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The soybean peptide aglycin regulates glucose homeostasis in type 2 diabetic mice via IR/IRS1 pathway☆

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Abstract

It has been previously reported that aglycin, a natural bioactive peptide isolated from soybean, is stable in digestive enzymes and has an antidiabetic potential. With a view to explore the pharmacological activity of aglycin *in vivo*, studies have been conducted to examine its therapeutic effect in diabetic mice, in which it was administered intragastrically as an oral agent. Diabetes was induced in BALB/c mice fed with a high-fat diet and a single intraperitoneal injection of streptozotocin. With onset of diabetes, the mice were administered daily with aglycin (50 mg/kg/d) for 4 weeks. Blood glucose was monitored once a week. Subsequently, skeletal muscle was isolated for assessment in terms of levels of gene and protein IR, IRS1, Akt and glucose transporter 4 (GLUT4). In addition, C2C12 muscle cells as an *in vitro* diabetic model were used to investigate the effect of aglycin on glucose uptake. Treatment with aglycin was found to be significantly effective in controlling hyperglycemia and improving oral glucose tolerance. Furthermore, aglycin enhanced glucose uptake and glucose transporter recruitment to the C2C12 cell surface in 10 min *in vitro*. Consistent with these effects, aglycin restored insulin signaling transduction by maintaining IR and IRS1 expression at both the mRNA and protein levels, as well as elevating the expression of p-IR, p-IRS1, p-Akt and membrane GLUT4 protein. The results hence demonstrate that oral administration of aglycin can potentially attenuate or prevent hyperglycemia by increasing insulin receptor signaling pathway in the skeletal muscle of streptozotocin/high-fat-diet-induced diabetic mice.

Keywords: Aglycin; Type 2 diabetes mellitus; Hypoglycemia; Insulin signaling

1. Introduction

Type 2 diabetes mellitus (T2DM) is a multifactorial metabolic disorder disease, which results from defects in both insulin secretion and insulin action [1,2]. Insulin stimulates uptake, utilization and storage of glucose in cells throughout the body by inducing multiple signaling pathways in the tissues that express the transmembrane insulin receptor [3], especially in skeletal muscle that accounts for 75% of whole-body insulin-stimulated glucose uptake [4,5]. The reduced responsiveness of cells to insulin is due to defective intracellular signaling processes [3]. It is therefore highly desirable to explore the agents that would be beneficial in restoring insulin signaling pathway of the skeletal muscle to treat diabetes.

Even though oral antidiabetic agents such as thiazolidinedione and biguanide that enhance insulin sensitivity in both peripheral and hepatic tissues are effective in the treatment of hyperglycemia [6], these chemical agents normally show unwanted effects during long-term medication. Previous studies have indicated that amino acids and peptides are key regulators in various cellular and intercellular physiological responses and therefore possess enormous potential for the treatment of various pathologic conditions [7]. However, most peptides in clinical settings are only administered by injection [8,9]. Since systemic long-term medication by oral administration is the preferred mode for treating diabetes, the development of oral antidiabetic peptide therapeutics from natural source that are stomach juice resistant is therefore expected.

Soybean peptides have been widely used as a natural health food and supplement. It should be good for preventing obesity and diabetes because long-term feeding of soy peptide induced weight loss in obese mice [10]. In healthy and diabetic animal models,

Abbreviations: Akt, protein kinase B; GLUT4, glucose transporter 4; OGTT, oral glucose tolerance test; IR, insulin receptor; IRS1, insulin receptor substrate 1.

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soybean peptides decreased blood glucose by increasing insulin sensitivity and improving glucose tolerance [11,12]. Aglycin is a 37-amino-acid peptide isolated from soybean. Structurally, it has a high stability with six cysteines embedded in three disulfide bonds [13]. It is also resistant to digestion by trypsin, pepsin, Glu-C and bovine rumen fluid [14]. Encouraged with these findings of aglycin, the present investigations were designed to examine the possibility of preventing diabetes by oral administration of aglycin and the underlying mechanisms.

2. Methods and materials

2.1. Animals

Male BALB/c (stock number: 0004771) mice weighing 18–22 g each were used and maintained under specific pathogen-free conditions at the experimental animal center of Tongji Medical College, Huazhong University of Science and Technology, China. The animals were kept in cages at 23°C±2°C with 12-h of light/dark and tap water *ad libitum*. The experiments were all carried out according to the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

2.2. Chemicals

Aglycin is a 37-residue polypeptide with a single chain, containing six half-cystine residues at positions 3, 7, 15, 20, 22 and 32, an N-terminal alanine and a C-terminal glycine. The molecular mass was 3742.3 Da, and amino acid sequence was ASCNGVCSPFEMPPCGSSACRCIPVGLVVGYCRHPSG (37 residues) (Fig. 1A). Aglycin is a kind of white amorphous powder. High-performance liquid chromatographic (HPLC) analysis showed that the purity of aglycin was above 99%. The peptide was provided by the School of Life Science and Technology, Huazhong University of Science and Technology.

Anti-IR, anti-IRS1, anti-Akt, anti-glucose transporter 4 (GLUT4) and anti-actin antibody were purchased from Santa Cruz Biotechnology (CA, USA). Glucose and sodium chloride were purchased from the Beijing Chemical Reagents Company (Beijing, China). Insulin was obtained from Eli Lilly (Indianapolis, IN, USA). The primers were synthesized by AuGCT Biotechnology (China). ^{3}H-2-deoxy-D-glucose was provided by ICN Biochemical (Canada). The mouse muscle myoblast cell line C2C12 was from ATCC (USA).

The blood glucose level was tested by glucose analyzer (OneTouch Ultra, Johnson & Johnson, USA). Insulin concentration was detected using ultrasensitive mouse insulin enzyme-linked immunosorbent assay (ELISA) kit for small sample volumes (Crystal Chem., Chicago, IL, USA).

