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ARTICLE

Increased skeletal muscle glucose uptake by rosemary extract through AMPK activation

Madina Naimi, Theodoros Tsakiridis, Theocharis C. Stamatatos, Dimitris I. Alexandropoulos, and Evangelia Tsiani

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Abstract: Stimulation of the energy sensor AMP-activated kinase (AMPK) has been viewed as a targeted approach to increase glucose uptake by skeletal muscle and control blood glucose homeostasis. Rosemary extract (RE) has been reported to activate AMPK in hepatocytes and reduce blood glucose levels in vivo but its effects on skeletal muscle are not known. In the present study, we examined the effects of RE and the mechanism of regulation of glucose uptake in muscle cells. RE stimulated glucose uptake in L6 myotubes in a dose- and time-dependent manner. Maximum stimulation was seen with 5 μg/mL of RE for 4 h (184% ± 5.07% of control, $p < 0.001$), a response comparable to maximum insulin (207% ± 5.26%, $p < 0.001$) and metformin (216% ± 8.77%, $p < 0.001$) stimulation. RE did not affect insulin receptor substrate 1 and Akt phosphorylation but significantly increased AMPK and acetyl-CoA carboxylase phosphorylation. Furthermore, the RE-stimulated glucose uptake was significantly reduced by the AMPK inhibitor compound C, but remained unchanged by the PI3K inhibitor, wortmannin. RE did not affect GLUT4 or GLUT1 glucose transporter translocation in contrast with a significant translocation of both transporters seen with insulin or metformin treatment. Our study is the first to show a direct effect of RE on muscle cell glucose uptake by a mechanism that involves AMPK activation.

Key words: muscle cells, metabolism, energy regulation, nutrition, insulin resistance.

Résumé : La stimulation de la kinase activée par le capteur énergétique AMP constituerait selon des études une approche ciblée pour accroître la captation de glucose dans les muscles squelettiques et pour contrôler l'homéostasie du glucose sanguin. Selon des études, l'extrait de romarin (« RE ») active l'AMPK dans les hépatocytes et abaisse la concentration sanguine de glucose in vivo, mais ses effets sur le muscle squelettique ne sont pas connus. Dans cette étude, nous examinons les effets de RE et le mécanisme de la régulation de la captation du glucose dans les cellules musculaires. RE stimule la captation du glucose dans les myotubes L6 en fonction de la dose et de la durée d'administration. On obtient une stimulation maximale au moyen de 5 μg/mL de RE durant 4 h (184 ± 5,07 % du contrôle, $p < 0,001$), soit une réponse comparable à une stimulation maximale provenant de l'insuline (207 ± 5,26 %, $p < 0,001$) et de la metformine (216 ± 8,77 %, $p < 0,001$). RE n'influence pas la phosphorylation du substrat 1 du récepteur de l'insuline et de l'Akt, mais accroit significativement la phosphorylation de l'AMPK et de l'acétyl-CoA carboxylase. De plus, la captation du glucose stimulée par RE est significativement réduite par le composé C, inhibiteur de l'AMPK, mais demeure inchangée par l'inhibiteur P13K, la wortmannine. RE n'influence pas la translocation du transporteur de glucose GLUT4 ou GLUT1, mais l'administration d'insuline et de metformine suscite la translocation significative de ces deux transporteurs. Notre étude est la première à révéler un effet direct du RE sur la captation musculaire du glucose par un mécanisme impliquant l'activation de l'AMPK. [Traduit par la Rédaction]

Mots-clés : cellules musculaires, métabolisme, régulation énergétique, nutrition, insulinorésistance.

Introduction

Skeletal muscle tissue accounts for approximately 80% of insulin-mediated glucose uptake in the postprandial state and therefore plays a predominant role in maintaining glucose homeostasis (Saltiel 2001; Tripathy and Chavez 2010). Insulin increases muscle glucose uptake by increasing translocation of intracellular stored GLUT4 glucose transporters to the plasma membrane through the phosphatidylinositol-3 kinase (PI3K) and Akt signalling pathway (Taniguchi et al. 2006). Impaired PI3K-Akt signalling causes insulin resistance (Tripathy and Chavez 2010) leading to type 2 diabetes mellitus (T2DM).

The energy sensor AMP-activated protein kinase (AMPK) is a serine/threonine kinase activated by increased AMP/ATP ratio and/or through its upstream kinases, LKB1 and calmodulin-dependent protein kinase kinase (CaMKKs) (Towler and Hardie 2007). Importantly, muscle AMPK is activated by exercise/contraction (Towler and Hardie 2007) and various compounds including metformin (Zhou et al. 2001), thiazolidineones (Fryer et al. 2002) and the polyphenols resveratrol (Breen et al. 2008) and naringenin (Zygmunt et al. 2010), leading to increased glucose uptake. In recent years, AMPK has become an attractive pharmacological target for the treatment of diabetes.

Diabetes is a global health problem with more than 50% of diabetes patients in Africa and Asia, areas with huge economical constrains. Plants and herbs that activate AMPK and increase skeletal muscle glucose uptake could provide the much-needed low-cost and effective medication for the treatment of diabetes.

Rosemary (*Rosmarinus officinalis* L.) is an aromatic evergreen shrub/plant indigenous to the Mediterranean region and South

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America reported to have antimicrobial, anticancer, and antioxidant (Cheung and Tai 2007) properties. Additionally, effects on lipid metabolism (Ibarra et al. 2011; Afonso et al. 2013) and plasma glucose levels (Erenmemisoglu et al. 1997; Bakırel et al. 2008; Ramadan et al. 2013) have been reported. Rosemary extract (RE) at concentrations of 2, 10, and 50 μg/mL inhibited gluconeogenesis in HepG2 hepatocytes in vitro, an effect that is comparable to that of metformin, indicating its potential antidiabetic effects (Tu et al. 2013). Furthermore, RE administration decreased plasma glucose levels in streptozotocin-induced diabetic rats (Erenmemisoglu et al. 1997; Eman 2012; Ramadan et al. 2013), alloxan-induced diabetic rabbits (Bakırel et al. 2008), genetic (Vaquero et al. 2012) and dietary (Ibarra et al. 2011; Afonso et al. 2013) animal models of obesity and insulin resistance. Despite the promising data from these studies, the direct effects of RE on skeletal muscle have not been examined previously and its mechanism of action is not known.

In the present study, we examined the effects of RE on muscle cells. RE increased glucose uptake in L6 myotubes and increased AMPK phosphorylation. Our data demonstrate for the first time a direct effect of RE on muscle cells, indicating its potential to be used in the treatment of diabetes and providing a rationale for further research investigating in detail its effects and mechanism of action.

Materials and methods

Materials

Minimum essential media (α-MEM), fetal bovine serum, trypsin, and antibiotic-antimycotic were purchased from GIBCO Life Technologies (Burlington, Ont., Canada). Insulin receptor substrate 1 (IRS-1), Akt, AMPK, acetyl-CoA carboxylase (ACC) (Total and Phospho-specific) antibodies, horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody, and LumiGLOW reagents were from NEB (Mississauga, Ont., Canada). The 9E10 anti-myc monoclonal antibody was from Santa Cruz (Santa Cruz, Calif., USA) and HRP-conjugated donkey antimouse IgG from Jackson ImmunoResearch Labs (West Grove, Pa., USA). Insulin (Humulin R) was from Eli Lilly (Indianapolis, Ind., USA). Bovine serum albumin and compound C were from Calbiochem (Gibbstown, N.J., USA). Bradford protein assay reagent, polyvinylidene difluoride (PVDF) membranes, molecular weight protein standards, and electrophoresis reagents were from BioRad. [^{3}H]-2-deoxy-D-glucose was from PerkinElmer (Boston, Mass., USA). Whole dried rosemary (*Rosmarinus officinalis* L.) leaves were purchased from Compliments/Sobeys (Mississauga, Ont., USA).

Preparation of rosemary extract

A methanol RE was prepared as described previously (Tu et al. 2013). Whole dried rosemary leaves were grounded (5 g) and steeped overnight (16 h) in 30 mL dichloromethane-methanol (1:1) followed by a filtration the next day. After the filtration, the liquid solvent (A) was collected and set aside while the leaves were boiled in MeOH (30 mL) for 30 min. The liquid solvent obtained after the boiling was combined with the liquid solvent A. The combined solvent was removed from the final extract by rotary evaporation and the green powder obtained was dissolved in dimethyl sulfoxide (DMSO) at 10 mg/mL, aliquoted and stored at –20 °C until used.

Cell culture, treatment, and glucose uptake assay

L6 rat muscle cells (parental, GLUT4myc, and GLUT1myc overexpressing) were grown in α-MEM as previously described (Breen et al. 2008; Zygmunt et al. 2010). Prior to experiments, the cells were serum deprived for 3 h followed by treatments as indicated in each figure. At the end of the treatment, the cells were rinsed with HEPES-buffered saline followed by [^{3}H]-2-deoxy-D-glucose uptake measurements (Breen et al. 2008; Zygmunt et al. 2010). All experiments were assayed in triplicate and performed at least 3 times. The Bio-Rad protein assay was used to measure cellular protein levels.

Immunoblotting

Protein samples (15 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane, blocked for 1 h with 5% (*w*/*v*) dry milk in Tris-buffered saline, and incubated overnight at 4 °C with the primary antibody followed by detection with HRP-conjugated anti-rabbit or anti-mouse secondary antibody and ChemiGLO reagent and visualizion using FluoroChem software (ThermoFisher).

GLUT4myc and GLUT1myc translocation assay

GLUT4myc and GLUT1myc overexpressing myotubes were treated, fixed with 3% paraformaldehyde for 10 min at 4 °C, incubated with 1% glycine, blocked with 10% goat serum in phosphate buffered saline, exposed to anti-myc antibody followed by incubation with peroxidase-conjugated donkey anti-mouse IgG. Cells were washed, and the *o*-phenylenediamine dihydrochloride reagent was added for 30 min at room temperature. The reaction was stopped with 3N HCl. The supernatant was collected and absorbance was measured.

Methodological approaches

To investigate the effects of RE on muscle glucose uptake, L6 myotubes were exposed to different concentrations of RE for 4 h, and the dose–response was determined. Time-course experiments were also performed. The concentrations of RE used were based on previous studies (Tu et al. 2013). A vehicle control (DMSO) was always included.

