Genipin stimulates glucose transport in C₂C₁₂ myotubes via an IRS-1 and calcium-dependent mechanism

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Abstract

Genipin, a compound derived from *Gardenia jasminoides* Ellis fruits, has been used over the years in traditional Chinese medicine to treat symptoms of type 2 diabetes. However, the molecular basis for its antidiabetic effect has not been fully revealed. In this study, we investigated the effects of genipin on glucose uptake and signaling pathways in C₂C₁₂ myotubes. Our study demonstrates that genipin stimulated glucose uptake in a time- and dose-dependent manner. The maximal effect was achieved at 2 h with a concentration of 10 μ M. In myotubes, genipin promoted glucose transporter 4 (GLUT4) translocation to the cell surface, which was observed by analyzing their distribution in subcellular membrane fraction, and increased the phosphorylation of insulin receptor substrate-1 (IRS-1), AKT, and GSK3 β . Meanwhile, genipin increased ATP levels, closed K_{ATP} channels, and then increased the concentration of calcium in the cytoplasm in C₂C₁₂ myotubes. Genipin-stimulated glucose uptake could be blocked by both the PI3-K inhibitor wortmannin and calcium chelator EGTA. Moreover, genipin increases the level of reactive oxygen species and ATP in C₂C₁₂ myotubes. These results suggest that genipin activates IRS-1, PI3-K, and downstream signaling pathway and increases concentrations of calcium, resulting in GLUT4 translocation and glucose uptake increase in C₂C₁₂ myotubes.

Key Words

- genipin
- myotubes
- glucose uptake
- insulin signaling

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Introduction

The prevalence of diabetes is dramatically increasing in the world, and according to the International Diabetes Federation, at present, the number of diabetic patients worldwide is ~366 million, a figure expected to rise to 600 million by 2030. Magnetic resonance spectroscopy studies on humans suggest that a defect in insulin-stimulated glucose transport in skeletal muscle is the

primary metabolic abnormality in insulin-resistant patients with type 2 diabetes. Although skeletal muscle takes up the important role in maintaining glucose homeostasis, few drugs treating diabetes have targeted this tissue. It is desirable to find novel small active molecules that could increase glucose uptake in skeletal muscle, and thus improve insulin resistance.

An extract from *Gardenia jasminoides* Ellis fruits has been used over the years in traditional Chinese medicine (TCM) to treat symptoms of type 2 diabetes (Danbuo 1984, Wang 1991). Geniposide is one of the major iridoid glycosides in the fruits of dried gardenia and is hydrolyzed to form aglycone genipin by h-D-glucosidase in the intestine and the liver. Besides being used to prepare a series of blue colorants in the food industry, genipin is also an effective cross-linking reagent for biological tissue fixation. According to previous studies, genipin and geniposide have been identified as being involved in various bioactivities, including modulation of proteins (Kuo *et al.* 2004, 2005), antitumor (Koo *et al.* 2004a, Kim *et al.* 2005, 2012), anti-inflammation (Koo *et al.* 2004b, 2006, Nam *et al.* 2010, Jeon *et al.* 2011), immunosuppression (Chang *et al.* 2005), antithrombosis (Suzuki *et al.* 2001, Wang *et al.* 2009), and protection of hippocampal neurons (Yamazaki *et al.* 2001, Lee *et al.* 2006). While it has been used for the relief of type 2 diabetes symptoms in TCM, the mechanism remains unknown. It was reported that genipin increased mitochondrial membrane potential, increased ATP levels, closed K_{ATP} channels, and stimulated insulin secretion in pancreatic islet cells (Zhang *et al.* 2006). However, in another study (Zhou *et al.* 2009), genipin was shown to cause suppression of insulin signal transduction through hyperactivation of c-Jun N-terminal kinase (JNK) and subsequent serine phosphorylation of insulin receptor substrate-1 (IRS-1), thus impairing insulin-stimulated glucose uptake in 3T3-L1 adipocytes. With these contradictory reports, the role of genipin in insulin resistance needs to be further explored; and as skeletal muscle accounts for 75–80% of the whole body insulin-stimulated glucose uptake, we examine its effect on glucose uptake in C_2C_{12} myotubes.

Glucose transport, a rate-limiting step in glucose metabolism, can be activated in peripheral tissues by two distinct pathways. One is insulin signaling pathway via PI3-K/Akt. Insulin accelerates glucose transport via the phosphorylation and activation of Akt, leading to glucose transporter 4 (GLUT4) translocation to the plasma membrane (PM). The other is by muscle contraction/exercise through the activation of AMPK (Krook *et al.* 2004). Besides these two signaling pathways, it has long been considered that the rise in intracellular Ca^{2+} is a critical mediator of increased glucose transport in skeletal muscle (Jessen & Goodyear 2005, Lanner *et al.* 2006). This has been proposed mostly on the basis of inhibition of the stimulation of glucose transport during insulin treatment, hypoxia, and contraction by agents that are thought to block Ca^{2+} channels (e.g. verapamil) or lower Ca^{2+} efflux

from the sarcoplasmic reticulum (e.g. dantrolene). Additionally, several studies have indicated that calcium is necessary for the recruitment of GLUT4 to the PM and have shown that the rates of glucose transport can be increased in mammalian muscle when cytoplasmic Ca^{2+} concentrations are raised using agents such as W-7, caffeine, and Ca^{2+} ionophores (Mitani *et al.* 1996, Jensen *et al.* 2007). Besides, intracellular calcium, PI3-K, and AMPK are known to stimulate glucose uptake; hence, we also included these factors to check their effect on genipin-induced glucose uptake.

In a word, in this study, we examined the effects of genipin on glucose transport and the intracellular distribution of glucose transporters in C_2C_{12} myotubes. Then, we explored the possible molecular mechanism by examining the main signal pathway involved in glucose uptake and the concentration of the intracellular calcium.

Materials and methods

Materials

Genipin was obtained from Wako Chemicals (Richmond, VA, USA). DMEM and fetal bovine serum (FBS) were purchased from Gibco (Invitrogen Corporation), wortmannin, cytochalasin B, 2-deoxy-D-[1- 3H] glucose, 5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide (AICAR), and EGTA from Sigma, and humulin R from Eli Lilly. Polyclonal anti-Akt antibody, anti-phospho-Akt (Thr308 and Ser473) antibody, anti-phospho-PKC ζ/λ (Thr410/403) antibody, anti-phospho-GSK3 β (Ser9) antibody, and anti-phospho-AMPK α (Thr172) antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). IRS-1 antibody and IR β antibody were purchased from Santa Cruz Biotechnology, Inc. and anti-phosphotyrosine antibody (PY20) from Upstate Biotechnology (Lake Placid, NY, USA).

Cell culture

Mouse skeletal muscle cells (C_2C_{12} myoblasts) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C with 95% air and 5% CO_2 . Myotube differentiation was induced as described previously (Kuang *et al.* 2009). In brief, C_2C_{12} cells were cultured until 75% confluence and differentiation medium (DMEM supplemented with 2% horse serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin) was then added and changed every other day. Experiments

were conducted in well-differentiated C₂C₁₂ myotubes for 4 days cultured in differentiation medium. Genipin was dissolved in DMSO. Genipin application was described in figure legends.

2-Deoxyglucose uptake assay

Glucose transport assay was performed by measuring [³H] 2-DOG uptake. Cells were cultured in 24-well plates and washed three times in Krebs–Ringer's phosphate buffer (20 mM HEPES, 137 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, and 2 mM pyruvate, pH 7.4). Cells were then incubated in the above medium containing 0.1 mM 2-DOG and 0.5 μCi [³H] 2-DOG (10 mCi/mmol) for 20 min and followed by three times of quick wash with ice-cold PBS containing 10 mM glucose. The cells were lysed in 0.1 M NaOH and subsequently solubilized in scintillation liquid (TritonX-100:methylbenzene, 1:2.5). [³H] 2-DOG was measured by liquid scintillation counter. Nonspecific glucose uptake in the presence of 10 μM cytochalasin B was subtracted.

Subcellular fractionation

To investigate GLUT4 translocation, PM and low-density microsomes (LDM) were obtained according to the protocol as described by Shisheva *et al.* (1994) and Tortorella & Pilch (2002) with modification. Briefly, cells were washed in ice-cold PBS and scraped in HES buffer (20 mM Tris/HCl, pH 7.4, 255 mM sucrose, 1 mM EDTA, and 1× protease inhibitor mixture) on ice and homogenized. The homogenates were centrifuged at 19 000 *g* for 20 min. The supernatants were further centrifuged at 180 000 *g* for 90 min to obtain LDM fraction and the pellets were further centrifuged at 100 000 *g* for 60 min to obtain PM fraction from the interface of a 1.12 M sucrose cushion. The purified fractions were then resuspended in HES buffer and quantitated using BCA Protein Assay Reagent (Pierce Product, Rockford, IL, USA).

Western blotting

The cell lysates were extracted with lysis buffer (RIPA, 1× PBS, 1% Nonidet P-40, 5 mM EDTA, 0.1% SDS, 1 mM sodium orthovanadate, 1% PMSF, complete protease inhibitor cocktail, and complete phosphatase inhibitors). The whole-cell lysates were centrifuged at 12 000 *g* at 4 °C for 20 min to remove the insoluble

materials. The lysates were loaded onto SDS–PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked and incubated with different antibodies, followed by incubation with HRP-conjugated secondary antibodies. GAPDH protein levels were used as a control for equal protein loading. The proteins were visualized with enhanced ECL reagents according to the manufacturer's protocol (Amersham Pharmacia). In some experiments, the intensities of blots were quantitated using a scan densitometer.

Immunoprecipitation

For immunoprecipitation, supernatants were incubated with indicated antibodies at 4 °C for 16 h followed by incubation with protein A-agarose beads for 2 h. The beads were washed in lysis buffer three times, boiled with Laemmli sample buffer for 5 min, and then loaded onto SDS–PAGE.

Measurement of intracellular reactive oxygen species

For analysis of intracellular reactive oxygen species (ROS), the redox-sensitive fluorescent probe DCFH-DA was used, as described previously (Kuang *et al.* 2009). Cells were incubated with 5 μM DCFH-DA for 30 min at 37 °C. The harvested cells were immediately analyzed by a flow cytometry.

ATP detection by luciferin/luciferase assay

The cell lysates were extracted with cell lysis reagent (Beyotime Institute of Biotechnology, China). The whole-cell lysates were centrifuged at 12 000 *g* at 4 °C for 10 min to remove the insoluble materials. The detection buffer (100 μl) was added to the tube and allowed to stand at room temperature for ~3 min. During this period, any endogenous ATP will be hydrolyzed, thereby decreasing the background. Then 100 μl of the lysates were added to 100 μl of detection buffer, swirling and quantifying in a luminometer. In parallel, a standard curve from 0.1 to 10 μmol/l ATP was performed using the same kit. Lineal range was obtained from 0.1 to 10 μmol/l ATP. Sample lectures were interpolated in standard curve to detect ATP concentrations in each condition.

Electrophysiological recordings

Whole-cell current-clamp recordings in dispersed C₂C₁₂ myotubes were conducted using a patch clamp amplifier

Figure 1

Glucose uptake in C_2C_{12} myotubes. (A) 2-Deoxyglucose (2-DOG) uptake with genipin treatment at different concentrations for 2 h. (B) 2-DOG uptake with 10 μ M genipin treatment for different duration. 2-DOG uptake increase is represented as the fold of the vehicle treatment (control). The data are mean $\pm$ S.E.M. of three independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with the control.

