Astragaloside IV facilitates glucose transport in C2C12 myotubes through the IRS1/AKT pathway and suppresses the palmitate-induced activation of the IKK/IκBα pathway

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Abstract. Astragaloside IV is a monomer isolated from *Astragalus membranaceus* (Fisch.) Bunge, which is one of the most widely used plant-derived drugs in traditional Chinese medicine for diabetes therapy. In the present study, we aimed to examine the effects of astragaloside IV on glucose in C2C12 myotubes and the underlying molecular mechanisms responsible for these effects. Four-day differentiated C2C12 myotubes were exposed to palmitate for 16 h in order to establish a model of insulin resistance and ^{3}H glucose uptake, using 2-Deoxy-D-[1,2-^{3}H(N)]-glucose (radiolabeled 2-DG), was detected. Astragaloside IV was added 2 h prior to palmitate exposure. The translocation of glucose transporter 4 (GLUT4) was evaluated by subcellular fractionation, and the expression of insulin signaling molecules such as insulin receptor β (IRβ), insulin receptor substrate (IRS)1/protein kinase B (AKT) and inhibitory κB kinase (IKK)/inhibitor-κBα (IκBα), which are associated with insulin signal transduction, were assessed in the basal or the insulin-stimulated state using western blot analysis or RT-PCR. We also examined the mRNA expression of monocyte chemotactic protein 1 (MCP-1), interleukin 6 (IL-6), tumor necrosis factor α (TNFα) and Toll-like receptor 4 (TLR4). Taken together, these findings demonstrated that astragaloside IV facilitates glucose transport in C2C12 myotubes through a mechanism involving the IRS1/AKT pathway, and suppresses the palmitate-induced activation of the IKK/IκBα pathway.

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Introduction

Skeletal muscle accounts for 40% of body weight in humans, and 75% of infused glucose is cleared by skeletal muscle (1,2); thus, skeletal muscle serves as the major site of insulin-dependent glucose uptake. Due to the key role played by skeletal muscle in glucose homeostasis, deleterious factors which provoke reductions in glucose uptake by skeletal muscle, described as skeletal muscle insulin resistance, may lead to decreases in the disposal rate of serum glucose (3).

The etiology of impaired insulin signaling in obese individuals is multifactorial and appears to be associated with at least two major events: a state of chronic, low-grade inflammation and the accumulation of intramyocellular lipids (4). Under physiological conditions, serum free fatty acids (FFAs) are an important fuel source for skeletal muscle, and 98-99% of FFAs bind to bovine serum albumin (5). Therefore, physiological concentrations of FFAs are in the μmol/l range (5). Constantly elevated serum levels of FFAs result in the transportation of FFAs into skeletal muscle cells, and subsequently stored as triglyceride. Whenever the accumulation of triglycerides and/or hydrolysis of triglycerides exceeds the oxidation capacity, incomplete lipid metabolic products, such as acyl-CoA, diacylglycerol and ceramide, are generated. These products inhibit the activation of critical molecules involved in insulin signaling, such as protein kinase B (AKT) and insulin receptor substrate (IRS)1 (6), reduce insulin sensitivity, leading to insulin resistance in skeletal muscle. Previous studies of muscle from diabetic rodents and human subjects demonstrated that pharmacological agents, such as 5-aminoimidazole-4-carboxamide riboside (AICAR) (7), metformin (8), thiazolidinediones (TZD) (9,10) and ciliary neurotrophic factor (11), increase muscle glucose uptake, and that this response is maintained.

Astragaloside IV, a 3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosylcycloastragenol purified from *Astragalus membranaceus* (Fisch.) Bunge, is one of the most widely used plant-derived drugs in traditional Chinese medicine for diabetes therapy (10). The saponin astragaloside IV has been reported to exert various pharmacological effects; astragaloside IV inhibited hepatic glycogen phosphorylase (GP) and glucose-6-phosphatase (G6P) activities thereby decreasing serum glucose levels in diabetic mice (12), attenuated lipolysis and reduced insulin resistance induced by tumor necrosis

factor α (TNFα) in 3T3-L1 adipocytes (13), improved the symptoms of metabolic syndrome in fructose-fed rats (14), prevented human cardiovascular pathological changes and protected against cardiovascular injury in rats (15-18) improved renal function (19-22), reduced the progression of peripheral neuropathy (23), and attenuated inflammatory responses by suppressing the nuclear factor-κB (NF-κB) pathway (24-26). Xu *et al* (27) reported that other astragalosides, astragaloside II and isoastragaloside I, elevated serum levels of adiponectin and alleviated insulin resistance and glucose intolerance in obese mice. Our research team has previously found that astragaloside IV decreases serum FFA and glucose levels in mice fed a high-fat diet, and improves insulin sensitivity (13). In order to examine the mechanisms through which astragaloside IV decreases serum glucose concentrations, we selected the major organ responsible for glucose clearance, skeletal muscle, for *in vitro* study. Thus, we found that astragaloside IV increases basal and insulin-stimulated glucose uptake in C2C12 myotubes through the IRS1/AKT pathway and suppresses the palmitate-induced activation of the inhibitory κB kinase (IKK)/inhibitor-κBα (IκBα) pathway.

