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Potentiation of neuronal insulin signaling and glucose uptake by resveratrol: the involvement of AMPK

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Abstract:
Resveratrol (RSV), a polyphenolic phytoestrogen, has been shown to activate the serine/threonine kinase 5'-adenosine monophosphate-activated protein kinase (AMPK) and to stimulate insulin signaling and glucose uptake in skeletal muscle cells. A direct effect of RSV on neuronal insulin signaling, however, has not been demonstrated. Here, we report that RSV stimulates glucose uptake and potentiates insulin signaling in Neuro-2A (N2A) cells, which is characterized by the increased phosphorylation of protein kinase B (Akt) and glycogen synthase kinase-3β (GSK-3β). Furthermore, RSV activates AMPK in N2A cells, which can be prevented using a specific pharmacological inhibitor, Compound C. Compound C abrogates RSV-induced Akt and GSK-3β phosphorylation and glucose uptake. Thus, we demonstrate that RSV potentiates insulin signaling and glucose uptake *via* AMPK activation in neuronal cells.

Key words:
resveratrol, AMPK, neuronal insulin signaling, glucose uptake

Abbreviations: 2-DOG – 2-deoxy-D-glucose, Akt – protein kinase B, AMPK – 5'-adenosine monophosphate-activated protein kinase, GSK-3β – glycogen synthase kinase-3β, N2A – Neuro-2A, PI3K – phosphatidylinositol-3-kinase, RSV – resveratrol

Introduction

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene, RSV) is a phytoestrogen that can be isolated from red wine, grapes, peanuts and mulberries [1]. RSV has shown a variety of beneficial physiological effects including anti-oxidant [21], cardioprotective, anti-atherosclerotic [1] and neuroprotective properties [30] and has even been reported to prolong the life span of mice [2]. In addition, RSV has recently gained attention as an anti-diabetic agent because it has been shown to increase insulin sensitivity [25] and stimulate glucose uptake in myotubes by 5'-adenosine monophosphate-activated protein kinase (AMPK) activation [5, 19]. The effect of RSV on neuronal insulin signaling, however, has not been reported.

Insulin plays an integral role in the brain, so it is not surprising that dysregulated insulin signaling has

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been linked to multiple complications [28]. Therefore, studies investigating the roles of therapeutic agents that activate neuronal insulin signal transduction have become essential. Such studies, however, have not been addressed as much as those concentrated on peripheral tissues. In neurons, the PI3K-Akt-GSK3β and Ras-Raf-MEK-Erk1/2 pathways govern the abovementioned actions of insulin [8, 20]. The interaction of insulin with its receptor activates phosphatidylinositol-3-kinase (PI3K), which then leads to Akt-mediated GLUT4 translocation and glucose uptake [3].

AMPK is a fuel-sensing kinase consisting of catalytic α (α1, α2) and regulatory β (β1, β2) and γ (γ1, γ2, γ3) subunits. This kinase has emerged as an important pharmacological target for the treatment of type 2 diabetes. The activity of AMPK is induced by elevated AMP:ATP levels, exercise, anti-diabetic drugs like metformin and thiazolidinediones, and RSV [12]. The high metabolic activity of neurons renders AMPK a crucial energy regulator [9, 24]. In neurons, AMPK plays a role in cytoprotection under ischemic and pathological conditions [9]. Even the activation of AMPK attenuates complications of neurodegenerative diseases such as Alzheimer's disease [29].

Therefore, the objective of the present study is to elucidate the effect(s) of RSV on insulin signaling and glucose uptake in neuronal cells and to investigate any involvement of AMPK.

Materials and Methods

Resveratrol (5-[(1E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol), Compound C (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]pyrimidine), [^{3}H]2-deoxy-D-glucose (2-DOG), Minimum Essential Medium (MEM), and anti-rabbit IgG coupled to alkaline phosphatase were from Sigma Chemical Co. (MO, USA). Bovine insulin was purchased from Calbiochem (CA, USA). Fetal bovine serum (FBS) and trypsin-EDTA were purchased from Gibco BRL (NY, USA). Antibodies against pAMPK (Thr172), AMPK, pAkt (Ser473), Akt, pGSK-3β (Ser9), and GSK-3β were from Cell Signaling Technology (MA, USA). All other reagents were from Sigma Chemical Co. (MO, USA) unless stated otherwise.

Cell culture and treatment

Neuro-2A (N2A), a mouse neuroblastoma cell line, was cultured in MEM supplemented with 10% fetal bovine serum (FBS) with antibiotics (penicillin 100 IU/ml and streptomycin 100 μg/ml) in 5% CO_2 at 37°C. Cells at 70% confluence were serum-starved in MEM for 24 h, treated with various concentrations of RSV for 2 h, and then incubated with 100 nM insulin for 30 min. Compound C-treated cells were pre-incubated with 50 μM of compound C or vehicle (DMSO) for 30 min before addition of RSV.

Preparation of cell lysates and immunoblotting

After N2A cells were lysed in cell lysis buffer, the protein concentration was estimated, and western blotting was performed as previously reported [15].

Glucose uptake assay

A glucose uptake assay was performed as previously reported from our laboratory with minor modifications [16]. Briefly, cells were washed with serum-free MEM, and then stimulated with or without 100 nM insulin for 30 min. Next, 2-DOG (0.1 μCi/ml [^{3}H]-radiolabeled 2-DOG in 1 μM unlabeled 2-DOG) was added, and the cells were incubated for 15 min. Cells were washed in ice-cold PBS three times and then solubilized in 0.1 M NaOH. After the protein concentration was measured, [^{3}H] in the samples was measured using a liquid scintillation counter.

Densitometry

Densitometry of the western blots was performed using a Gel Doc 2000 apparatus and Quantity One 1-D analysis software, both from Bio-Rad (CA, USA). Bands from positive control groups were assigned the value of 1.0, and the background value was set as 0.0 for each experiment [15].

Statistical analysis

The data are expressed as the mean ± SEM. For the comparison of two groups, a p-value was calculated using a two-tailed unpaired Student's *t*-test. In all cases, $p < 0.05$ was considered statistically significant.

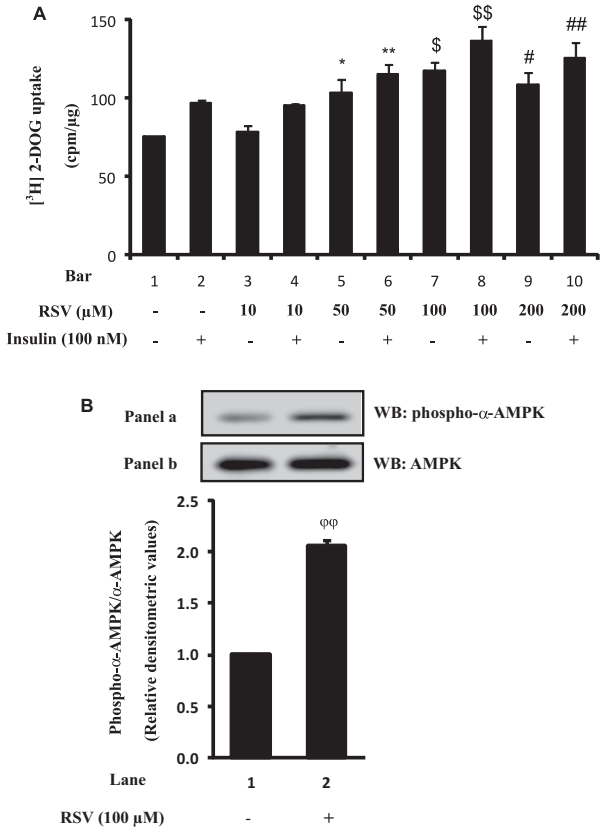

Fig. 1. The effect of RSV on glucose uptake and AMPK activation in N2A cells: (**A**) After proliferation, cells were serum starved and treated with or without RSV (10 µM, 50 µM, 100 µM and 200 µM) for 2 h, followed by stimulation with or without insulin. Glucose uptake was determined by measuring [^{3}H]2-DOG. Bars represent glucose uptake in cpm/µg. (**B**) After proliferation, the cells were serum starved and treated with or without RSV (100 µM) for 2 h. The protein lysates (25 µg) were subject to western blotting and probed with an anti-phospho-α-AMPK (Thr172) antibody. The blots were stripped and re-probed with an anti-α-AMPK antibody. Bars represent the relative densitometric values of phospho-α-AMPK/α-AMPK. All experiments were performed in triplicate, and representative figures are shown. Reported values are the mean ± SEM. * $p < 0.01$, $^{\$}$ $p < 0.01$, $^{\#}$ $p < 0.01$, $^{\varphi\varphi}$ $p < 0.01$ compared to untreated samples; ** $p < 0.01$, $^{\$\$}$ $p < 0.05$, $^{\#\#}$ $p < 0.05$ compared to insulin-stimulated samples

Results and Discussion

To study the effect of RSV on glucose uptake, N2A cells were treated with RSV at various concentrations (10 µM, 50 µM, 100 µM, 200 µM) for 2 h. The addition of RSV increased glucose uptake in a dose-dependent manner, with maximum uptake occurring with 100 µM RSV. RSV (100 µM) alone increased glucose uptake by 56.5 ± 0.06% (Fig. 1A, lane 7 *vs.* lane 1, $p < 0.01$), and RSV with insulin stimulation increased uptake by 42 ± 0.12% (Fig. 1A, lane 8 *vs.* lane 2, $p < 0.01$). Moreover, 100 µM RSV did not affect cell viability or morphology (data not shown). These data corroborate previous studies showing RSV promotes maximum glucose uptake in C2C12 and L6 myotubes at 100 µM after 1–2 h of incubation [5, 19].

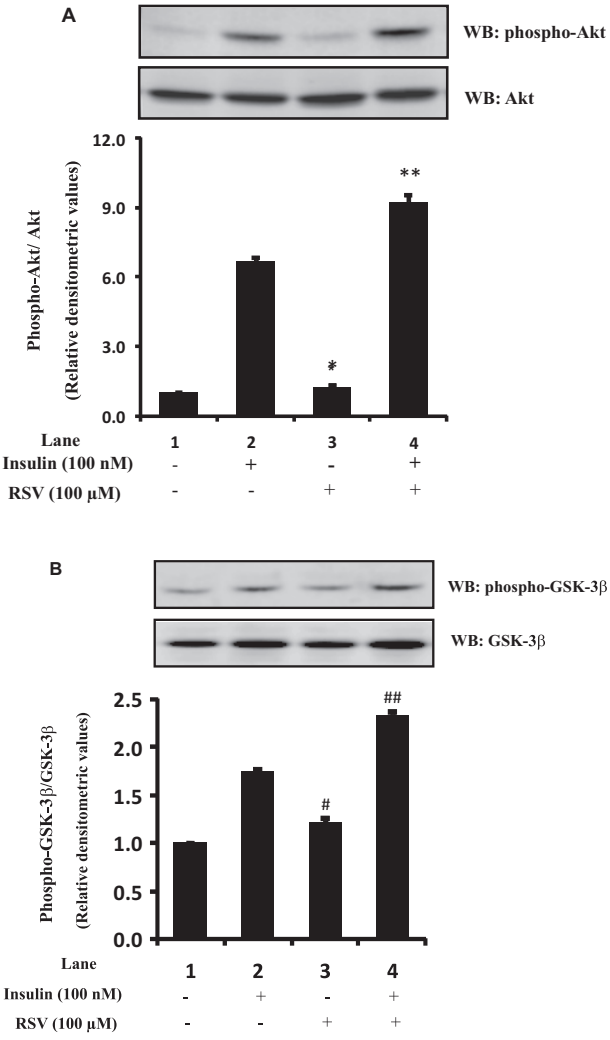

Fig. 2. The effect of RSV on Akt and GSK-3β phosphorylation in N2A cells: After proliferation, cells were serum starved and treated with or without RSV (100 µM) for 2 h and stimulated with or without 100 nM insulin for 30 min. The protein lysates (25 µg) were subject to western blotting and probed with either an (**A**) anti-phospho-Akt (Ser473) antibody or (**B**) anti-phospho-GSK-3β (Ser9) antibody. The blots were stripped and re-probed with an (**A**) anti-Akt antibody or (**B**) anti-GSK-3β antibody. Bars represent the relative densitometric values of (**A**) phospho-Akt/Akt or (**B**) phospho-GSK-3β/GSK-3β. All experiments were performed 3 times, and representative figures are shown. The reported values are the mean ± SEM. * $p < 0.05$ and $^{\#}$ $p < 0.05$ compared to untreated samples; ** $p < 0.01$ and $^{\#\#}$ $p < 0.01$ compared to insulin-stimulated samples