(EPC10; HEKA Elektronik, Lambrecht, Germany). Stimulation and recordings were controlled by PULSE software. Capacitive transients and series resistance errors were minimized before recording. External medium for current clamp contained 140 mM NaCl, 5.6 mM KCl, 1.2 mM $MgCl_2$, 2.6 mM $CaCl_2$, 10 mM HEPES, and 2 mM glucose (pH 7.4 with NaOH). Patch electrodes were fabricated from borosilicate glass and had a resistance of 2.5–5 M Ω while filled with internal recording solution containing 70 mM K_2SO_4 , 10 mM NaCl, 10 mM KCl, 21 mM $MgCl_2$, 10 mM HEPES, and 40 mM sucrose (pH 7.2 with KOH). Currents flowing through K_{ATP} channels were monitored using alternate 10 mV pulses of 200 ms duration from a holding potential of -70 mV. Pulses were applied every 2 s. All experiments were carried out at room temperature (22–24 $^{\circ}C$).

Measurement of intracellular calcium concentrations ($[Ca^{2+}]_i$)

C_2C_{12} myotubes were grown on coverslips and loaded with 5 μ M Fura-2/AM for 30 min at 37 $^{\circ}C$ in a solution containing 119 mM NaCl, 4.75 mM KCl, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.18 mM KH_2PO_4 , 2.54 mM $CaCl_2$, 3 mM glucose, and 20 mM HEPES (pH 7.4). The coverslip was placed in a superfusion chamber under IX71 inverted microscope (Olympus). The $[Ca^{2+}]_i$ was measured using the Video-Imaging-System (Till Photonics, Munich, Germany). Cells were illuminated by alternative excitation light of 340 and 380 nm wavelength, which was produced by a monochromator (Till Photonics). The images were captured with emission light of 510 nm wavelength using an image-intensifying CCD camera (SensiCam, PCO, Kelheim, Germany) and processed using an image-processing system (TillVision, Till Photonics). The ratio images were captured at intervals of every 10 s. Calcium concentrations were indicated as ratio of

Figure 2

Immunoblotting of GLUT4 in PM and LDM of C_2C_{12} myotubes. (A) GLUT4 distribution in PM and LDM fractions in C_2C_{12} myotubes treated with 10 μ M genipin for 0.5 and 2 h, and with 100 nM insulin for 30 min respectively. (B) Relative quantities of GLUT4 in LDM and PM fractions are represented as percentage of total LDM and PM. The data are shown as mean $\pm$ S.E.M. of three independent experiments.

F340/F380. Cells were continuously superfused with the solution as described earlier throughout the experiment. All drugs were applied through superfusion.

Calculations and statistical analysis

Data are presented as mean $\pm$ S.E.M. Comparisons were made using one-way ANOVA followed by Tukey's *post hoc* test for multiple comparison. *P* value <0.05 was considered as being significantly different.

Results

Genipin increases glucose uptake in C₂C₁₂ myotubes dose and time dependently

We first examined the dose- and time-dependent effect of genipin on glucose uptake in C₂C₁₂ myotubes. For short treatment with genipin, cells were incubated in serum-free media for 6 h and then with the indicated concentrations of genipin for 2 h (Fig. 1A). Glucose uptake was induced in a concentration-dependent manner, a maximum of

Figure 3

Immunoblotting of IRβ, IRS-1, and downstream signaling molecules in C₂C₁₂ myotubes. (A) Immunoprecipitation of IRβ following an incubation of genipin at 10 μM for 0.5 h and insulin at 100 nM for 30 min. Anti-IRβ antibody used for pull-down and anti-phosphotyrosine antibody (PY20) for immunoblotting. ****P* < 0.001 compared with control. (B) Immunoprecipitation of IRS-1 following an incubation of genipin at 10 μM for 0.5 and 2 h. Anti-IRS-1 antibody used for pull-down and anti-phosphotyrosine antibody (PY20) for immunoblotting. A ratio between phosphorylated form and total form of IRβ and IRS-1 was presented in a bar graph. ***P* < 0.01 compared with control. (C) 2-DOG