2.3. Determination of aglycin in BALB/c mice using reversed-phase (RP)-HPLC method

For oral delivery, aglycin was dissolved in saline at a concentration of 5 mg/ml and given at a dose of 50 mg/kg to BALB/c mice. Sixty minutes after peptide administration, blood samples (n=6) were withdrawn and collected in sterile heparinized plastic centrifuge tubes. Samples were extracted as follows: 200 μl of peptide/plasma solution was added to 400 μl acetonitrile in the tube; tubes were vortexed for 10 min and then centrifuged for 10 min at 12,000g; 500 μl of the supernatant was transferred to a clean tube and evaporated at 37°C under a stream of nitrogen. Dried samples were redissolved in 50 μl 30% acetonitrile, 0.1% trifluoroacetic acid before RP-HPLC analysis. The injection volume was 10 μl.

RP-HPLC were performed using a Zorbax C18 column (4.6×150 mm, 5-μm particles), with mobile phase A containing 0.1% trifluoroacetic acid in water and mobile phase B containing 0.1% trifluoroacetic acid in acetonitrile. Flow rate was 1 ml/min. A gradient was 30%–39% B, 0–20 min; and detection was at 214 nm.

2.4. Induction of type 2 diabetes in BALB/c mice [15,16]

After a 1-week acclimation to our facilities, mice were randomly divided into two groups. The normal control mice were fed with chow diet. The diabetic mice were induced by feeding with a high-fat diet consisting of 40% fat, 41% carbohydrate and 18% protein for 5 weeks, and then injected with a single dose of streptozotocin (STZ) [100

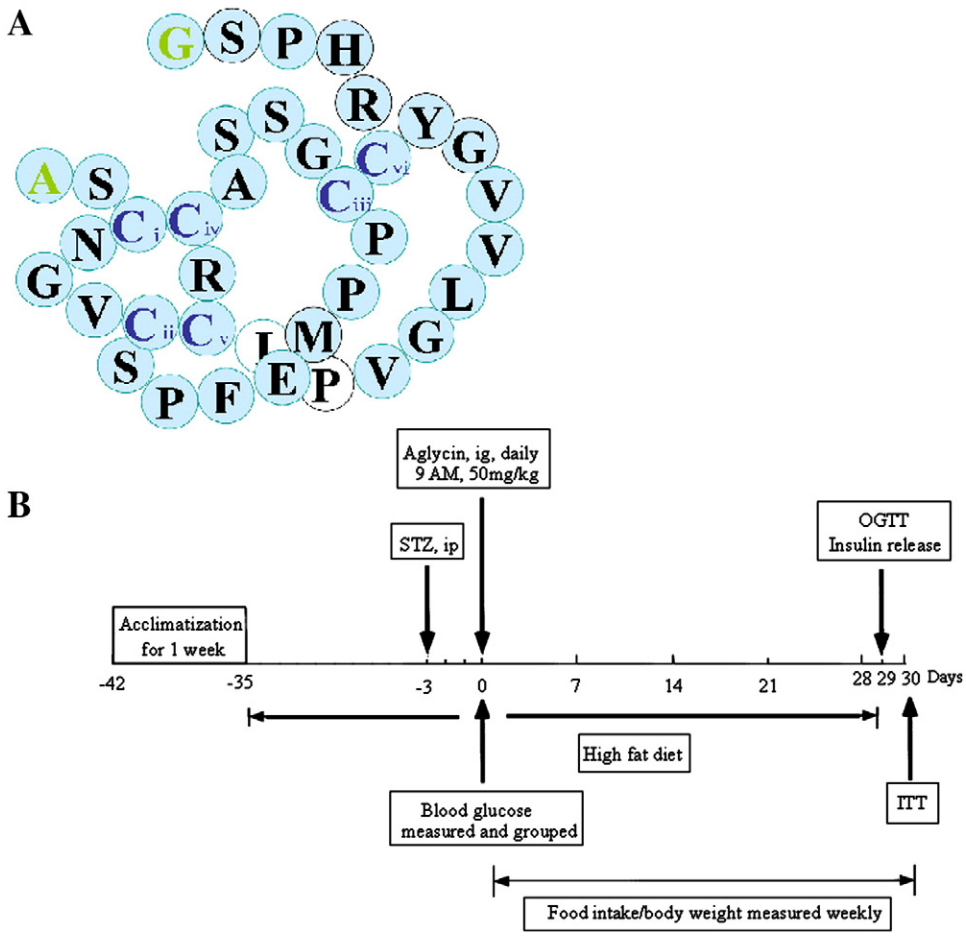


Fig. 1. Primary structure of aglycin (A) and experimental design (B).

mg/kg, intraperitoneally (ip)] without fasting. This diet continued until the end of the experiment. During this period, the mice had free access to food and water except special operations for experiment. Three days later, the glucose concentrations of model mice after fasting for 6 h were measured to make sure that diabetes was induced in all the mice with blood glucose concentration above 7.8 mmol/L.

2.5. Animal treatments

To evaluate antidiabetic effect of aglycin *in vivo*, aglycin (50 mg/kg/d) was orally administered to diabetic mice daily at 9:00 a.m. Metformin (100 mg/kg/d) was used as positive drug. The model and normal control mice were treated with the same volume of saline every day. The injection of aglycin on days 0, 7, 14, 21 and 28 were taken after completion of blood glucose in order to avoid any short-term drug effect. The dosage of aglycin was determined according to previous experimental results (data not shown). This regimen lasted for 4 weeks. Body weight and food consumption were monitored to evaluate variables among groups. The blood glucose was measured on days 0, 7, 14, 21 and 28 at 9:00 a.m. by the glucose analyzer. The regimens used in this study are shown in Fig. 1B.

2.6. Oral glucose tolerance test (OGTT) and insulin release in vivo

On day 29, eight mice in each group were subjected to OGTT. After fasting for 6 h, mice were given 2.5 g/kg glucose orally. A total of 20 μl of blood samples from each mouse were collected from the caudal vein at time 0, 30, 60, 90 and 120 min after glucose loading for the determination of glucose level. The values of area under the glucose-time curve (AUC) were calculated. Blood samples were centrifuged, and the plasma was stored at −20°C for the determination of insulin concentration by using ultrasensitive ELISA kit for small sample volumes.