To investigate the mechanism of action of RE, we examined the involvement of PI3K in RE-stimulated glucose uptake using its well-established inhibitor, wortmannin. Similarly, the role of AMPK was examined using its inhibitor, compound C. Furthermore, SDS-PAGE was performed to examine the total and phosphorylated levels of IRS-1, Akt, AMPK, and ACC. Using GLUT4 and GLUT1 overexpressing L6 cells, the effects of RE on glucose transporters were examined by measuring plasma levels of GLUT4 and GLUT1 glucose transporters.

Statistical analysis

The significance of the differences between groups was determined using ANOVA followed by Tukey's post hoc analysis. Statistical significant was assumed at $p < 0.05$. SAS version 9.0 software was used for calculations.

Results

To investigate whether RE has direct effects on muscle cell glucose uptake, L6 myotubes were treated with 0.1, 1.0, 5.0, 10, and 20 μg/mL of RE for 4 h. RE at 0.1 and 1.0 μg/mL did not affect glucose uptake (119% ± 4.73% and 129% ± 6.54% of control, $p > 0.05$, respectively) (Fig. 1A). However, RE at 5, 10, and 20 μg/mL significantly increased glucose uptake (184% ± 5.05%, 178% ± 7.84% and 145% ± 5.05% of control, $p < 0.001$, respectively). Microscopic examination of cell morphology did not reveal any changes by any treatment. The appearance of nuclei, cells, and the entire cell monolayer was exactly the same in control and treated cells. Furthermore, cell viability as assessed by trypan blue staining was not affected by RE treatment. The RE-induced glucose uptake was time-dependent, with no significant increases seen at 0.5 and 1 h (115% ± 4.10%, 123% ± 5.21%, $p > 0.05$) of exposure while the increase was significant at 2, 4, 6 and 16 h (154% ± 5.93%, 184% ± 5.07%, 197% ± 7.28% and 157% ± 8.66% of control, $p < 0.001$, respectively) (Fig. 1B). Importantly, the increase in glucose uptake seen with RE (5 μg/mL, 4 h, 184% ± 5.07% of control, $p < 0.001$) was comparable to the increase seen with maximum insulin (100 nmol/L, 30 min, 207% ± 5.26% of control, $p < 0.001$) or metformin (2 mmol/L, 16 h, 216% ± 8.77%, $p < 0.001$) stimulation (Fig. 2).

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Fig. 1. Rosemary extract (RE) increases muscle glucose uptake. Serum deprived L6 myotubes were incubated with the indicated concentrations of RE for 4 h (A), with 5 μg/mL of RE for the indicated time (B), followed by [^{3}H]-2-deoxy-D-glucose uptake measurement. Results are the mean ± SE of 6–8 independent experiments. **, $p < 0.01$; ***, $p < 0.001$ vs. control.

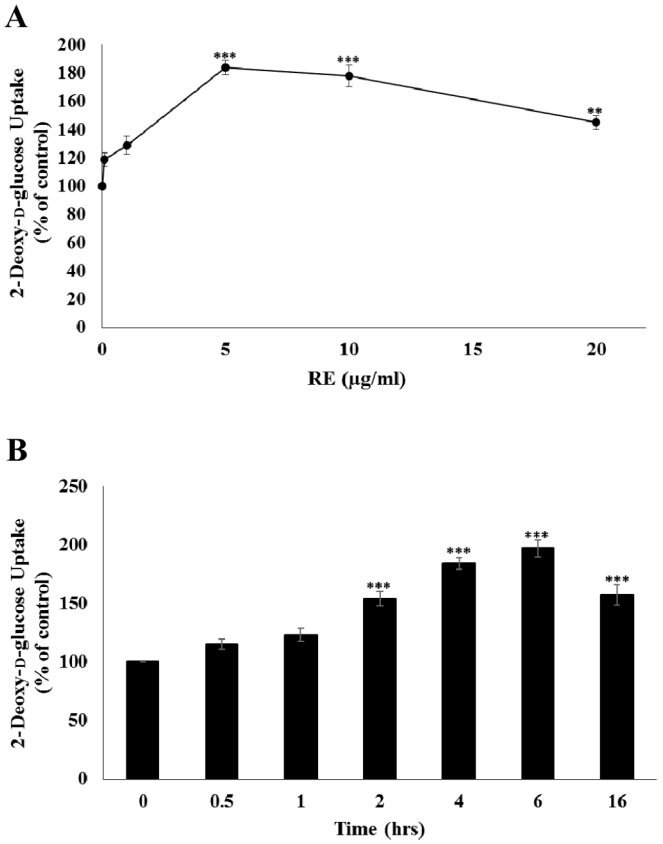
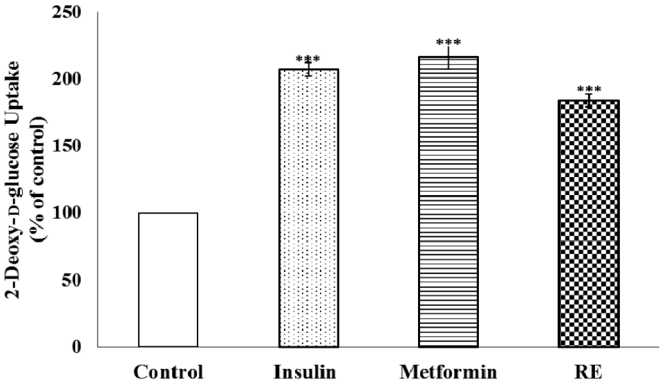


Fig. 2. Effects of rosemary extract (RE) on glucose uptake are comparable to effects of insulin and metformin. Serum deprived L6 myotubes were incubated with 5 μg/mL RE (4 h), 2 mmol/L metformin (16 h) or 100 nmol/L insulin (30 min), followed by [^{3}H]-2-deoxy-D-glucose uptake measurement. Results are the mean ± SE of 6–8 independent experiments. ***, $p < 0.001$ vs. control.



To investigate whether PI3K may be involved in the RE-stimulated glucose uptake, we used the PI3K inhibitor, wortmannin. Wortmannin did not affect the RE response (RE: 208% ± 13.62%, wortmannin + RE: 201% ± 9.32% of control) while it inhibited the insulin response (insulin: 210% ± 9.47%; wortmannin + I: 140% ± 6.08% of control) (Fig. 3A). Furthermore, RE did not affect the phosphorylation and expression of Akt, a downstream target of PI3K, in contrast with a significant phosphorylation/activation seen with insulin (260% ± 11.5% of control, $p < 0.05$) (Figs. 3B, 3C). In addition, treatment with RE did not affect the tyrosine phosphorylation of IRS-1 (Tyr895), a downstream target of the insulin receptor, in contrast with a significant phosphorylation/activation seen with insulin (234 ± 18.9 of control, $p < 0.05$) (Figs. 3D, 3E). The total levels of IRS-1 were not changed by any treatment (Figs. 3D, 3E). These data indicate that the IRS1-PI3K-Akt signalling cascade involved in insulin-stimulated glucose uptake is not involved in the RE-stimulated glucose uptake.

We showed previously that the polyphenols resveratrol and naringenin activate AMPK (Breen et al. 2008; Zygmunt et al. 2010), and we hypothesized that RE, which is reported to contain polyphenols, activates AMPK. Indeed, treatment with RE (5 μg/mL for 2 or 4 h) resulted in a significant increase in AMPK-Thr172 phosphorylation, an indicator of activation (RE 2 h: 157.3% ± 11.3%, RE 4 h: 190.3% ± 17.6% of control, $p < 0.05$), (Figs. 4A, 4B). As expected, metformin, 5-aminoimidazole-4-carboxamide-1-4-ribofuranoside (AICAR), and resveratrol significantly increased AMPK phosphorylation (196% ± 27.5%, 238% ± 28.4%, and 212% ± 29.3% of control, respectively) while insulin treatment did not have any effect (Figs. 4A, 4B). Activation of AMPK leads to downstream phosphorylation of ACC and phosphorylation of ACC has been established as an indicator of AMPK activity. We therefore examined the effect of RE on ACC phosphorylation. Treatment with 5 μg/mL RE for 4 h resulted in a significant increase in ACC-Ser79 phosphorylation (328% ± 36.9% of control, $p < 0.05$) (Figs. 4C, 4D) clearly indicating AMPK activation. The total levels of ACC were unchanged by any treatment. Interestingly, the use of compound C, an inhibitor of AMPK, significantly reduced the RE-stimulated glucose uptake (RE: 208% ± 8.11%; compound C + RE: 142% ± 2.40% of control) and the metformin-stimulated glucose uptake (metformin: 216% ± 8.77%, compound C +metformin: 106.2% ± 5.59% of control) (Fig. 5) indicating AMPK involvement in the action of both.

Insulin increases glucose transport in L6 cells by causing GLUT translocation. To elucidate the mechanism of RE-stimulated glucose uptake, we measured plasma membrane levels of GLUT4 and compared it with the effects of insulin and metformin. Using GLUT4myc overexpressing L6 cells, we found an increase in plasma membrane GLUT4myc levels by insulin and metformin (217% ± 11.26%, 175% ± 7.18% of control, respectively; both $p < 0.001$), while RE had no effect (106% ± 3.71% of control, $p > 0.05$), (Fig. 6A). Similarly, RE did not affect GLUT1myc plasma membrane levels (113% ± 5.52% of control, $p > 0.05$), in contrast with a significant increase seen with insulin (139% ± 1.44%, $p < 0.05$) and metformin (134% ± 7.8% of control, $p < 0.05$), (Fig. 7A). RE increased glucose uptake in both GLUT4myc and GLUT1myc L6 overexpressing cells (172% ± 7.19%, 167% ± 8.82% of control, respectively; $p < 0.001$) (Figs. 6B, 7B). Taken together, these results indicate that RE stimulates muscle cell glucose uptake by a mechanism that is independent of GLUT4 and GLUT1 translocation.

Discussion

The present study is the first to show that RE directly stimulates glucose uptake in L6 muscle cells to levels comparable to insulin and the anti-diabetic drug metformin. This effect was dose- and time-dependent and did not require the presence of insulin as the cells were incubated in media without any insulin. Significant stimulation was seen with 5–10 μg/mL RE, which are concentrations shown previously to inhibit gluconeogenesis in hepatocytes (Tu et al. 2013; Yun et al. 2013) and used in other in vitro studies examining its anticancer properties (Cheung and Tai 2007; Ngo et al. 2011).

Insulin stimulates glucose uptake rapidly within 30 min while RE required a longer incubation time (2 h) and a significant in-

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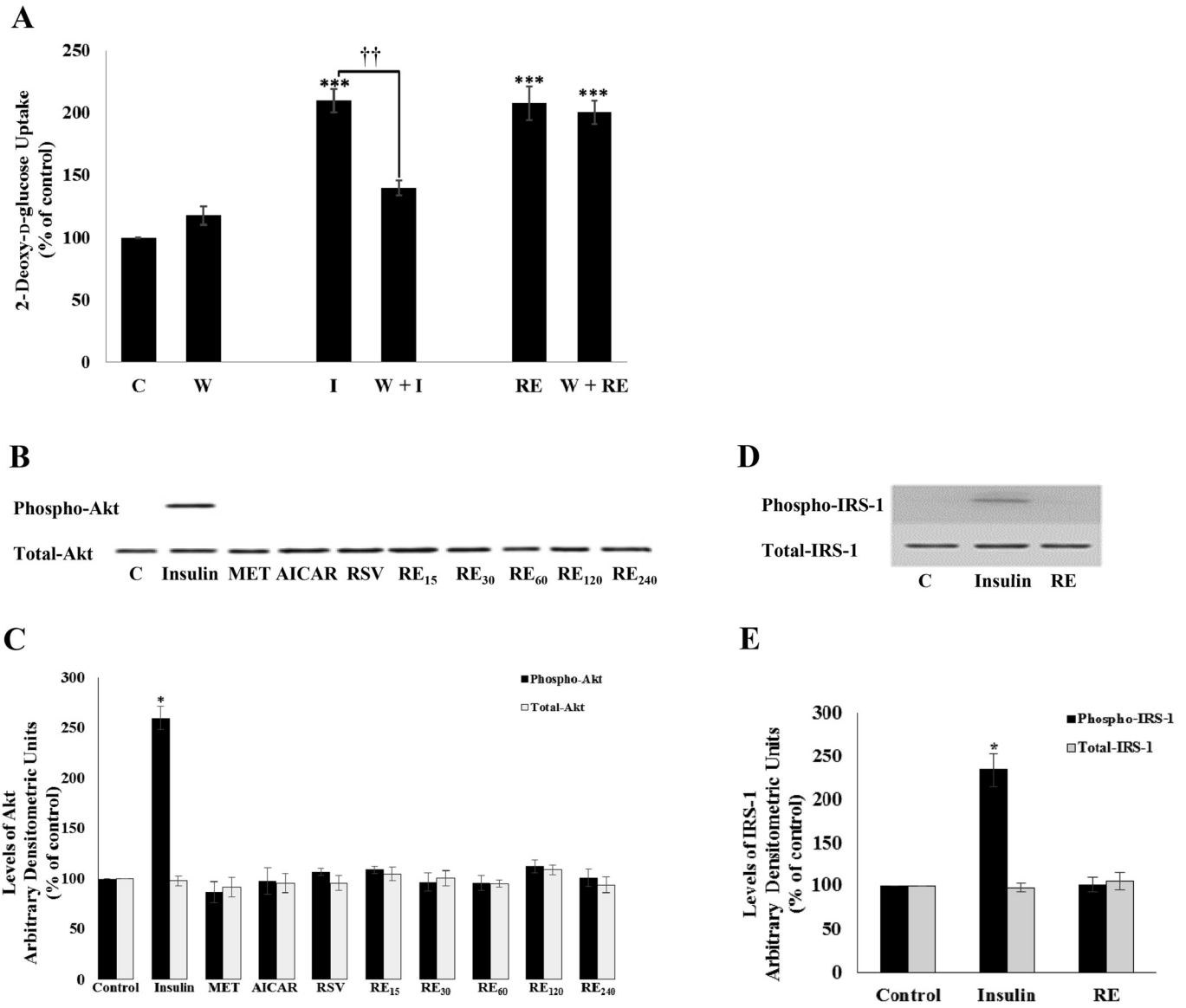
Fig. 3. Rosemary extract (RE)-induced glucose uptake is independent of IRS1-PI3K-Akt signalling. Cells were incubated in the absence or presence of 100 nmol/L wortmannin for 15 min followed by treatment with or without 5 μg/mL of RE for 4 h or 100 nmol/L insulin for 30 min and [^{3}H]-2-deoxy-D-glucose uptake measurements (A). Whole cell lysates from cells treated with 100 nmol/L insulin (15 min), 2 mmol/L metformin (MET) (120 min), 2 mmol/L 5-aminoimidazole-4-carboxamide-1-4-ribofuranoside (AICAR) (120 min), 100 μmol/L resveratrol (RSV) (120 min), or 5 μg/mL RE for the indicated times (B, C, D, E) were prepared, resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and immunoblotted for total and phosphorylated (phospho) Akt (Ser473) and insulin receptor substrate 1 (IRS-1) (Tyr895). Representative immunoblots (B, D). Immunoblots were scanned and the values are arbitrary densitometric units compared with control (C, E). Results are the mean ± SE of 4–7 independent experiments. *, $p < 0.05$; ***, $p < 0.001$ vs. control; ††, $p < 0.01$ vs. insulin alone.



crease was maintained after 16 h of exposure. Interestingly the time-dependent action of RE is similar to metformin, which has a kinetically prolonged onset of action (Owen et al. 2000; Zhou et al. 2001) in comparison with insulin.

RE contains many polyphenols with carnosol, carnosic acid, and rosmarinic acid being the ones found in high concentrations (Moreno et al. 2006; Bai et al. 2010). It is possible that the observed effects of RE are mediated by one, a combination of a few, or all of these polyphenols. A recent study in L6 muscle cells found a significant increase in glucose uptake by 5 to 20 μmol/L carnosic acid treatment (Lipina and Hundal 2014). Additionally, carnosic acid was shown to attenuate palmitate-induced lipid accumulation and insulin resistance in HepG2 hepatocytes (Wang et al. 2012). Unfortunately, there are no studies examining the effects of the other major RE polyphenols, rosmarinic acid, and carnosol alone in any insulin target tissues. In addition, there are no studies examining the combined effects of these polyphenols on glucose homeostasis.

Our data indicate that the effect of RE on glucose uptake is PI3K-independent since wortmannin, an irreversible inhibitor of PI3K, did not affect the RE-stimulated glucose uptake in contrast with significant inhibition of the insulin response. RE had no effect on IRS-1 or Akt phosphorylation while it significantly increased phosphorylation of AMPK at its Thr172 residue and ACC at its Ser79 residue, which are established indicators of activation (Towler and Hardie 2007). Furthermore, inhibition of AMPK with compound C (an ATP-competitive inhibitor of AMPK) significantly reduced RE-stimulated glucose uptake, indicating that AMPK is

Fig. 4. Rosemary extract (RE) phosphorylates/activates muscle AMP-activated kinase (AMPK). L6 myotubes were treated without or with 5 μg/mL of RE for 15, 30, 60, 120, or 240 min, 2 mmol/L metformin (MET) (120 min), 2 mmol/L 5-aminoimidazole-4-carboxamide-1-4-ribofuranoside (AICAR) (120 min), or 100 μmol/L resveratrol (RSV) (120 min). Whole cell lysates were prepared, resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and immunoblotted for total and phosphorylated (phospho) AMPK (Thr172) and acetyl-CoA carboxylase (ACC) (Ser79). Representative immunoblots (A, C). The immunoblots were scanned and the values are arbitrary densitometric units compared with control untreated cells (B, D). Data are the mean ± SE of 3–4 experiments. *, $p < 0.05$ compared with control. C, control.

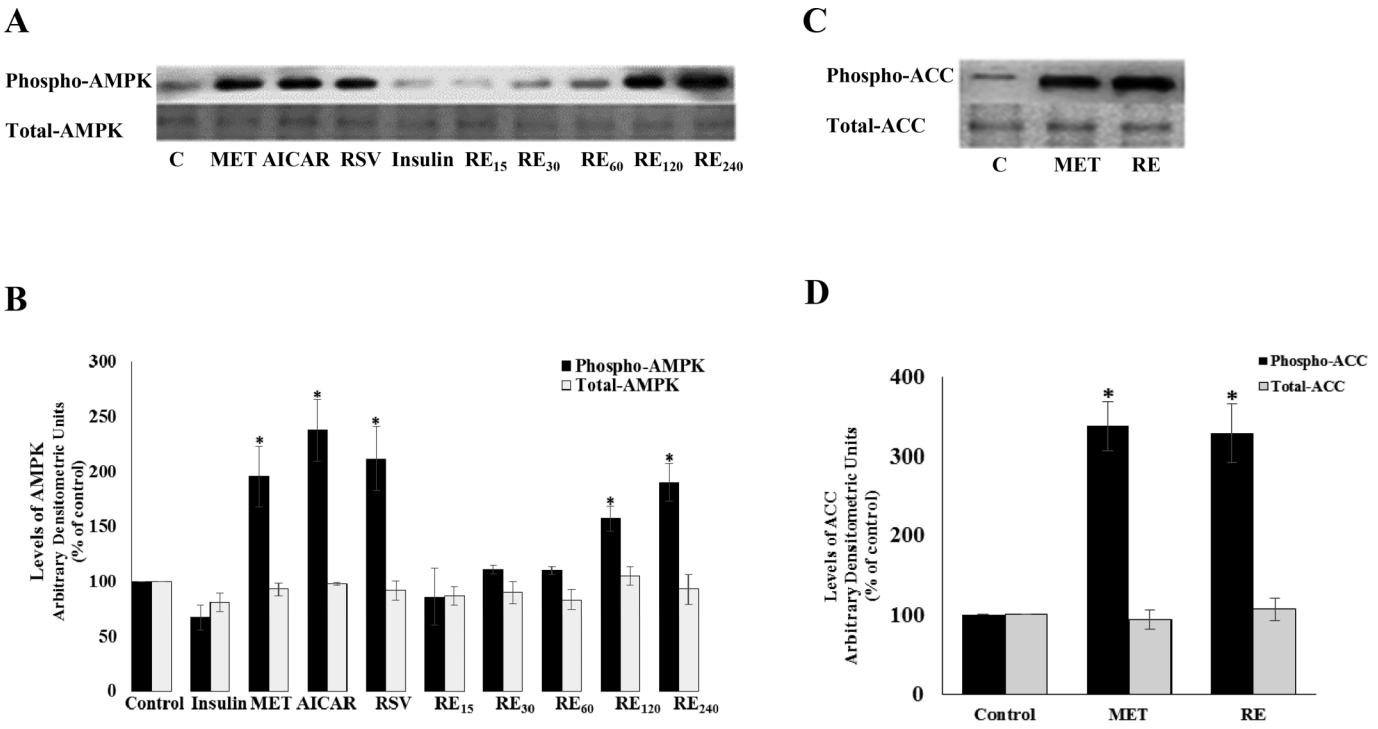
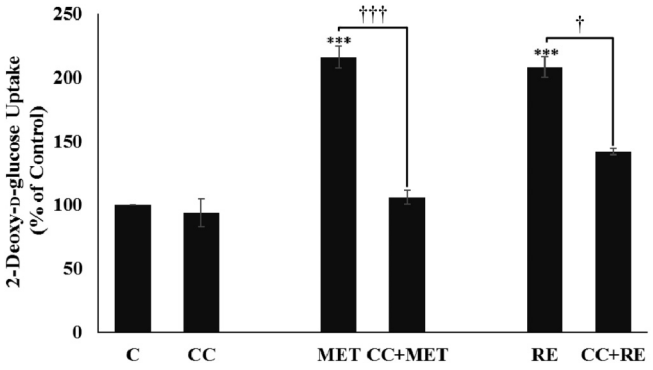


Fig. 5. AMPK is involved in rosemary extract (RE)-induced glucose uptake. L6 myotubes were incubated in the absence or presence of 25 μmol/L compound C (CC) for 30 min followed by the addition of 2 mmol/L metformin (MET) for 2 h or 5 μg/mL of RE for 4 h and [^{3}H]-2-deoxy-D-glucose uptake measurements. Data are the mean ± SE of 3–4 experiments. ***, $p < 0.001$ compared with control. †, $p < 0.05$ vs RE alone; †††, $p < 0.001$ vs. MET alone.



involved in this action of RE. In agreement with our study, RE increased AMPK phosphorylation in hepatocytes (Tu et al. 2013). Similarly, carnosic acid, a polyphenol found in high concentration in rosemary extract (Ho et al. 2000; Moreno et al. 2006; Costa et al. 2007), was shown to activate AMPK in hepatocytes in a time-dependent manner (Wang et al. 2012). We previously reported that the polyphenols resveratrol (Breen et al. 2008) and naringenin (Zygmunt et al. 2010) increased glucose uptake in muscle cells by a mechanisms involving AMPK activation. Our present study is the first to show activation of muscle cell AMPK by RE.

Activators of AMPK include an increase in intracellular AMP/ATP ratio and its upstream kinases LKB1 and CaMKK (Towler and Hardie 2007). RE or its bioactive compounds may allosterically modulate AMPK activity, increase the activity of upstream kinases (LKB1 and CaMKK), or alternatively cause an increase in the AMP/ATP ratio that is secondary to inhibition of the mitochondrial complex 1, an action similar to that reported for metformin (Owen et al. 2000).

Skeletal muscle glucose uptake is mediated by the glucose transporters (GLUTs). During basal conditions, GLUT1 is predominantly responsible for transporting glucose while GLUT4 becomes the major glucose transporter in skeletal muscle cells in response to insulin (Zhao and Keating 2007). Since RE increased muscle cell glucose uptake, we sought to determine its effects on GLUT1 and GLUT4 translocation. These transporters are expressed in L6 myotubes and have been shown to be affected by insulin and metformin. We measured plasma membrane GLUT1 and GLUT4 levels in L6 cells overexpressing GLUT1 or GLUT4, respectively, that were tagged with an exofacial c-myc epitope. RE did not cause significant increases in GLUT1 or GLUT4 translocation despite significant increases in glucose transport. In contrast, both insulin and metformin increased GLUT1 and GLUT4 translocation. It is of importance to note that previous work by our group indicated that the polyphenol resveratrol stimulates skeletal muscle glucose uptake without causing translocation of GLUTs (Breen et al. 2008). RE appears to have similar effects and deserves further attention and detailed elucidation of its mechanisms of action. It is possible that the polyphenolic constituents of RE may have a sole or combined effect to increase the (*i*) intrinsic activity of the GLUT1 and GLUT4 transporters, (*ii*) intrinsic activity of the GLUT3 glucose transporter also expressed in L6 cells, (*iii*) translocation of GLUT3, and (*iv*) stability/half-life of GLUTs by reducing their degradation.

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Fig. 6. Effect of rosemary extract (RE) on plasma membrane GLUT4 levels. L6 GLUT4*myc* overexpressing cells were treated with insulin (100 nmol/L, 20 min), metformin (MET) (1 mmol/L, 6 h), or RE (5 μg/mL, 4 h), followed by measurements of plasma membrane GLUT4 transporter levels (A) or glucose uptake (B). Results are means ± SE of 6–8 independent experiments performed in triplicate and expressed as percentage of control; ***, $p < 0.001$.