uptake and phosphorylation of AKT is abrogated by wortmannin (100 nM) in C₂C₁₂ myotubes treated with either genipin or insulin. Data are represented as mean $\pm$ S.E.M. of three independent experiments. ****P* < 0.001 compared with ge alone, ##*P* < 0.001 compared with insulin alone. (D) Immunoblotting of phospho-Akt (Ser473 and Thr308), phospho-PKCζ/λ (Thr410/403), and phospho-GSK3β (Ser9) in C₂C₁₂ myotubes treated with either ge or insulin (Ins). The representative immunoblots are shown from at least three independent experiments. C₂C₁₂ myotubes were treated with 10 μM genipin for 2 h and 100 nM insulin for 30 min respectively.

Figure 4

AMPK is not involved in genipin-induced glucose uptake. (A) Immunoblotting of phospho-AMPK (Thr172). The representative immunoblots are shown from at least three independent experiments. C_2C_{12} myotubes were treated with 10 μ M genipin for 2 h and 1 mM metformin (met) for 2 h respectively. (B) The effects of genipin + insulin or genipin + AICAR on 2-DOG uptake in C_2C_{12} myotubes. C_2C_{12} myotubes were treated with 10 μ M genipin for 2 h, 100 nM insulin for 30 min, and 2 mM AICAR for 2 h respectively. Data are represented as mean $\pm$ S.E.M. of three independent experiments. *** P < 0.001.

~1.43-fold induction at 10 μ M was observed. As assessed by MTT assay, genipin did not influence the viability of C_2C_{12} cells in the dose range between 0 and 100 μ M, but a severe toxicity was observed at higher concentrations (data not shown), and the 10 μ M concentration was used in subsequent experiments. We next determined a time-dependent effect; cells were maintained in serum-free media with genipin (10 μ M) for the indicated time (Fig. 1B), and genipin-induced activation reached the peak level at 2 h.

Genipin promotes GLUT4 translocation

To determine the GLUT4 translocation, PM and LDM fractions were harvested separately. Immunoblotting showed that GLUT4 translocated from the cytoplasm

to PM in genipin-treated C_2C_{12} myotubes (Fig. 2A). Relatively, the quantity of GLUT4 in PM fractionation was larger compared with LDM fractionation (Fig. 2B).

Genipin activates IRS-1 and its downstream signaling pathway and AMPK is not involved in genipin-induced glucose uptake

As genipin increased glucose uptake and GLUT4 translocation in C_2C_{12} myotubes, we next tried to explore whether genipin might induce GLUT4 translocation through the insulin signaling pathways. IR β and IRS-1 were immunoprecipitated respectively and then resolved by SDS-PAGE. Immunoblotting with anti-phosphotyrosine antibody (PY20) showed that phosphorylation of IR β did not increase (Fig. 3A), whereas phosphorylation of IRS-1 slightly increased by genipin (Fig. 3B). These results indicated that genipin could activate IRS-1 independent of

Figure 5

Effect of genipin on the level of ROS and ATP. (A) Cells were pretreated with the redox-sensitive fluorescence dye DCFH-DA for 30 min and then treated with genipin at 10 μ M for 10, 20, 30 min respectively. (B) Cells were treated with genipin at 10 μ M for 15, 30, and 60 min respectively. The level of ATP was measured as described earlier. * P < 0.05 compared with control. *** P < 0.001 compared with control.

insulin receptor activation. PI3-K activation is crucial in insulin signaling; the addition of wortmannin, a PI3-K inhibitor, abrogated genipin-mediated glucose uptake (Fig. 3C). The downstream signaling cascade of PI3-K was further activated by genipin, as the phosphorylation of Akt/PKB on ser473 and Thr308 and phosphorylation of PKC- ζ/λ and GSK3 β were increased (Fig. 3D). These results indicated that genipin treatment led to the activation of IRS-1, PI3-K, and downstream signaling. AMPK is known to regulate insulin-independent glucose uptake in C₂C₁₂ myotubes; however, we found that genipin did not increase phosphorylated AMPK (Fig. 4A). In order to further clarify the signaling pathways involved in genipin-induced glucose uptake, we examined whether the effects of genipin + insulin or genipin + AICAR are additive for 2-deoxyglucose uptake in C₂C₁₂ myotubes. The results showed that genipin + AICAR are additive and genipin + insulin are not additive for 2-deoxyglucose uptake in C₂C₁₂ myotubes (Fig. 4B). These results suggested that IRS-1 and its downstream signaling pathway are involved in genipin-induced glucose uptake and AMPK is not involved in genipin-induced glucose uptake.

