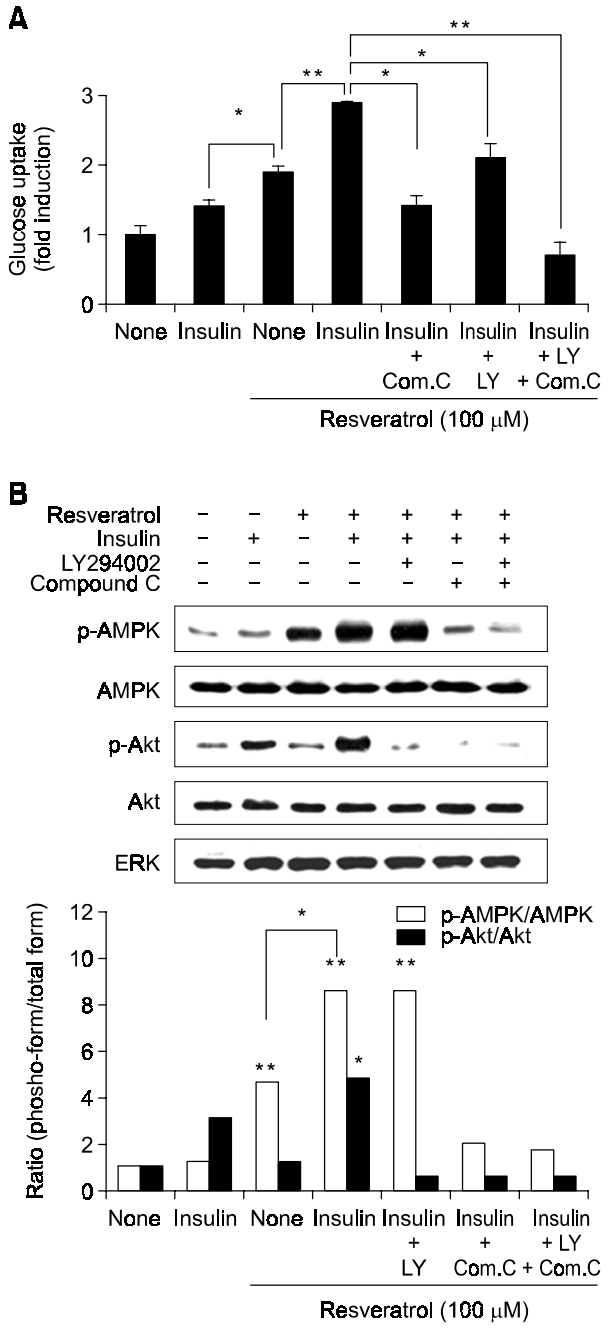

Figure 4. Resveratrol improves insulin sensitivity via activation of AMPK. (A) C2C12 cells were pretreated with or without 20 μM compound C (com. C) or 25 μM LY294002 (LY) for 30 min and then exposed to 100 μM t-RVT for 1 h in the presence or absence of insulin (100 nM). Glucose uptake was measured as described in Materials and Methods. The results represent the means ± S.E. for two independent assay in triplicate (*$P < 0.05$, **$P < 0.01$; compared with control, or between two groups as indicated). (B) The phosphorylation level of AMPK-Thr^{172} (pAMPK) and Akt-Thr^{473} (pAkt) as well as the level of AMPK and Akt were determined by immunoblot assay. ERK protein levels were used as a control for equal protein loading. A ratio between phosphorylated form and total form of AMPK and Akt was presented in a bar graph.

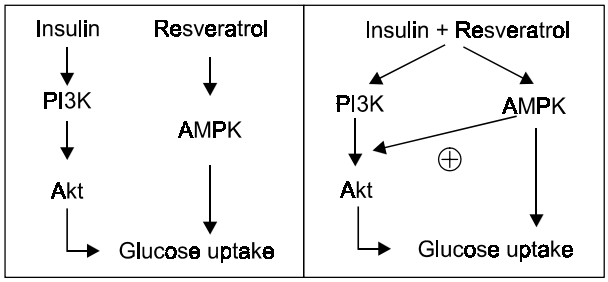

Figure 5. Proposed scheme for t-RVT-stimulated signaling in C2C12 cells. In the absence of insulin, the effect of t-RVT on glucose transport is primarily dependent on AMPK activation, which does not involve PI-3 kinase/Akt pathway. However, in the presence of insulin, signals from insulin and t-RVT appear to be interconnected and amplified, leading to increased insulin sensitivity.

results suggest that AMPK activation is required for the t-RVT-induced
increases of glucose uptake, whereas PI-3 kinase/Akt was not involved.

Effect of t-RVT on insulin sensitivity

We next attempted to study whether or not t-RVT has an insulin-sensitizing potential (Figure 4). To this end, C2C12 cells were pretreated with or without 20 μM compound C or 25 μM LY294002 for 30 min and then exposed to 100 μM t-RVT for 1 h in the presence or absence of insulin (100 nM). The effect of insulin on glucose transport was greater in C2C12 cells that had been incubated with t-RVT, indicating that t-RVT increases the sensitivity of C2C12 cells to insulin. This increase in insulin sensitivity was inhibited by compound C (Figure 4A). Furthermore, the phosphorylation of Akt-Ser^{473} by insulin was enhanced by t-RVT, which was attenuated by compound C (Figure 4B). These results indicate that t-RVT increases insulin sensitivity by activating AMPK, which eventually leads to activation of Akt signal pathway.