Materials and methods

Materials. Astragaloside IV, palmitate and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and horse serum were purchased from Gibco Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from PAA Laboratories, Pasching, Austria). Fatty acid-free bovine serum albumin (BSA) was purchased from Calbiochem (San Diego, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit and rabbit anti-mouse IgG were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Phosphorylated (p)-IRS1 [tyrosine (Y)612] was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). An antibody against GAPDH was obtained from Novus Biologicals (Littleton, CO, USA). Antibodies against p-AKT [serine (Ser)473 and threonine (Thr)308], AKT, p-insulin receptor β (IRβ), glucose transporter 4 (GLUT4), p-IKKα/IKKβ and IκBα were purchased from Cell Signaling Technology, Inc. 2-Deoxy-D-[1,2-^{3}H(N)]-glucose (radiolabeled 2-DG) was purchased from Amersham (Buckinghamshire, UK), and purified human insulin was obtained from Eli Lilly (Indianapolis, IN, USA). SYBR® Premix Ex Taq™ was purchased from Takara Bio, Dalian, China). SuperScript III was purchased from Invitrogen (Carlsbad, CA, USA).

Cell culture. The mouse myoblast cell line C2C12 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in DMEM supplemented with 10% FBS. When the cells reached 70% confluence, the medium was replaced with differentiation medium containing DMEM and 2% horse serum, which was replaced every other day. After 4 days, the differentiated C2C12 cells had fused into myotubes. Lipid-containing media were prepared by the conjugation of FFA with FFA-free BSA, according to a method previously described by Itani *et al* (6) with some modifications. Briefly, palmitate was dissolved in 0.1 N NaOH and diluted in DMEM containing 2% (wt/vol) fatty acid-free BSA. The myotubes were incubated for 16 h in serum-free DMEM containing 2% BSA in either the presence or absence of palmitate. The cells were then incubated with 100 nM insulin for 30 min. Following incubation, RNA and total proteins were extracted from the myotubes as described below. Astragaloside IV was added 2 h prior to palmitate exposure.

2-DG transport assay. Glucose uptake into the C2C12 myotubes was measured using radiolabeled 2-DG, according to a method previously described by Perrini *et al* (28) with some modifications. Prior to performing the glucose transport assays, the cells were washed twice with HEPES-buffered saline (HBS). The glucose transport assays were performed by incubating the cells with HBS containing radiolabeled 2-DG (0.5 μCi/ml) at room temperature. The cells with prior exposure to insulin (see below) were incubated in glucose uptake assay medium supplemented with insulin. After 30 min, 2-DG uptake was terminated by three rapid washes with ice-cold phosphate-buffered saline (PBS) + glucose. The cells were lysed in 0.1 N NaOH, and then ^{3}H was counted using a liquid scintillation spectrophotometer (Beckman Instruments, Fullerton, CA, USA).

Measurement of mRNA levels. Total RNA was isolated using TRIzol reagent. The total RNA isolated by this method is undegraded and free of protein and DNA contamination. In order to perform amplification, the following sense and antisense primer sequences were used for amplification: GLUT4, 5'-GTGACTGG AACACTGGTCCTA-3' and 5'-CCAGCCACGTTGCATTG TAG-3'; Toll-like receptor 4 (TLR4), 5'-TCTTCTCCTGCCTG ACACCA-3' and 5'-TCTTCTCCTGCCTGACACCA-3'; monocyte chemotactic protein 1 (MCP-1), 5'-GGCCTGTTGTTCA C AGTTGC-3' and 5'-AGCCGACTCATTGGGATCAT-3'; tumor necrosis factor (TNF)α, 5'-CACAAGATGCTGGGACAGTGA-3' and 5'-TCCTTGATGGTGGTGCATGA-3'; and interleukin-6 (IL-6), 5'-CAGCCACTGCCTTCCCTACT-3' and 5'-CAGTGCATCAT CGCTGTTCAT-3'. Preliminary experiments were performed with various amounts of cDNA to determine the non-saturating conditions of PCR amplification for all the genes studied. Therefore, under these conditions, the relative quantification of mRNA was assessed by the RT-PCR method used in this study. The expression of specific mRNAs are presented relative to the expression of the control gene [adenine phosphoribosyltransferase (APRT), 5'-CGGCAAGATCGACT ACATCG-3' and 5'-CCAGCTCAGCCTTCCCATAC-3'].

Subcellular fractionation. The subcellular fractionation of the C2C12 cells was performed as described by Tortorella and Pilch (29) with some modifications. The C2C12 cells were washed three times in ice-cold PBS, pH 7.4 (in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4 and 1.8 KH_2PO_4) and then the cells were sheared through a 22-gauge needle 15 times in HES buffer [255 mM sucrose, 4 mM disodium EDTA, 20 mM HEPES pH 7.4, 10 μM leupeptin, 1 μM pepstatin, 1 μM aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 mM benzamidine]. The homogenate was centrifuged at 19,000 x g for 20 min. The pellet was saved and fractionated further in order to extract the following fractions: crude 'plasma membrane' (PM) fraction (P1) and crude nuclear (N)/endoplasmic reticulum (ER) fraction. In order to obtain the PM fraction, the pellet was resuspended in HES,

layered onto a 1.12 M sucrose cushion in 20 mM HEPES and 1 mM disodium EDTA, and centrifuged at 100,000 x g for 1 h. The sucrose cushion interface was collected and pelleted at 40,000 x g for 20 min. This PM-containing pellet (P1) was resuspended in PBS plus protease inhibitors. All centrifugations were performed at 4°C by a Beckman Ultraspeed centrifuge (Beckman Coulter, Brea, CA, USA). GLUT4 was detected by western blot analysis as described below.

Western blot analysis. The C2C12 cells grown on six-well plates were washed with ice-cold PBS and then scraped into homogenizing buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM Na_3PO_4, 100 mM NaF, 2 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. The lysates were centrifuged at 13,000 rpm for 30 min. Soluble proteins were quantitated using the bicinchoninic acid kit (Pierce Chemical Co., Rockford, IL, USA) with BSA as the standard and adjusted to 2 μg/μl. Aliquots of homogenate were solubilized in Laemmli sample buffer and the protein was subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Whatman; GE Healthcare, