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SHORT COMMUNICATION

4-Hydroxyisoleucine stimulates glucose uptake by increasing surface GLUT4 level in skeletal muscle cells via phosphatidylinositol-3-kinase-dependent pathway

Natasha Jaiswal · Chandan K. Maurya · K. Venkateswarlu · P. Sukanya · Arvind K. Srivastava · Tadigoppula Narender · Akhilesh K. Tamrakar

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Abstract
Purpose To determine the effect of 4-Hydroxyisoleucine (4-HIL), an unusual amino acid isolated from the seeds of *Trigonella foenum-graecum*, on glucose uptake and the translocation of glucose transporter 4 (GLUT4) to plasma membrane in skeletal muscle cells and to investigate the underlying mechanisms of action.
Methods Rat skeletal muscle cells (L6-GLUT4*myc*) were treated with 4-HIL, and the effect on glucose uptake was determined by measuring the incorporation of radio-labeled 2-deoxy-[^{3}H]-D-glucose (2-DG) into the cell. Translocation of GLUT4*myc* to plasma membrane was measured by an antibody-coupled colorimetric assay.
Results The prolonged exposure (16 h) of L6-GLUT4-*myc* myotubes to 4-HIL caused a substantial increase in the 2-DG uptake and GLUT4 translocation to the cell surface, without changing the total amount of GLUT4 and GLUT1. Cycloheximide treatment reversed the effect of 4-HIL on GLUT4 translocation to the basal level suggesting the requirement of new protein synthesis. The 4-HIL-induced increase in GLUT4 translocation was completely abolished by wortmannin, and 4-HIL significantly increased the basal phosphorylation of AKT (Ser-473), but did not change the mRNA expression of AKT, IRS-1, GLUT4, and GSK3β.
Conclusion Results suggest that 4-HIL stimulates glucose uptake in L6-GLUT4*myc* myotubes by enhancing translocation of GLUT4 to the cell surface in a PI-3-kinase/AKT-dependent mechanism.

Keywords Insulin resistance · 4-Hydroxyisoleucine · GLUT4 translocation · Skeletal muscle

N. Jaiswal · C. K. Maurya · A. K. Srivastava · A. K. Tamrakar (✉)
Division of Biochemistry, CSIR-Central Drug Research Institute, Lucknow 226001, India
e-mail: akhileshcdri@gmail.com; akhilesh_tamrakar@cdri.res.in

K. Venkateswarlu · P. Sukanya · T. Narender (✉)
Division of Medicinal and Process Chemistry, CSIR-Central Drug Research Institute, Lucknow 226001, India
e-mail: t_narendra@cdri.res.in

Introduction

Type 2 diabetes mellitus (T2DM) is one of the most prevalent and fastest growing diseases, characterized by a combination of pancreatic-impaired insulin secretion and peripheral and hepatic insulin resistance [1]. Insulin resistance is characterized by a reduced ability of target tissues, such as liver, skeletal muscle, and adipose, to respond to insulin [2]. Skeletal muscle is the principal site for post-prandial glucose utilization and disposal, and has a paramount role in energy balance. Insulin resistance in skeletal muscle is associated with blunted responsiveness of insulin-signaling pathway, characterized by decreased insulin-stimulated glucose uptake as a consequence of altered translocation of glucose transporter 4 (GLUT4) from intracellular compartment to plasma membrane [3].

Although numerous oral antidiabetic drugs exist, there is no promising therapy for T2DM. Current therapeutics for diabetes are often associated with drawbacks like poor pharmacokinetic properties, secondary failure, undesirable side effects, and in many cases, the precise mechanism of action remains to be completely clarified [4]. Therapeutic approaches with natural products provide a fruitful source for searching safe, effective, and relatively inexpensive new remedies for diabetes mellitus [5, 6]. An ideal approach addressing defect in both pancreatic insulin

secretion and peripheral insulin action is of great interest for the treatment of T2DM [7].