2.7. Insulin tolerance test

On day 30, for the insulin tolerance test, a group of mice was fasted for 2 h and injected with human insulin (0.5 U/kg) subcutaneously, and blood samples were collected from the caudal vein at time 0, 30, 60 and 120 min for the determination of glucose concentration. The values of AUC were calculated.

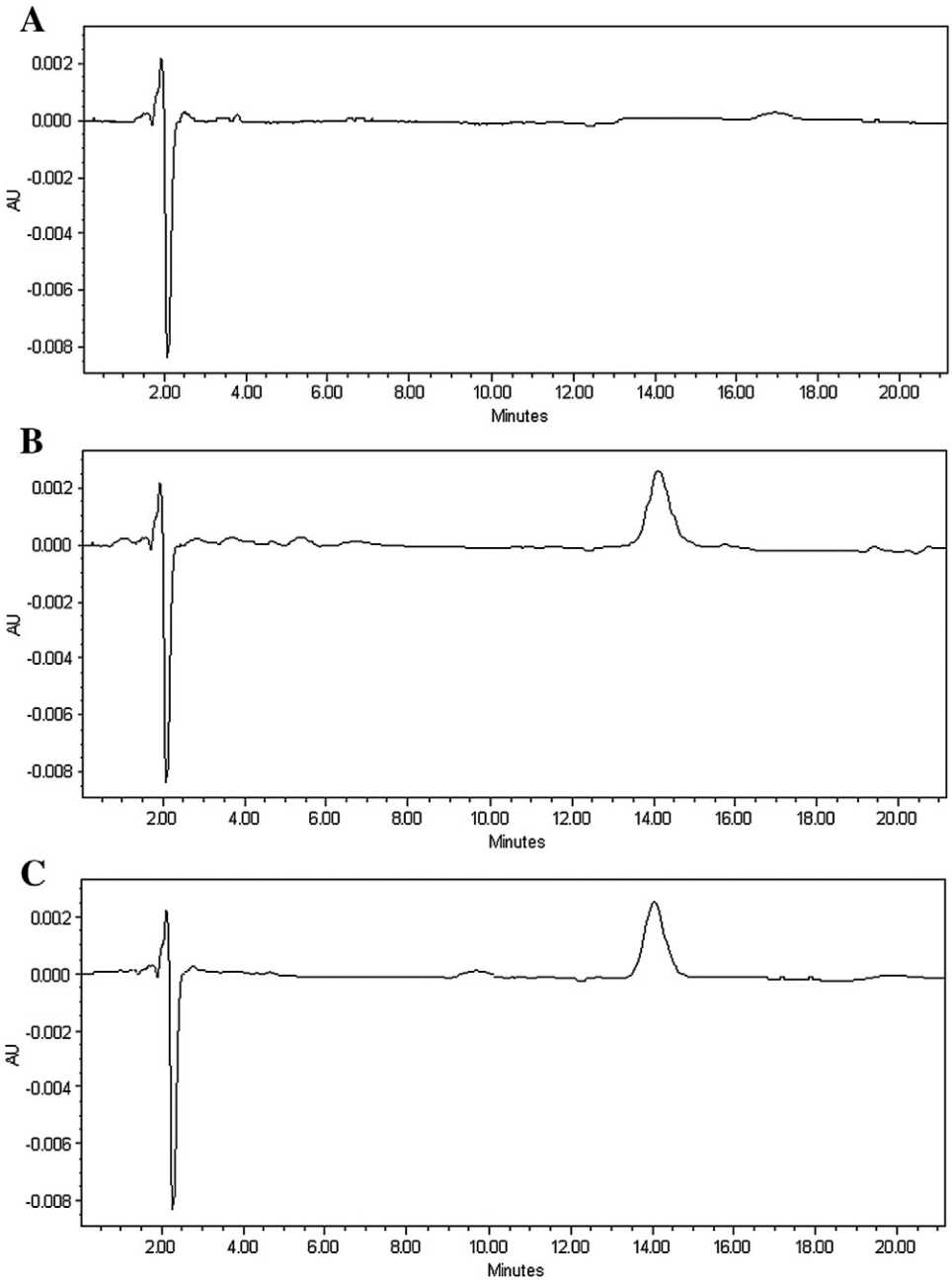


Fig. 2. Plasma concentration of aglycin after oral administration in BALB/c mice. Sixty minutes after oral administration of aglycin, the plasma concentration of aglycin was determined using RP-HPLC method. (A) Blank plasma; (B): blank plasma plus with aglycin; (C) plasma sample at 60 min.

2.8. Ex vivo insulin signaling studies by immunoblotting and quantitative real-time polymerase chain reaction (RT-PCR)

In order to evaluate the effect of aglycin on insulin signaling, on day 29, after a 12-h fast, another group of mice was intraperitoneally injected with 1 U/kg of human insulin and killed 10 min later [17]. Hind-limb skeletal muscles were dissected and frozen in lipid nitrogen for immunoblotting and RT-PCR analysis of insulin signaling.

The skeletal muscle was homogenized in different lysis buffers to obtain different proteins including total protein, membrane protein and phosphorylated protein. The protein content was measured by the Bradford assay, and 50 μg of protein was subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. After probing with specific antibodies — IR, p-IR, IRS1, p-IRS1, Akt, p-Akt and GLUT4 (Santa Cruz Biotechnology, CA, USA) — immunoreactive bands were detected by a gel analysis system (Elurochem 5500, Alpha Innotech, USA).

Total RNA was isolated from the skeletal muscle of mice in Trizol reagent, and cDNA was synthesized from total RNA by RT using the Super Script First-Strand Synthesis System kit (Invitrogen Life Technology, CA, USA). Quantitative detection of specific mRNA transcripts was carried out by RT-PCR detection system (SLAN, Hongshi, Shanghai, China). The primer pairs were as follows: IR (forward, 5′GCCGCTCCTATCTCTGGTAT3′; reverse, 5′ GAGTGATGGT GAGGTTGTGTTTG3′), IRS1 (forward, 5′AGTGGTGGAGTTGAGTTGGG C3′; reverse, 5′GAAGAGGCTGTGGAGATGGA3′), Akt (forward, 5′TAGGCATCCCTTCCTTACG3′; reverse, 5′GACACAATCTCCGCACCATA3′) and GLUT4 (forward, 5′TTCCTTCTATTTGCC GTCCTC3′; reverse, 5′ CTGTTTTGCCCCTCAGTCATT3′). All samples for RT-PCR were assayed in triplicate, and data were normalized to the relative levels of β-actin mRNA transcripts in the same experiment.

2.9. Cell culture and 2-deoxyglucose (2-DOG) uptake

To evaluate whether aglycin could directly enhance glucose uptake in skeletal muscle, we performed the study in insulin-resistant C2C12 skeletal muscle cells.

The C2C12 skeletal muscle cells were cultured in Dulbecco's modified Eagle's medium supplemented with 4.5 mg/ml glucose, penicillin 100 IU/ml, streptomycin 100 μg/ml and 10% fetal bovine serum (Sigma Chemical, St Louis, MO, USA) in 5% CO_2 at 37°C. When the cells achieved 70% confluency, they were differentiated by lowering the serum concentration to 2% horse serum for 3 days. To develop insulin resistance state, the C2C12 cells were differentiated in an equal mixture of two serum-free media (MCDB 201 and Ham's F-12 medium) in the absence and chronic presence of 100 nM insulin for 3 days as described previously [18]. The media were changed after every 12 h. Aglycin was dissolved in 0.1% (v/v) dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA). On day 4, the cells were co-cultured with 0.1 mM aglycin or vehicle (the same concentration of DMSO without aglycin) for 24 h. The C2C12 myotubes were fully differentiated by day 3, the characteristics of which were checked by testing the expression of various muscle markers, like myosin, myo D and myogenin. After treatment with aglycin, the cells were washed twice with the Krebs–Ringer phosphate buffer (KRP). The cells were further incubated in KRP buffer containing 100 nM insulin without glucose for 15 min. 2-DOG (0.5 μCi of 2-deoxy-D-[^{3}H] glucose) was added, and cells were incubated for 10 min. The reaction was terminated by placing the plates on ice and adding ice-cold phosphate-buffered saline (PBS). After washing three times with PBS, the cells were dissolved in 0.1% SDS. Trace activities were determined by liquid scintillation counter.

2.10. Statistical analysis

All results were presented as means±SD. The one-way analysis of variance (SPSS version 11.0, SPSS Inc., Chicago, IL, USA) was performed to analyze data, and a *P* value of less than .05 was considered to be statistically significant.

3. Results

3.1. Plasma concentration of aglycin after oral administration in BALB/c mice

The bioanalysis was performed to measure plasma aglycin levels in BALB/c mice orally dosed with 50 mg/kg. Intact aglycin was

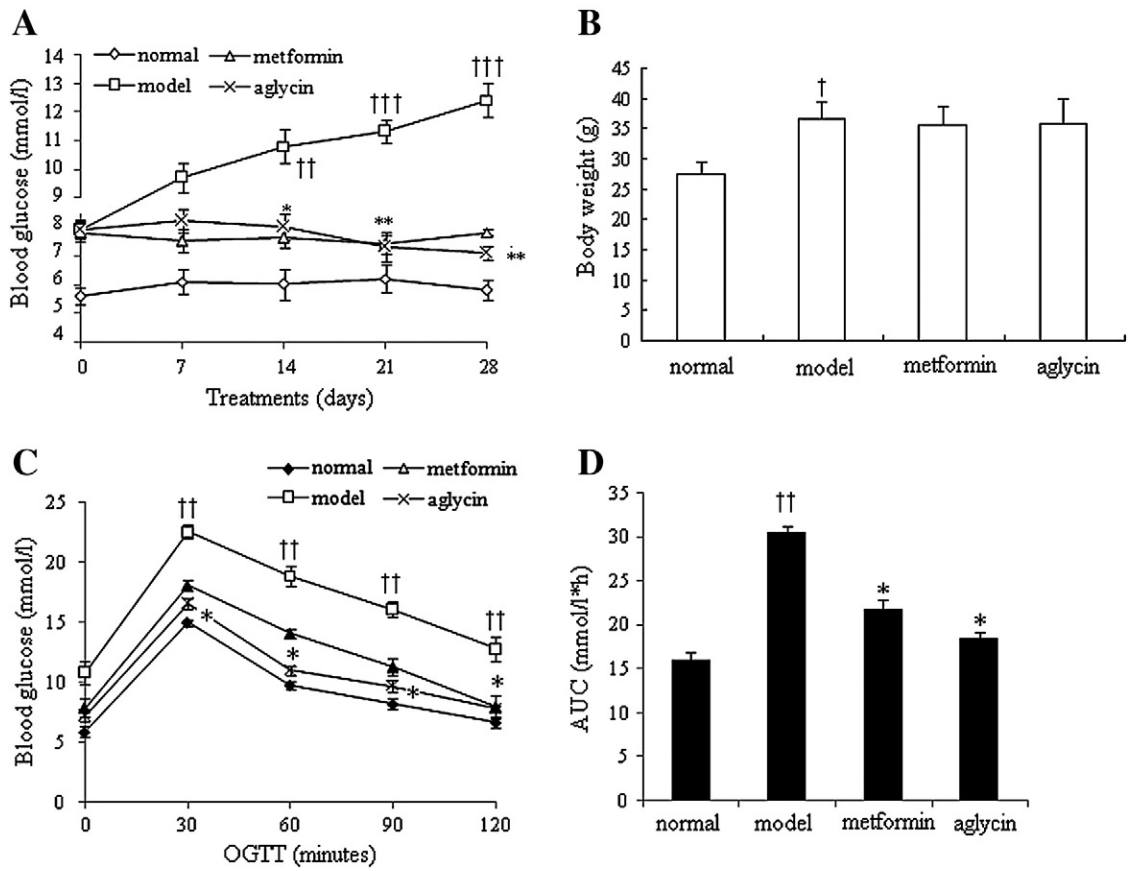


Fig. 3. Effects of aglycin on fasting blood glucose (A), body weight changes (B) and oral glucose tolerance (C, D) in diabetic mice. (A) On days 0, 7, 14, 21 and 28, fasting blood glucose concentration were measured 24 h after treatment with aglycin. (B) The final body weights of all groups were compared. (C, D) OGTT was performed on day 29 after aglycin was omitted for 24 h. The mice received 2.5 g/kg glucose orally after a 6-h fasting period, and blood samples were collected from the caudal vein at t=0, 30, 60, 90 and 120 min for the determination of glucose concentration (C). The AUCs are shown in the right panels. (D) †, ††, †††P<.05, .01, .001 vs. normal group. *, **P<.05, .01 vs. model group (n=16 for each group).

detected in plasma by RP-HPLC after oral administration for 60 min, which indicated that aglycin was resistant to enzymatic hydrolysis in the gastrointestinal tract and was absorbed into the systemic circulation (Fig. 2).

3.2. Aglycin reduced blood glucose levels after long-term treatment

Before initiation of the treatment, there was no significant difference in the blood glucose among different groups. After injection with STZ and feeding with high-fat diet, the BALB/c mice developed hyperglycemia significantly and had a steady increase in blood glucose compared with the normal group. The model mice showed a noticeable higher fasting blood glucose compared with normal mice (day 28, normal vs. model: 5.8±0.4 vs. 12.4±0.6 mmol/L, $P<.001$). Although blood glucose levels of aglycin-treated mice were higher than the normal control (day 28, normal vs. aglycin: 5.8±0.4 vs. 7.1±0.2 mmol/L), those between the aglycin group and the model one were significantly different (aglycin vs. model: day 21: 7.3±0.5 vs. 11.3±0.4 mmol/L; day 28: 7.1±0.2 vs. 12.4±0.6 mmol/L, $P<.01$), respectively. Moreover, aglycin and metformin had similar effect on diabetic mice (Fig. 3A). The body weight of mice fed with high-fat diet increased significantly compared with that of ones fed with chow food. Both metformin and aglycin had no effect on body weight control (Fig. 3B). In addition, the amount of food intake was almost the same among all groups fed with high-fat diet.

3.3. Aglycin improved OGTT and insulin tolerance, but had no significant effect on insulin release

Impaired glucose tolerance is one major characteristic of insulin resistance. In OGTT, as compared to normal control, model mice had impaired glucose tolerance, while the metformin-treated mice had improved glucose tolerance. Aglycin and metformin had similar effect on glucose tolerance improvement in diabetic mice when the glycemic response was expressed as the AUC (Fig. 3C and D, aglycin vs. metformin: 18.5±0.7 vs. 21.9±0.9 mmol/L*h, $P>.05$).

To evaluate the effect of aglycin on peripheral insulin action, we carried out an insulin tolerance test in diabetic mice by determining blood glucose concentration after subcutaneous injection of insulin (Fig. 4A). After insulin loading, the AUC value in the model mice was much higher compared with that in the normal mice (Fig. 4B, model mice vs. normal mice: 22.2 ± 1.5 mmol/L*h vs. 9.7±0.8 mmol/L*h, $P<.01$). Compared with those in the model mice, metformin and aglycin lowered the AUC values by 45.0% and 45.9%, respectively.

In order to assess the effect of aglycin on insulin secretion, we measured the insulin concentration during OGTT. As shown in Fig. 4C, during the course of OGTT, the model mice had an abnormal insulin response to oral glucose challenge compared with the normal mice. However, insulin secretion in aglycin-treated mice was not affected.

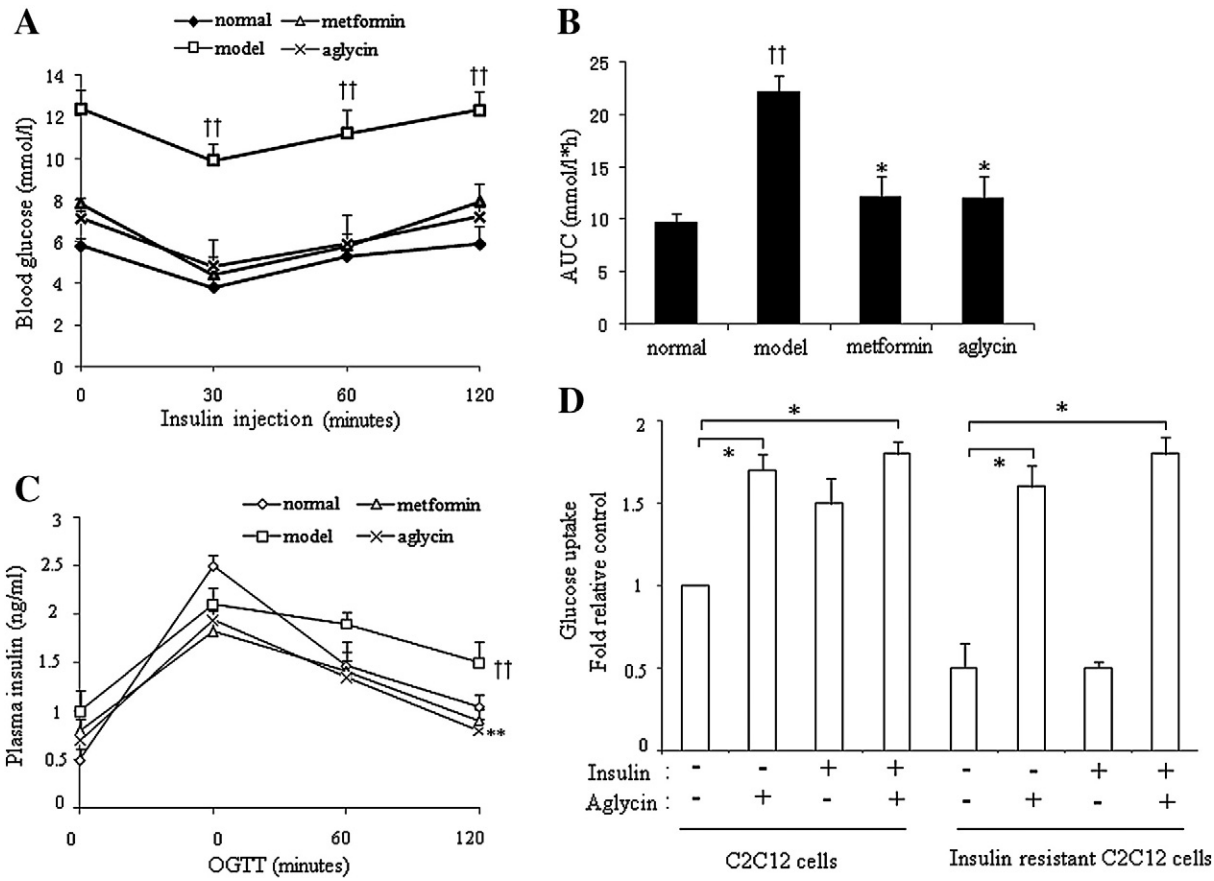


Fig. 4. Effect of aglycin on insulin tolerance (A, B), insulin release *in vivo* (C) and glucose uptake in C2C12 cells (D). (A, B) Insulin tolerance test was performed on day 30 after aglycin was omitted for 48 h. The mice were injected with insulin (0.5 U/kg) subcutaneously after being fasted for 2 h. Blood samples were collected from the caudal vein at $t=0$, 30, 60 and 120 min for the determination of glucose concentration. The AUCs are shown in the right panels (B). ††$P<.01$ vs. normal group. *, **$P<.05$, 0.01 vs. model group ($n=8$ for each group). (C) On day 29, the plasma insulin concentration was measured during OGTT using an insulin ELISA kit. ††$P<.01$ vs. normal group. **$P<.01$ vs. model group ($n=8$ for each group). (D) C2C12 cells, differentiated in the absence or chronic presence of insulin, were washed with KRP buffer and then simulated by insulin. A total of 0.1 mM aglycin was added during the last 24 h of differentiation. Glucose uptake was measured as described in Methods. Results are expressed as the fold change in glucose uptake normalized to control based on four independent experiments. *$P<.05$.

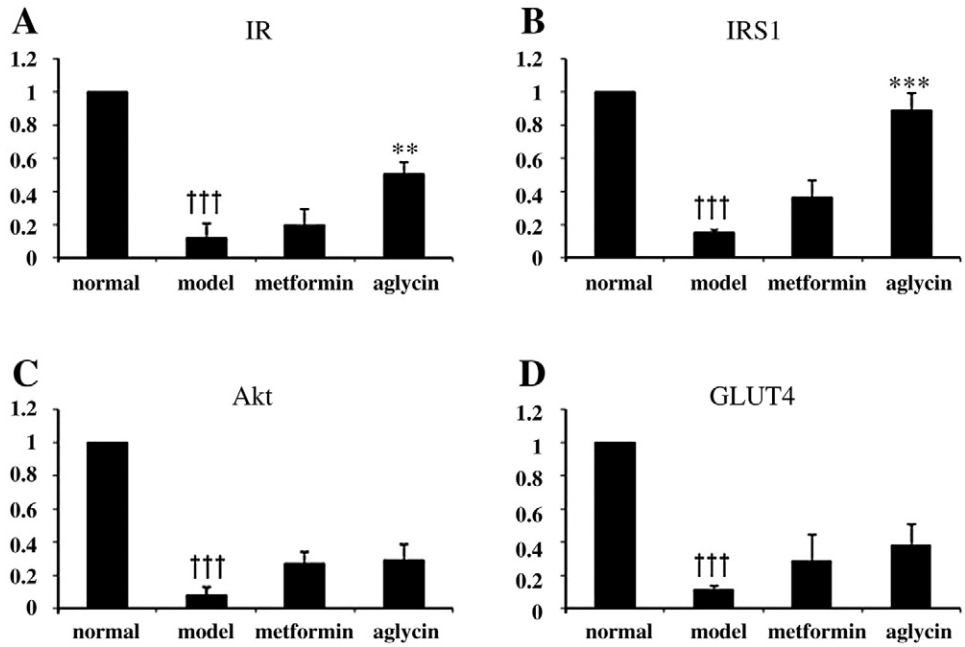


Fig. 5. Effects of aglycin on the gene expression in skeletal muscle using absolute quantification RT-PCR method. On day 29, mice were fasted overnight and injected with human insulin (20 U/kg body weight ip) to stimulate insulin signaling. Then mice were killed 10 min later. Skeletal muscle was obtained for analysis of gene expression. Actin was used as endogenous control to normalize for mRNA levels. †††P<.001 vs. model group. *, **, ***P<.05, .01, .001 vs. normal group (n=8 for each group).

3.4. Aglycin restored insulin signaling in diabetic mice

For examining effects of aglycin on insulin signaling in the skeletal muscle of diabetic mice, hypothesis-driven targeted insulin signaling gene and protein expression events known to influence insulin sensitivity within skeletal muscle were compiled by using RT-PCR and Western blotting methodology. Hyperexpression of IR, IRS1 and Akt in insulin-sensitive tissues created sensitivity to insulin-elicited disposal of blood glucose. As shown in Figs. 5 and 6, IR, IRS1 and Akt gene and protein expression were down-regulated in model mice compared with that in normal mice. A significant increase in the expression of IR and IRS1 gene, as well as total IR and IRS1 protein, was found in the skeletal muscle of aglycin-treated mice as compared with that of model mice. Aglycin could not affect Akt gene and total Akt protein expression. However, compared with that in the model mice, tyrosine phosphorylation of IR and IRS1, and serine phosphorylation of Akt expressions were remarkably enhanced by aglycin. There were no changes in either gene expression or activating tyrosine and serine phosphorylation of IR, IRS1 and Akt in the muscle of metformin-treated mice.