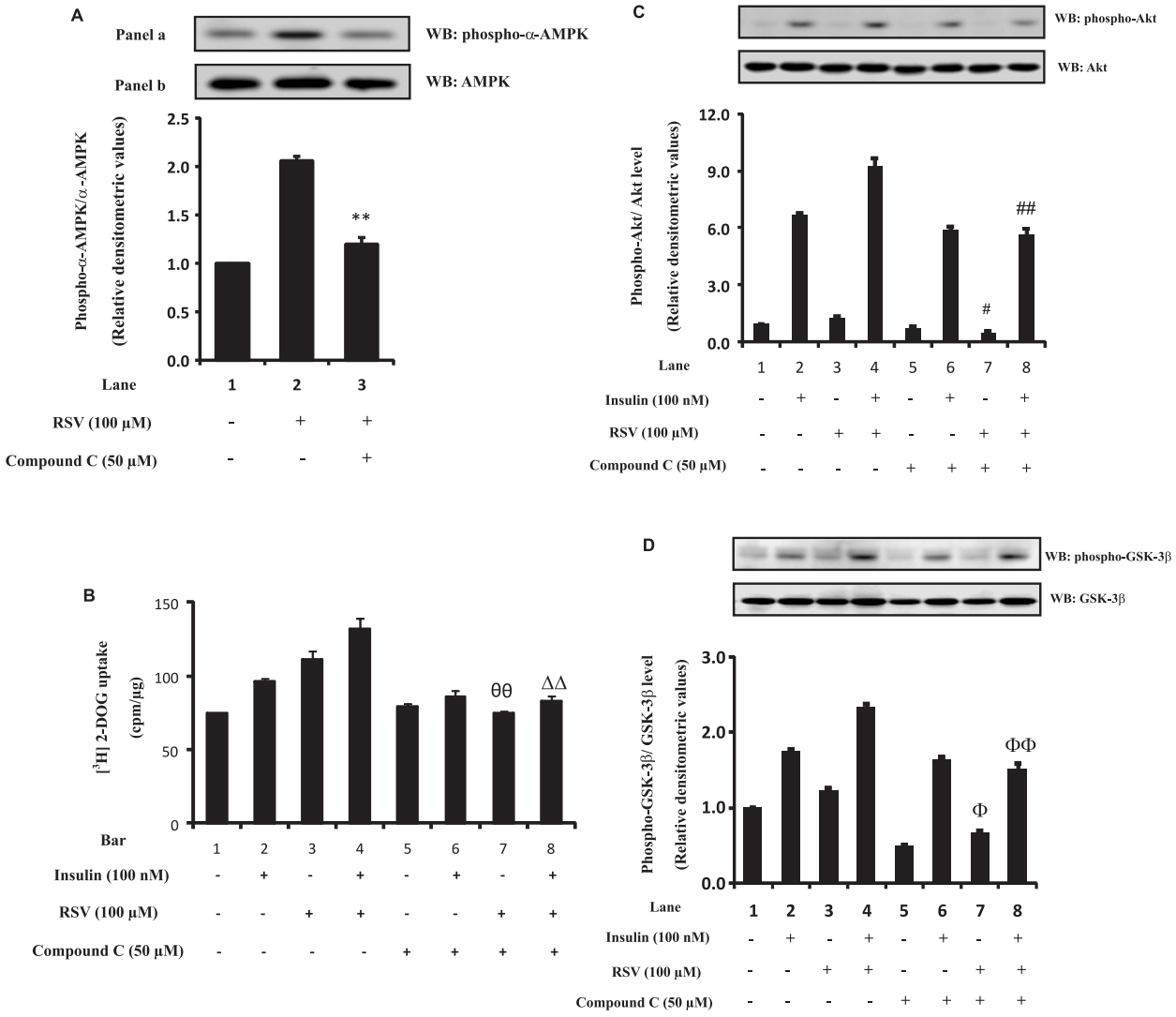

Fig. 3. The effect of AMPK inhibition on RSV-mediated Akt and GSK-3β phosphorylation and glucose uptake in N2A cells: (**A**) After proliferation, cells were serum starved and incubated with or without 50 μM Compound C for 30 min followed by treatment with or without 100 μM RSV for 2 h and then stimulated with or without 100 nM insulin. The protein lysates (25 μg) were subject to western blotting and probed with an anti-phospho-α-AMPK (Thr172) antibody. The blots were stripped and reprobed with an anti-α-AMPK antibody. Bars represent the relative densitometric values of phospho-α-AMPK/α-AMPK. (**B**) After proliferation, cells were serum starved and incubated with or without 50 μM Compound C for 30 min followed by treatment with or without 100 μM RSV for 2 h and then stimulation with or without 100 nM insulin. Glucose uptake was determined using [^{3}H]2-DOG. Bars represent glucose uptake in cpmμg^{-1}. (**C**) The protein lysates (25 μg) were subjected to western blotting and probed with an anti-phospho-Akt (Ser473) antibody. The blots were stripped and reprobed with anti-Akt antibody. Bars represent the relative densitometric values of phospho-Akt/Akt. (**D**) The protein lysates (25 μg) were subjected to western blotting and probed with an anti-phospho-GSK-3β (Ser9) antibody. The blots were stripped and reprobed with an anti-GSK-3β antibody. Bars represent the relative densitometric values of phospho-GSK-3β/GSK-3β. All experiments were performed 3 times, and representative figures are shown. The reported values are the mean ± SEM. ** $p < 0.01$, $^{\theta\theta}$ $p < 0.01$, $^{\#}$ $p < 0.05$ and $^{\Phi}$ $p < 0.05$ compared to 100 μM RSV-treated samples; $^{\Delta\Delta}$ $p < 0.01$, $^{\#\#}$ $p < 0.01$ and $^{\Phi\Phi}$ $p < 0.01$ compared to 100 μM RSV and 100 nM insulin-stimulated samples