Trigonella foenum-graecum (Leguminosae family) is an annual herbaceous plant commonly known as fenugreek and is widely distributed across Asia, Africa, and Europe. Fenugreek seeds have been traditionally used to decrease hyperglycemia in diabetic patients [8]. One of the active ingredients has been identified as 4-hydroxyisoleucine (4-HIL) that is exclusively present in the fenugreek plant. 4-HIL has shown to exhibit antidiabetic and antidyslipidemic effects in animal models of diabetes [9–12]. Furthermore, 4-HIL was proven to possess glucose-dependent insulinotropic [13, 14], and insulin-sensitizing effects in in vivo rodent model [7]. In present study, we investigated the influences of 4-HIL on glucose uptake and GLUT4 translocation in clonal skeletal muscle cells to explore the possible mechanisms of its antidiabetic effect. Results indicate the 4-HIL promotes glucose uptake in skeletal muscle cells via the enhancement of GLUT4 translocation to cell surface through a phosphatidylinositol-3-kinase (PI-3-K)/AKT-dependent pathway.

Materials and methods

Materials

Alpha MEM, fetal bovine serum, trypsin, and antibiotic/antimycotic solution were from Gibco, USA. 2-Deoxy-D-[^{3}H]-glucose (2-DG) was from GE Healthcare, UK. Antibodies to GLUT4 (IF8) and GLUT1 were from Santa Cruz Biotechnology, Inc. (USA) and antibody to phospho-AKT (Ser-473) was from Cell Signaling Technology (USA). All other chemicals, unless otherwise mentioned were from Sigma–Aldrich, USA.

Isolation of 4-HIL

4-HIL was isolated and characterized from the seeds of *T. foenum-graecum* as described previously [11].

Cell culture

L6 skeletal muscle cells stably expressing rat GLUT4 with a *myc* epitope inserted in the first exofacial loop (L6-GLUT4*myc*) were a kind gift of Dr. Amira Klip, Program in Cell Biology, The Hospital for Sick Children, Toronto, Canada. Cells were maintained in α-MEM and used for experimentation as described previously [16, 20].

Glucose transport

Determination of 2-Deoxy-D-[^{3}H]-glucose (2-DG) transport in L6-GLUT4*myc* myotubes was performed by incubating cells for 5 min in HEPES-buffered saline containing 10 μM 2-DG (0.5 μCi/ml 2-[^{3}H] DG) at room temperature, followed by cell lysis and measurement of radioactivity incorporated by scintillation counting, as described previously [16]. Nonspecific uptake was determined in the presence of cytochalasin B (25 μM) during the assay.

GLUT4 translocation

GLUT4*myc* levels at the cell surface of intact myotubes were measured by an antibody-coupled colorimetric assay as previously described [16]. Essentially, after indicated treatments cells were fixed in 3 % paraformaldehyde and quenched in 100 mM glycine. Following blocking with 5 % skimmed milk, cells were incubated with anti-*myc* antibody solution for 1 h. After labeling, excess antibodies were removed by extensive washing in PBS. Cell surface GLUT4-bound antibodies were probed by HRP-conjugated secondary antibodies followed by the detection of bound HRP by O-phenylenediamine dihydrochloride reagent. The fraction of GLUT4*myc* at the cell surface, measured in triplicate, was expressed as fold induction with respect to unstimulated cells.

Cell lysates and immunoblotting

L6-GLUT4*myc* myotubes were treated as indicated, and cell lysates were prepared as previously described [16, 17]. Equal amounts of protein were immunoblotted with antibodies to phospho-AKT (Ser-473), GLUT4 or GLUT1. Densitometric quantification of protein bands was performed using National Institute of Health (NIH) Image J software. To validate equal loading in each lane and normalize the blots for protein levels, Actinin-1 was used as internal loading control.

RNA extraction and gene expression analysis

Total RNA was extracted from L6-GLUT4*myc* myotubes, following the exposure to 4-HIL by TRIZOL (invitrogen). Semi-quantitative reverse transcriptase-PCR was performed by two-step RT-PCR kit (Qiagen Inc.) using gene-specific primers.

Statistical analysis

Values are given as mean ± SE. Analysis of statistical significance of differences in measurements between samples was done by one-way ANOVA with Dunnets post hoc test (GraphPad Prism version 3). $p < 0.05$ was considered statistically significant.