Genipin increases the level of ROS and ATP in C₂C₁₂ myotubes

We have found that genipin activated IRS-1 and its downstream signaling pathway. However, its molecular mechanism needs to be further investigated. It was reported that genipin could inhibit uncoupling protein 2 (UCP2) in pancreatic cells (Zhang *et al.* 2006). UCP3, with 73% homology to UCP2, is highly expressed in skeletal muscle (Argyropoulos *et al.* 1998, Bezaire *et al.* 2007). Costford *et al.* (2006) reported that insulin resistance was improved in *Ucp3* null mice. The function of UCP3 is to diminish the production of ATP and ROS by mediating mild uncoupling. Because skeletal muscle accounts for 75–80% of whole body insulin-stimulated glucose uptake, we examined whether genipin would increase glucose uptake in C₂C₁₂ myotubes with UCP3 inhibited. C₂C₁₂ myotubes were treated with DMSO vehicle or with 10 μ M genipin; it was found that 10 μ M genipin increased cellular ATP and ROS concentrations within 30 min of incubation (Fig. 4A and B).

The rise of intracellular calcium is necessary for genipin-induced glucose uptake

In pancreatic cells, ATP closes PM K_{ATP} channels resulting in depolarization of the cell, leading to the opening of

Figure 6

The rise of intracellular calcium is necessary for genipin-induced glucose uptake. (A) Effect of genipin (10 μ M) on K_{ATP} channel currents in C₂C₁₂ myotubes. Electrophysiological recordings were performed as described in the Materials and methods section. Currents flowing through K_{ATP} channels were monitored using alternate 10 mV pulses of 200 ms duration from a holding potential of -70 mV. (B) Effect of genipin (10 μ M) on the concentration of intracellular calcium in C₂C₁₂ myotubes. C₂C₁₂ myotubes grown on coverslips were loaded with 5 μ M Fura-2/AM for 30 min at 37 $^{\circ}$ C as described in the Materials and methods section. (C) 2-DOG uptake is abrogated by EGTA (2 mM) in C₂C₁₂ myotubes treated with genipin. Data are represented as mean $\pm$ S.E.M. of three independent experiments. *** P < 0.001 compared with genipin alone.

voltage-gated calcium channels, ultimately stimulating insulin secretion. In this study, we assessed whether genipin would close K_{ATP} channels. Currents were recorded in response to alternate 10 mV voltage steps from a holding potential of -70 mV, using the whole-cell patch clamp configuration (Fig. 6A). This result proved increasing ATP level by genipin could in turn close K_{ATP} channels in C_2C_{12} myotubes. To determine whether genipin changes the intracellular free calcium concentration after K_{ATP} channels are closed in C_2C_{12} myotubes, using Fura-2/AM as an indicator, we further measured its concentration and found that $[Ca^{2+}]_i$ was significantly increased. This result indicated that genipin could increase $[Ca^{2+}]_i$ by closing K_{ATP} channels in C_2C_{12} myotubes. Furthermore, 2-DOG uptake was abrogated by EGTA (2 mM) in genipin-treated C_2C_{12} myotube. This indicates that the rise of intracellular calcium is necessary for genipin-induced glucose uptake (Fig. 6).

Discussion

Our data demonstrate that genipin activates IRS-1, PI3-K, and downstream signaling pathways and increases the concentration of calcium, resulting in GLUT4 translocation and increased glucose uptake in C_2C_{12} myotubes. Although many beneficial properties of genipin have been reported. Thus, to our knowledge, this is the first report demonstrating genipin increases insulin sensitivity.