Enhanced expression and posttranslational activation of key insulin signaling components — IR and IRS1 — in the experiments above prompted us to investigate its downstream player, GLUT4. The gene, total protein and membrane protein expression of GLUT4 in the skeletal muscle were diminished significantly in model mice. Although GLUT4 at the transcriptional and total protein level varied less, membrane GLUT4, the actual transporter in glucose transport, was enhanced markedly in the skeletal muscle of aglycin-treated mice. Our results suggest that aglycin promotes GLUT4 translocation to the cell surface upon insulin or other stimuli, which is in accord with an increase in the function of GLUT4 to reduce blood glucose. Metformin showed no effect on GLUT4 gene and protein expression (Figs. 5 and 6).

3.5. Effect of aglycin on glucose uptake in C2C12 cells

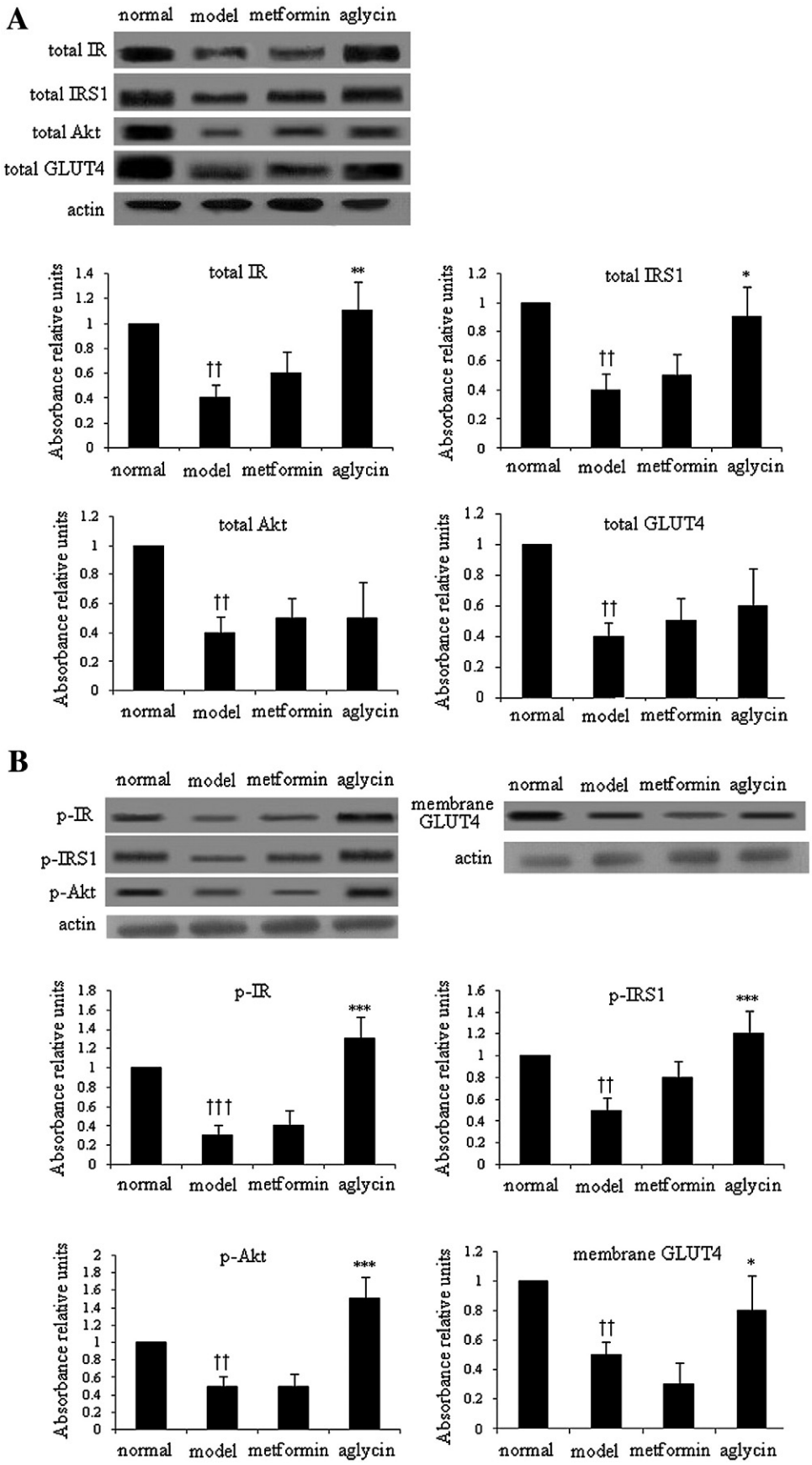
We examined the effect of aglycin on the basal and insulin-stimulated glucose uptake in normal and insulin-resistant C2C12 cells, respectively. Compared with normal C2C12 cells, the glucose uptake was increased 75% in the cells stimulated with insulin, but not in insulin-resistant C2C12 cells in our study. Therefore, aglycin resulted in enhancement of basal glucose uptake as well as insulin-stimulated glucose uptake in both normal and insulin-resistant C2C12 cells (Fig. 4D).

4. Discussion

Since aglycin has a potential as an oral antidiabetic peptide [14], it is desirable to determine the therapeutic effect of aglycin on blood glucose in diabetic mice by administering it intragastrically. Indeed, in our work, aglycin treatment was effective in preventing hyperglycemia in a diabetic animal model with impaired glucose tolerance and insulin resistance, which were induced in BALB/c mice that were fed with a high-fat diet and received a single intraperitoneal injection of STZ [19]. We found that aglycin administration increased the mRNA expressions of IR and IRS1, which corresponded to elevation in protein and activity of IR and IRS. In addition, protein levels of p-Akt and membrane GLUT4 were also better maintained.