Discussion

The present study demonstrates that t-RVT stimulated glucose uptake and improved insulin sensitivity by activating AMPK in C2C12 skeletal muscle cells. Although many beneficial properties of t-RVT have been reported, t-RVT has not been focused as an antidiabetic agent. Thus, to our knowledge this is the first report demonstrating antidiabetic potential of t-RVT.

Our results are schematically diagrammed in Figure 5. In the absence of insulin, the effect of

t-RVT on glucose uptake seems to be primarily dependent on AMPK activation, which does not involve PI3- kinase/Akt. However, when cells were exposed to insulin and t-RVT together, signals from two stimuli appear to be interconnected and amplified. First, insulin activates PI-3 kinase/Akt pathway (Figure 4B). Second, AMPK activation by t-RVT definitely potentiated the insulin-induced Akt activation (Figure 4B), leading to improvement of insulin sensitivity. Quite similar to our results, a couple of recent studies demonstrated that α-lipoic acid as well as metformin has been reported to improve insulin sensitivity by activating AMPK (Kumar and Dey, 2002; Lee *et al*., 2005). Despite many efforts, the mechanism by which AMPK increase insulin sensitivity remains almost unknown. In fact, AMPK has been reported to lead to the phosphorylation of insulin receptor substrate-1 on Ser 789 in C2C12 myotubes when exposed to AICAR, a known AMPK activator (Jakobsen *et al*., 2001). However, this phosphorylation does not alone affect PI-3 kinase/Akt, but augments insulin-stimulated glucose uptake, indicating the existence of the complex signaling network.

Indeed, the data on the interaction between AMPK and PI-3 kinase/Akt signal pathway is highly controversial. There are two major mechanisms enhancing glucose transport in skeletal muscle. One is insulin signaling pathway via PI-3 kinase/Akt. Insulin accelerates glucose transport via the phosphorylation and activation of Akt leading to glucose transporter 4 (GLUT4) translocation to the plasma membrane (Wang *et al*., 1999). The other is AMPK signaling pathway, which is activated by such stimuli as exercise, hypoxia, and hyperosmolarity (Czech and Corvera, 1999). Initially, AMPK has been proposed to stimulate GLUT4 translocation independent of PI-3 kinase/Akt pathway (Russell *et al*., 1999). However, some other reports suggest that AMPK functions upstream of Akt signaling (Ouchi *et al*., 2004). In contrast, AMPK has been reported to be activated via PI-3 kinase pathway in vascular endothelial cells (Zou *et al*., 2004). The opposite was found in a report suggesting Akt negatively regulates AMPK phosphorylation in the heart (Kovacic *et al*., 2003). In the present study, our data support a possibility that AMPK can potentiate the insulin signal by activating Akt, thus increasing the complexity of understanding how these two signals are interconnected. Moreover, our present data shows that insulin can slightly activate AMPK (Figure 4B), which is in contrast to a previous report demonstrating that AMPK can be inactivated by insulin and Akt (Kovacic *et al*., 2003). Therefore, interaction between these two signals appears to be highly cell type- and text-dependent. As a result, further studies are required to elucidate the precise mechanism interconnecting these two signal pathways.

At this point, we do not understand the mechanism by which t-RVT activates AMPK. However, t-RVT may share common signaling pathway with leptin or adiponectin for activating AMPK and/or improving insulin sensitivity. Leptin, a peptide hormone that decreases food intake and increases energy expenditure through its actions in the hypothalamus, regulate energy homeostasis through direct actions in peripheral tissue by regulating AMPK (Minokoshi *et al*., 2002). In skeletal muscle, fatty acid oxidation is regulated by AMPK-acetyl-CoA carboxylase (ACC)-malonyl-CoA axis. ACC produces malonyl-CoA, which inhibits carnitine palmitoyltransferase 1, the rate-limiting step in mitochondrial fatty acid oxidation. Thus, the activation of AMPK by leptin inhibits ACC, decreases malonyl-CoA levels, and leads to stimulation of fatty acid oxidation. Likewise, adiponectin, another adipocyte-derived hormone has been reported to stimulate glucose uptake by activating AMPK in C2C12 cells (Yamauchi *et al*., 2002). Furthermore, in a good agreement with our findings showing that t-RVT increases glucose uptake via a PI-3 kinase-independent pathway (Figure 3), the effect of adiponectin on glucose transport is not mediated by PI-3 kinase/Akt pathway. Thus, it is possible that t-RVT may activate AMPK via leptin or adiponectin signaling pathway. In addition, t-RVT may regulate the expression of genes involved in glucose uptake. As demonstrated in Figure 1B, glucose uptake was increased in a biphasic manner; a transient and rapid induction was observed in 1 h treatment of t-RVT, and second induction was observed in 6-12 h. As mentioned above, a transient and rapid glucose uptake may be induced by t-RVT via activating leptin or adiponectin signal pathways, and translocation of glucose transport 4 to the plasma membrane may be responsible for this induction. AMPK is also known to increase the expression of glucose transporter 1 (Kahn *et al*., 2005), and therefore, second activation by t-RVT may be mediated by the gene expression of glucose transporter 1. We are currently investigating this possibility.

Acknowledgement

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