UCPs are inner mitochondrial membrane transporters that dissipate mitochondrial proton gradient, thus uncoupling oxidative phosphorylation. Stored energy is therefore expended as heat, instead of storing in the form of ATP. UCP3 is also suggested to limit the production of ROS by mediating a mechanism of mild uncoupling, resulting in a decrease in proton-motive force, thereby diminishing superoxide production (Echtay *et al.* 2003). Recent *in vivo* studies on ATP synthesis in skeletal muscle have revealed the rate of ATP synthesis in fasting *Ucp3* knockout mice is over fourfold higher and the ratio of ATP to ADP is 31% higher than in control mouse (Cline *et al.* 2001). Vidal-Puig *et al.* (2000) reported that mitochondria in UCP3-ablated skeletal muscle overproduced ROS *in vivo*. It has been reported that genipin inhibits UCP2-mediated proton leak from inner mitochondria and stimulates insulin secretion from pancreatic β cells; due to its high homology to UCP2, genipin should be able to inhibit UCP3-mediated proton leak as well (Zhang *et al.* 2006). Our results showed that treatment with genipin resulted in the increase of both ROS and ATP in C_2C_{12} myotubes. These results suggest that genipin might inhibit UCP3 in

myotubes, although we are still working to obtain the direct proof. The increase in ATP may explain the unchanged AMPK phosphorylation.

It has been known that H_2O_2 and other oxidants exert insulin-like effects for a long time. H_2O_2 can be produced in response to insulin signaling and acts as a positive regulator of early and late insulin signaling (Mahadev *et al.* 2001). Higaki *et al.* (2008) demonstrated that acute exposure to ROS stimulated glucose uptake in skeletal muscle through a PI3-K-dependent mechanism. Another study reported that mice lacking one (glutathione peroxidase 1 (GPX1)) of the key enzymes involved in the elimination of physiological ROS were protected from high-fat-diet-induced insulin resistance (Loh *et al.* 2009). The increased insulin sensitivity in *Gpx1* knockout mice

Figure 7

Possible mechanisms for the increase of glucose uptake by genipin in C_2C_{12} myotubes. First, genipin may increase the level of ROS, activate IRS-1, PI3-K, and downstream signaling pathways. Secondly, genipin may increase the level of ATP, close K_{ATP} channels, and increase the concentration of calcium. Both activated IRS-1/PI3-K/AKT and increases in the concentration of calcium increases the rates of translocation of GLUT4 to membranes and glucose uptake. However, whether the increases of both ROS and ATP in C_2C_{12} myotubes are the result of inhibiting UCP3 by genipin needs further investigation.

was attributed to insulin-induced PI3-K/Akt signaling and glucose uptake in muscle. These studies provide causal evidence for the enhancement of insulin signaling by ROS *in vivo*, indicating that treatment with genipin in C₂C₁₂ myotubes results in an increase of ROS, which is possibly relevant to IRS-1/PI3-K activation and glucose uptake increase in skeletal muscle.

It has been reported that in pancreatic islet cells, genipin increases mitochondrial membrane potential, increases ATP levels, closes K_{ATP} channels, and stimulates insulin secretion (Zhang *et al.* 2006). In our studies, we also found that genipin increased ATP levels, closed K_{ATP} channels, leading to increased concentration of calcium in cytoplasm in C₂C₁₂ myotubes. Ca²⁺ plays an important role in glucose uptake because genipin-mediated glucose uptake was abrogated by calcium chelator EGTA; this proves that the increase in glucose transport stimulated by genipin is mediated by changes in intracellular calcium levels.

In this study, we prove that genipin increases the levels of ROS and ATP, activates IRS-1, PI3-K, and downstream signaling pathways, and increases concentration of calcium, resulting in GLUT4 translocation and increased glucose uptake in C₂C₁₂ myotubes. We also suggest that the increases of ATP and ROS might be the result of inhibition of UCP3 (Fig. 7).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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