It is known that peptide agents have usually been administered by subcutaneous or intramuscular injection because of poor absorption

Fig. 6. Effects of aglycin on the protein expression in skeletal muscle using Western blotting method. On day 29, mice were fasted overnight and injected with human insulin (20 U/kg body weight ip) to stimulate insulin signaling. Then, mice were killed 10 min later. Skeletal muscle was obtained for analysis of protein expression. Representative immunoblots and quantification expressed as means±S.D. were shown. The band intensities of protein were quantified by densitometry. (A) Total IR, total IRS1, total Akt, total GLUT4. (B) p-IR: insulin-induced IR tyrosine phosphorylation. p-IRS1: insulin-induced IRS1 tyrosine phosphorylation. p-Akt: insulin-induced Akt serine phosphorylation. Membrane GLUT4: membrane GLUT4. †, ††, †††P<.05, .01, .001 vs. normal group. *, **, ***P<.05, .01, .001 vs. model group (n=8 for each group).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in the gastrointestinal tract [8,9]. However, many studies have also shown that peptides with molecular weight above 3000 Da have the ability to cross the intact intestinal wall and could be detected in plasma [20,21]. Actually, aglycin is a 3742.3-Da peptide and resists digestive enzymes [14]. These features indicated that aglycin would be a potential novel oral pharmacologic agent. Accordingly, in the present study, we showed that aglycin was resistant to enzymatic hydrolysis in the gastrointestinal tract and was absorbed into the systemic circulation. Our discovered pharmacodynamic effects of aglycin on glycemic control in diabetic mice also provided evidence supporting bioactivity of aglycin following oral delivery.

Prevention of aglycin against the loss of glucose tolerance and abnormal glucose levels in diabetic mice was hence in line with some studies that soybean peptide could improve glucose tolerance [22]. The possibility that aglycin maintained normoglycemia in these mice by increasing insulin secretion was ruled out because aglycin failed to increase insulin release in response to glucose challenge. However, glucose levels were lowered after insulin loading in aglycin-treated mice in the insulin tolerance test, indicating that glucose control induced by aglycin is largely mediated by enhancing glucose utilization and insulin sensitivity in peripheral insulin target sites. The results were also supported by our *in vitro* study that demonstrated aglycin directly promoted glucose uptake in insulin-resistant C2C12 cells, the state of which was developed with chronic presence of insulin [23].

Insulin resistance is largely associated with dysfunction of intracellular insulin signaling cascades and defect of multiple intracellular postreceptor in peripheral tissues such as skeletal muscles [24], which are the primary site for blood glucose disposal and a vital target tissue for the therapy of type 2 diabetes [25]. Furthermore, recent data have also suggested that soybean peptides improve insulin action via increasing the expressions of GLUT4 and insulin regulatory genes in diabetic animals [11,26]. Thus, it is critical to determine the role of insulin signaling and GLUT4 on glucose uptake in the skeletal muscle of aglycin-treated mice.

IR and IRS1 are two vital molecules involved in this insulin signaling pathway linking peripheral insulin action: IR recruits and phosphorylates cellular substrates to initiate signal transduction [27], while IRS1 controls insulin action by binding with upstream common proteins [28]. In addition, Akt is a key enzyme transmission of the insulin signal and an essential downstream signaling molecule [29], playing a vital role in insulin-stimulated GLUT4 translocation [30]. In our research, we observed that the expressions of IR and IRS1 in aglycin-treated mice were up-regulated at gene level as well as total and tyrosine phosphorylation protein ones. With respect to Akt, we did not find any changes in gene expression; nevertheless, we observed an increase in serine phosphorylation protein levels. This discrepancy between gene and protein expression may be due to posttranslational modification. Although the possibility exists that improved metabolic state associated with diabetes indirectly affects insulin signal activity, our findings indicated that aglycin had a direct effect on IR and IRS1 gene, leading to increase of posttranslational modification, especially phosphorylation.

Since several previous studies strongly suggest that the insulin resistance at skeletal muscle level is a defect in the insulin signaling pathways that regulate the translocation of GLUT4, their docking and fusion with the membrane [31,32], the GLUT4 protein that assesses insulin signaling is responsible for the entry of glucose to the muscle cells. GLUT4 can translocate to the cell membrane where it inserted, and results in the facilitated glucose uptake [33]. However, GLUT4 translocation could be impaired in the diabetic skeletal muscle followed by the reduction of autophosphorylation or substrate phosphorylation of the IR kinase, IRS1 and Akt [34–37]. In our study, we observed that the expression of plasma membrane GLUT4 protein was enhanced in aglycin-treated mice, yet no significant changes were found in gene and total protein levels. Together with increased expression of IR and IRS1, we suggest that aglycin promotes insulin action in skeletal muscles by enhancing insulin signaling pathway that leads to the translocation of GLUT4.

It was of interest to note that metformin treatment was effective on controlling hyperglycemia, but no significant effect was found on genes and proteins levels of IR, IRS1, Akt and GLUT4 in our research, which consistent with studies performed in diabetic human subjects [38]. Thus, it is indicated that the mechanism of aglycin enhancing glucose utilization differed from that of metformin.

In summary, this study investigated the antidiabetic effect of aglycin *in vivo* when administered orally to experimentally diabetic mice induced by STZ/high-fat diet. Our results suggest that aglycin exerts its effect by enhancing insulin signal at gene levels of IR and IRS1, which in turn promoting activation of IR, IRS1 and Akt at phosphorylation levels. Aglycin also increased the number of GLUT4 at the cell surface, thereby increasing glucose uptake in peripheral tissues (particularly skeletal muscle). Although the exact mechanism involved in the insulin action by aglycin remains to be elucidated, aglycin, as a natural and nontoxic soybean peptide, is still a promising candidate for the treatment of T2DM.

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