One of above studies have also demonstrated that 100 μM RSV activates AMPK in skeletal muscle cells [19]. Therefore, RSV-treated N2A lysates were subject to immunoblotting with an anti-phospho-α-AMPK antibody. By western blotting, 100 μM RSV increased AMPK phosphorylation by 106.3 ± 0.04% (Fig. 1B, panel a, lane 2 *vs.* lane 1, $p < 0.01$) as compared to untreated samples. No change in AMPK expression levels was observed with RSV treatment (Fig. 1B, panel b).

Akt phosphorylation has been associated with increased glucose uptake in skeletal muscle cells [6]

and 3T3-L1 adipocytes [17]. To identify the pathway through which RSV, alone or in combination with insulin, induced glucose uptake, we performed a western blot to probe for the phosphorylation of Akt, a key insulin signaling protein, and the downstream kinase GSK-3β. Cell lysates were immunoblotted with either an anti-phospho-Akt (Fig. 2A) or an anti-phospho-GSK-3β antibody (Fig. 2B). RSV alone increased the phosphorylation of both Akt and GSK-3β by 25.4 ± 0.08% (Fig. 2A, lane 3 *vs.* lane 1, $p < 0.05$) and 22.6 ± 0.03% (Fig. 2B, lane 3 *vs.* lane 1, $p < 0.05$), respectively, as compared to untreated samples. With insulin stimulation, RSV treatment further increased Akt and GSK-3β phosphorylation by 39.0 ± 0.28% (Fig. 2A, lane 4 *vs.* lane 2, $p < 0.01$) and 33.2 ± 0.05% (Fig. 2B, lane 4 *vs.* lane 2, $p < 0.01$), respectively, as compared to untreated insulin-stimulated samples. Enhanced AMPK activation and the increased levels of phosphorylated Akt and GSK-3β upon RSV treatment indicate that AMPK activation by RSV may influence the increased insulin sensitivity. Previous *in vitro* studies performed with muscle cells have shown that RSV induces insulin sensitivity through AMPK activation [5, 19]. Moreover, *in vivo* studies have indicated that RSV improves metabolism [2] and increases insulin sensitivity through AMPK activation [27] in mice. The latter study has revealed that mice deficient in AMPK fail to increase insulin sensitivity and glucose tolerance in response to RSV treatment, suggesting that AMPK plays a central role in mediating the effect of RSV.

To elucidate the role of AMPK in the above mentioned effects of RSV, we used Compound C, a specific inhibitor of AMPK [5]. To determine whether Compound C (50 μM) has any effect on RSV-mediated AMPK activation, cell lysates were immunoblotted with an anti-phospho-α-AMPK antibody (Fig. 3A). Compound C completely inhibited RSV-mediated AMPK phosphorylation (Fig. 3A, panel a, lane 3 *vs.* lane 2, $p < 0.01$) without affecting AMPK expression (Fig. 3A, panel b). Moreover, cell viability and morphology were not affected by Compound C treatment (data not shown). RSV-induced glucose uptake was abrogated by Compound C in samples without (Fig. 3B, bar 7 *vs.* bar 3, $p < 0.01$) and with (Fig. 3B, bar 8 *vs.* bar 4, $p < 0.01$) insulin stimulation. Similarly, Compound C abrogated RSV-induced Akt and GSK-3β phosphorylation without (Fig. 3C, lane 7 *vs.* lane 3, $p < 0.05$ and Fig. 3D, lane 7 *vs.* lane 3, $p < 0.01$, respectively) and with insulin stimulation (Fig. 3C, lane 8 *vs.* lane 4, $p < 0.01$ and Fig. 3D, lane 8 *vs.* lane 4, $p < 0.01$, respectively). The inhibition of AMPK by pre-incubation with Compound C abrogated the effect of RSV on the phosphorylation of Akt and GSK-3β followed by glucose uptake. This validates our hypothesis that AMPK participates in effect of RSV on the phosphorylation of Akt and GSK-3β and glucose uptake.