Results

4-HIL stimulates 2-DG uptake without affecting GLUT4 and GLUT1 proteins level

4-HIL increased glucose uptake in L6-GLUT4*myc* myotubes in a concentration-dependent fashion (Fig. 1a). 4-HIL increased basal glucose uptake in L6-GLUT4*myc* myotubes to a significant level at a minimum concentration of 5 μM ($p < 0.05$), while maximal stimulation was observed at 25 μM (1.5 ± 0.12-fold, $p < 0.01$ vs. control basal). Insulin alone enhanced glucose uptake by 2.0 ± 0.24-fold ($p < 0.001$ vs. control basal) in L6-GLUT4*myc* myotubes. Treatment with 4-HIL for 16 h before challenging the latter with insulin for 20 min had no significant effect on glucose uptake compared to control cells treated with insulin alone (Fig. 1a).

To investigate whether the 4-HIL-induced glucose uptake was accompanied by an increase in total amount of GLUT4 or GLUT1, cellular level of GLUT4 and GLUT1 proteins was assessed. Treatment with 4-HIL had no significant effect on total amount of GLUT4 or GLUT1 proteins either under basal or insulin-stimulated condition in L6-GLUT4*myc* myotubes (Fig. 1b, c).

4-HIL enhances surface GLUT4*myc* level in L6-GLUT4*myc* myotubes

In skeletal muscle, translocation and redistribution of the GLUT4 to the plasma membrane is a characteristic feature for increased glucose uptake [3]. Treatment of L6-GLUT4*myc* myotubes with 4-HIL enhanced the surface level of GLUT4*myc* in a dose-dependent manner under basal condition (Fig. 1d), with a maximal increase at 25 μM concentration (1.4 ± 0.22-fold, $p < 0.01$ vs. control basal). As expected, insulin enhanced cell surface GLUT4*myc* level by 2.0 ± 0.07-fold ($p < 0.001$) under control condition. Incubation with 4-HIL (16 h) had no significant effect on insulin response to stimulate cell surface GLUT4*myc* level compared to cells treated with insulin alone (Fig. 1d).

Incubation of L6-GLUT4*myc* myotubes with 25 μM 4-HIL resulted in the significant increase in the level of GLUT4*myc* at cell surface in a time-dependent fashion (Fig. 1e). Maximum stimulation by 4-HIL was observed after 16 h (1.38 ± 0.07-fold, $p < 0.01$ vs. control basal), and in all subsequent experiments, 16-h incubation at 25 μM concentration was used.

To examine whether the biological response in 4-HIL-treated cells was caused by a protein synthesis-dependent process, we determined the effect of cycloheximide (CHX, 1 μg/ml), an inhibitor of protein synthesis on 4-HIL-induced GLUT4*myc* translocation (Fig. 1f). In the presence of CHX, the effect of 4-HIL (16 h) was completely abolished (control, 1.0 ± 0.13-; 4-HIL, 1.36 ± 0.17-; 4-HIL and CHX, 0.95 ± 0.15-fold vs. control without CHX). CHX alone had no significant effect on GLUT4*myc* surface level (1.06 ± 0.12-fold vs. control without CHX). These results suggest that increase in GLUT4*myc* translocation by 4-HIL require synthesis of new proteins.

4-HIL-stimulated GLUT4*myc* translocation via PI-3-K/AKT-dependent mechanisms

To clarify the mechanism of the enhancement of GLUT4*myc* translocation to plasma membrane by 4-HIL, we measured 4-HIL-induced GLUT4*myc* translocation in the presence of wortmannin (WRT), a specific inhibitor for PI-3-K. In 4-HIL-treated cells, GLUT4*myc* translocation was completely inhibited in the presence of WRT (Fig. 2a). In parallel experiments, the presence of WRT completely reversed the insulin-induced GLUT4*myc* translocation to cell surface, to basal level (Fig. 2a).

Given that effect of 4-HIL to stimulate GLUT-4 translocation is PI-3-K-dependent; its effect on phosphorylation status of AKT (Ser-473), a post-PI-3-K step in the insulin-signaling pathway was investigated. Insulin alone significantly increased the phosphorylation of AKT ($p < 0.001$ vs. control basal). 4-HIL treatment (16 h) significantly increased the AKT (Ser-473) phosphorylation under basal condition ($p < 0.05$ vs. control basal) without affecting its insulin-stimulated level in L6-GLUT4*myc* myotubes (Fig. 2b). Results indicated the involvement of PI-3-K/AKT-dependent pathway in 4-HIL-induced GLUT4*myc* translocation. However, treatment with 4-HIL (16 h) had no significant effect on mRNA levels of IRS-1, AKT, GSK3β, or GLUT4 in L6-GLUT4*myc* myotubes, the major components of PI-3-K/AKT signaling cascade (Fig. 2c).