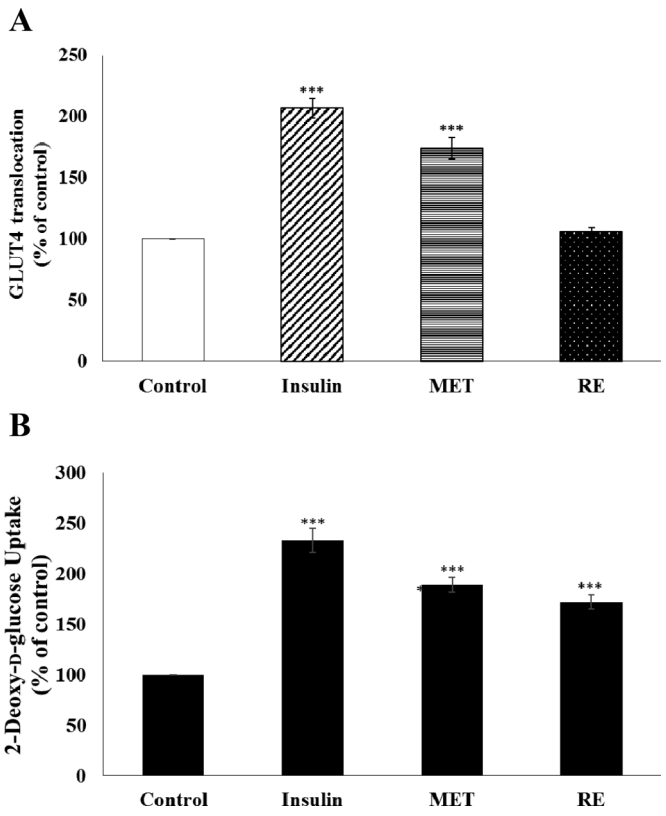
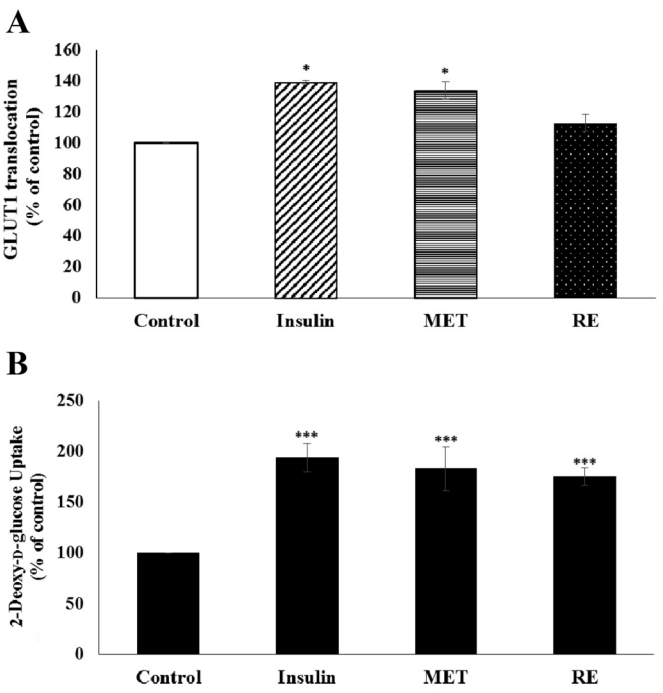


Fig. 7. Effect of rosemary extract (RE) on plasma membrane GLUT1 levels. L6 GLUT1*myc* overexpressing cells were treated with insulin (100 nmol/L, 20 min), metformin (MET) (1 mmol/L, 6 h), or RE (5 μg/mL, 4 h), followed by measurements of plasma membrane GLUT1 transporter levels (A) or glucose uptake (B). Results are means ± SE of 6–8 independent experiments performed in triplicate and expressed as percentage of control; *, $p < 0.05$; ***, $p < 0.001$.



Our findings that RE-induced muscle cell glucose uptake and AMPK phosphorylation was not accompanied by effects on GLUT translocation are in agreement with studies using AICAR, troglitazone, and berberine. Activation of AMPK with AICAR resulted in increased skeletal muscle glucose uptake without a parallel increase in GLUT4 translocation (Lemieux et al. 2003). Troglitazone, a thiazolidinedione class of anti-diabetic medication, increased glucose uptake and AMPK phosphorylation in L6 cells independent of GLUT translocation (Konrad et al. 2005). Berberine, a botanical alkaloid, increased glucose uptake in an AMPK-dependent manner without the involvement of the GLUTs (Yin et al. 2008). Additionally, AMPK activation was suggested to increase intrinsic GLUT4 activity in 3T3-L1 adipocytes (Yamaguchi et al. 2005) and GLUT1 activity in clone 9 cells (Barnes et al. 2002). It is possible that activation of AMPK by RE leads to increased plasma membrane GLUT activity.

Previous in vivo studies have demonstrated that RE protects against hyperlipidemia and hyperglycemia in genetic, chemically induced and dietary animal models of obesity and T2DM. Daily administration of RE significantly improved plasma glucose levels in streptozotocin-induced diabetic rats (Koga et al. 2006; Aljamal and Alqadi 2011; Khalil et al. 2012; Ramadan et al. 2013) and in alloxan-induced rabbits (Bakırel et al. 2008). Similarly, RE significantly protected mice against high-fat-diet–induced elevations in plasma glucose and lipid levels (Ibarra et al. 2011; Al-Sheyab et al. 2012; Afonso et al. 2013). In genetically obese (*ob*/*ob*) mice, carnosic acid, a polyphenolic component of RE, protected against fat-induced hyperglycemia and hyperlipidemia (Wang et al. 2011). Collectively, these studies demonstrate that RE and its polyphenolic components have anti-hyperglycemic properties in vivo. However, there are currently no studies that delineate the mechanism underlying the reported in vivo effects of RE. Our study is the first to show a direct effect of RE to increase muscle cell glucose uptake by a mechanism involving AMPK activation. Direct effects of RE on muscle tissue may explain its in vivo anti-hyperglycemic effects. Overall RE and its polyphenolic constituents deserve further systematic studies in animals and humans to evaluate their direct effects on insulin target tissues and their potential use to prevent insulin resistance.

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