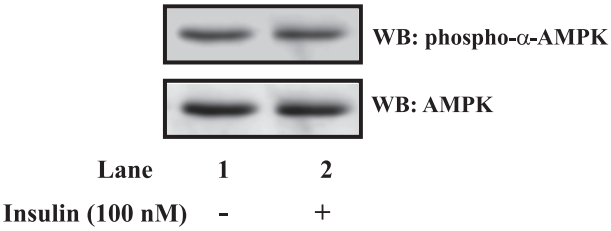

Fig. 4. The effect of 100 nM insulin on AMPK phosphorylation in N2A cells: After proliferation, cells were serum starved and treated with or without 100 nM insulin for 30 min. The protein lysates (25 μg) were subject to western blotting and probed with an anti-phospho-α-AMPK (Thr172) antibody. The blots were stripped and re-probed with an anti-α-AMPK antibody

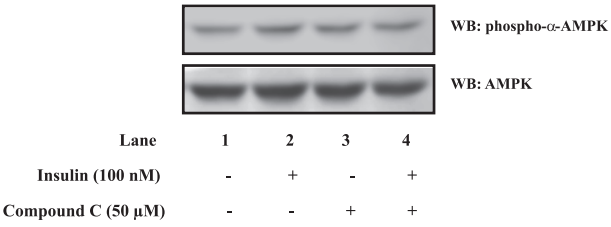

Fig. 5. The effect of Compound C on basal and insulin-induced AMPK phosphorylation. After proliferation, N2A cells were serum starved and incubated with or without 50 μM Compound C for 30 min followed by stimulation with or without 100 nM insulin. The protein lysates (25 μg) were subject to western blotting and probed with an anti-phospho-α-AMPK (Thr172) antibody. The blots were stripped and re-probed with anti-AMPK antibody

It is understood that AMPK is not an inevitable protein in the insulin signaling cascade [7, 11, 18, 26], and AMPK activation, by its known activators like AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) and RSV, has shown an effect on insulin signaling [5, 19, 23]. Our results suggest that this signaling cascade is present in neuronal cells because insulin treatment did not affect AMPK phosphorylation (Fig. 4) in our neuronal cell model, and AMPK activation by RSV potentiated neuronal insulin signaling,

which was evident by increased Akt and GSK-3β activation followed by increased glucose uptake. Moreover, Compound C inhibited RSV-induced AMPK activation seen by reduced Akt and GSK-3β phosphorylation and glucose uptake.

In this study we observed that the specific AMPK inhibitor, Compound C, did not affect insulin-induced Akt and GSK-3β phosphorylation or glucose uptake. Compound C, a competitive AMPK inhibitor [31], only inhibited RSV-induced AMPK phosphorylation without altering the basal or insulin-stimulated phosphorylation status of AMPK (Fig. 3A and Fig. 5). Similar observations have been reported previously regarding Compound C and a steady basal phosphorylation state of AMPK [10, 13, 14], which explains the ineffectiveness of Compound C against the insulin-induced Akt and GSK-3β phosphorylation and glucose uptake.

The ability of AMPK to increase insulin sensitivity and increase glucose uptake has been shown in C2C12, L6 myotubes, cardiomyocytes and 3T3-L1 adipocytes in earlier studies [4, 5, 19]. In muscle cells, it has been observed that AMPK activation imparts a synergistic effect on insulin-stimulated Akt activation and glucose uptake, suggesting that insulin sensitivity is affected by a connection between AMPK and Akt activation. However, the role of AMPK in regulating insulin sensitivity to neurons was unexplored. In neurons, AMPK acts as a multidimensional energy sensor that regulates an organism's metabolism by altering feeding behavior and locally regulating neuronal homeostasis [24]. Apart from its involvement in energy homeostasis [22], its cytoprotective effect [9], and its ability to differentiate neurons [8], our study suggests that AMPK is involved in increasing the insulin responsiveness and glucose uptake of neuronal cells.

In summary, RSV potentiates insulin sensitivity and glucose uptake in neurons by activating AMPK. Thus, our study provides a rationale for exploring the therapeutic potential of RSV and AMPK in complications associated with the dysregulation of neuronal insulin signaling.

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