Discussion

Insulin resistance results in decreased ability of insulin to stimulate glucose disposal in peripheral tissues [15]. A wide array of plant-derived molecules have been reported to increase glucose transport in skeletal muscle cells through their action on different cellular pathways leading to GLUT4 exocytosis to cell membrane [6, 16–18]. Such pharmacological agents could be helpful for treating T2DM. 4-HIL from *T. foenum-graecum* has been reported with glucose-dependent insulinotropic effect by a direct effect on pancreatic islets [13, 14] and insulin-sensitizing effect on muscle and liver tissues [7]. Here, we showed a concentration-dependent stimulation of basal glucose uptake with 4-HIL in L6-GLUT4*myc* myotubes. Moreover, there was no further significant increase in

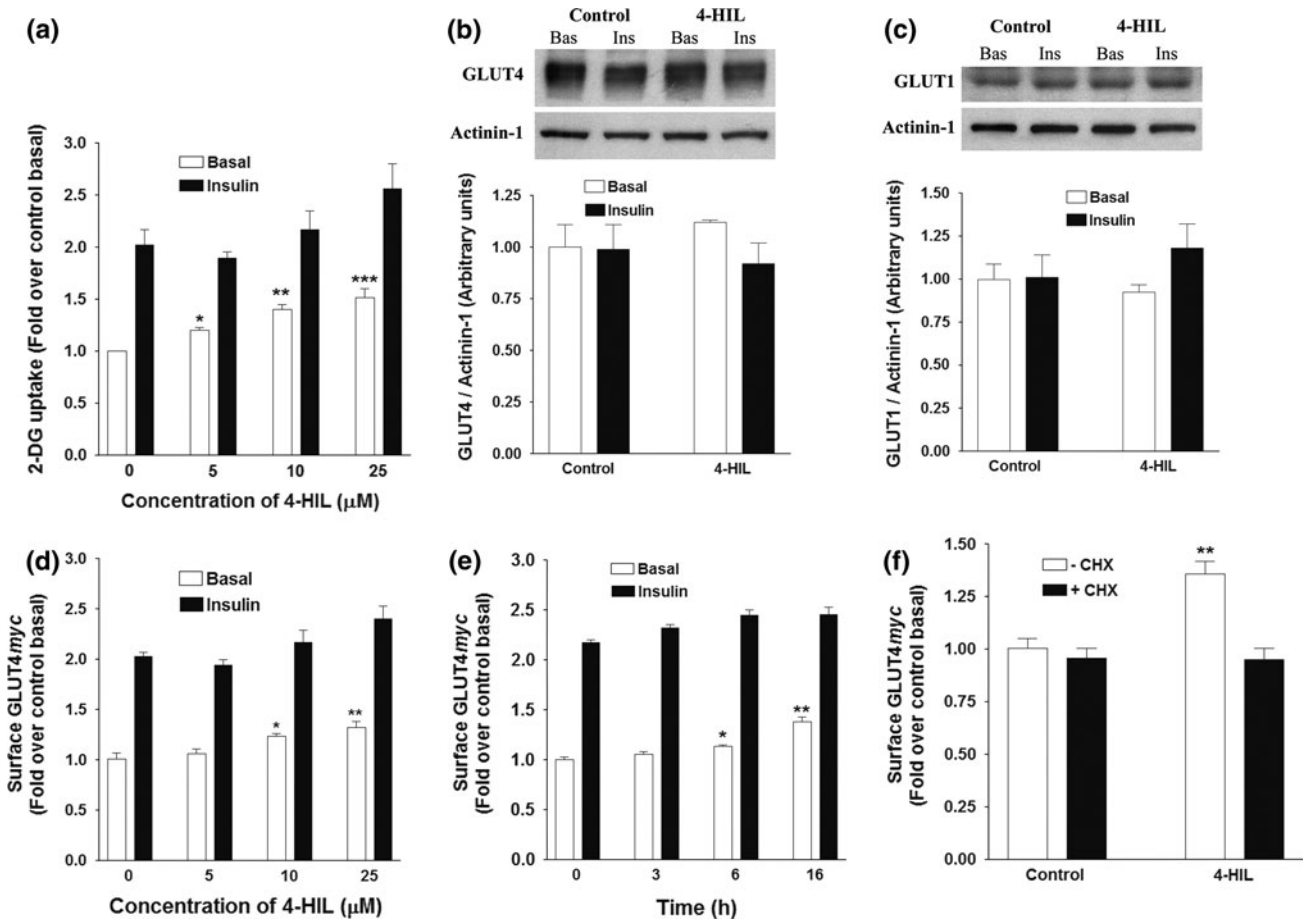

Fig. 1 Effect of 4-HIL on 2-DG uptake, glucose transporter expression, and GLUT4 translocation to cell surface in L6-GLUT4*myc* myotubes. Cells were incubated with indicated concentrations of 4-HIL for 16 h. After incubation, myotubes were left untreated or stimulated with 100 nM insulin for 20 min, followed by the determination of 2-DG uptake (**a**). After treatment with 4-HIL, cells were lysed for western analysis of glucose transporters. Shown are the representative immunoblots and densitometric quantification of GLUT4 (**b**) and GLUT1 (**c**). Cells were incubated with different concentrations of 4-HIL for 16 h (**d**) or 25 μM concentration of 4-HIL for various time period (**e**). After incubation, myotubes were left untreated or stimulated with 100 nM of insulin for 20 min, followed by the determination of the proportion of GLUT4*myc* at the cell surface (**d** and **e**). Cells were treated with 4-HIL in the presence of cycloheximide (CHX, 1 μg/ml) for 16 h. After incubation, surface level of GLUT4*myc* was measured (**f**). The values are mean ±SE of three independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. control. Bas, Basal; Ins, insulin treated

insulin-stimulated glucose transport upon preincubation with 4-HIL, indicating the likelihood that a complete insulin-dependent glucose uptake was already achieved. Treatment with 4-HIL had no significant effect on total amount of GLUT1 and GLUT4, the major glucose transporters responsible for glucose uptake in skeletal muscle cells.

In skeletal muscle, stimulation of glucose uptake is mostly attributed to increased translocation and redistribution of the GLUT4 to the plasma membrane [19]. L6-GLUT4*myc* cells express ectopically high quantities of GLUT4*myc* protein [20] and GLUT4*myc* is largely responsible for both basal and insulin-stimulated glucose uptake [21] in these cells. To explore the glucose uptake stimulatory effect of 4-HIL, we examined its effect on cell surface level of GLUT4*myc* and observed that similar to glucose uptake, 4-HIL increased surface GLUT4*myc* level under basal condition in a concentration-dependent fashion. Incubation with 4-HIL did not cause any further increase in insulin response to increase cell surface GLUT4*myc* density to a significant level, suggesting that signaling pathway activated by 4-HIL converges with that activated by insulin.

The PI-3-K/AKT pathway plays crucial role in insulin-stimulated GLUT4 translocation [22]. Binding of insulin to its receptor triggers tyrosine phosphorylation of insulin receptor substrate (IRS) proteins. This promotes binding of Src homology 2 domain-containing signaling proteins, which includes PI-3-K, Grb-2, and SHP2. Upon association with IRS proteins, PI-3-K is activated, leading to the activation of AKT via the generation of phosphatidylinositol 3,4,5-triphosphate [23]. Active AKT phosphorylates

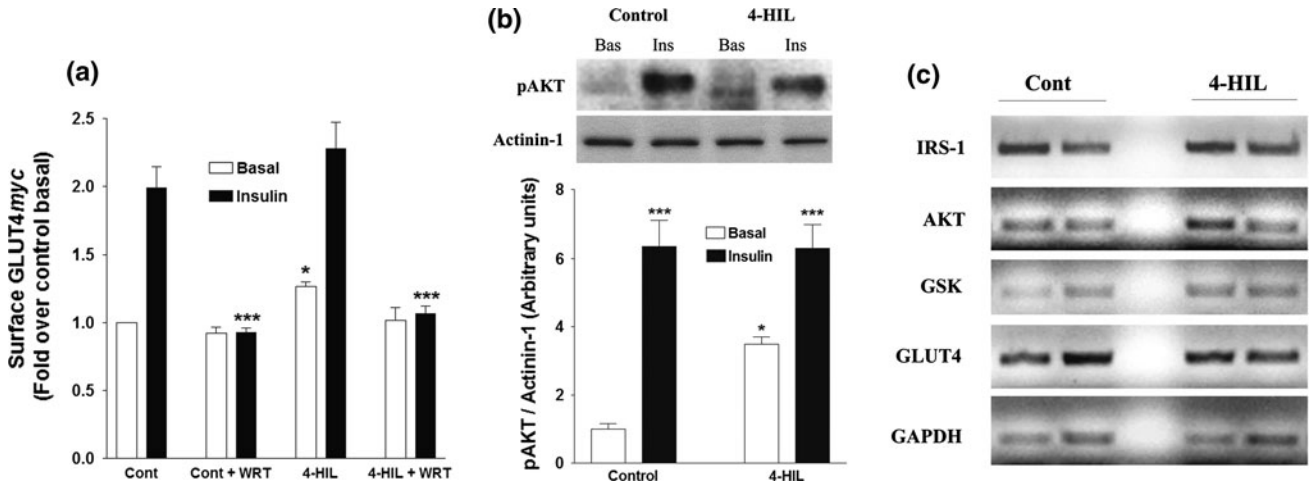

Fig. 2 4-HIL-induced GLUT4 translocation in L6-GLUT4*myc* myotubes is mediated via PI-3-K/AKT-dependent pathway. Cells were incubated with 4-HIL (25 μM) for 16 h with final one hour in the presence or absence of wortmannin (WRT, 50 nM). After incubation, myotubes were left untreated or stimulated with 100 nM insulin for 20 min, followed by the determination of the proportion of GLUT4*myc* at the cell surface (**a**). Cells were treated with 4-HIL for 16 h, followed by insulin treatment prior to cell lysis and immunoblotting for phosphor-AKT (Ser-473) (**b**). Gene expression analysis of the major components of PI-3-K/AKT pathway after treatment with 4-HIL for 16 h (**c**). Results shown are mean ±SE of three independent experiments, each performed in triplicate. $^{**}p < 0.01$, $^{***}p < 0.001$ relative to respective control condition. Cont, control; Bas, Basal; Ins, insulin treated

multiple downstream effectors that promote diverse biological responses, including the stimulation of glucose transport, protein, and glycogen synthesis, and the regulation of gene expression [22]. In the present study, PI-3-K inhibitor wortmannin reversed the insulin-stimulated GLUT4*myc* translocation to the basal level. The presence of wortmannin also blocks the 4-HIL-stimulated translocation of GLUT4*myc* to plasma membrane in L6-GLUT4*myc* myotubes, suggesting the involvement of PI-3-K-dependent pathway in 4-HIL-mediated biological response. To substantiate these findings, effect of 4-HIL on the activation of downstream-signaling molecule AKT was investigated. The activation of AKT requires its phosphorylation on Ser-473 [23]. Insulin significantly increased the phosphorylation of AKT on Ser-473 within 10 min. Treatment with 4-HIL also increased AKT (Ser-473) phosphorylation under basal condition but did not affect the insulin-stimulated AKT phosphorylation. These results are consistent with the increased glucose uptake and GLUT4*myc* translocation under basal condition.

The 4-HIL-mediated increase in GLUT4*myc* translocation was slow, and significant response was observed after 6 h of treatment, suggesting the requirement for de novo protein synthesis. Furthermore, the presence of protein synthesis inhibitor CHX prevented the HIL-induced increase in surface GLUT4*myc* level. Thus, the increase in surface GLUT4*myc* level with time apparently reflects the ongoing synthesis of protein(s) that affects GLUT4 translocation, although further investigation is necessary to identify the protein(s).

In summary, prolonged 4-HIL treatment stimulates glucose transport in L6-GLUT4*myc* myotubes by enhancing GLUT4 translocation from intracellular compartment to plasma membrane without changing the total amount of GLUT4 protein and its mRNA expression. 4-HIL exerts these effects through the involvement of PI-3-K/AKT-dependent pathway. The effects of 4-HIL on GLUT4 translocation provide a mechanism for its antidiabetic activity via improving glucose transport in skeletal muscle.

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Conflict of interest None.

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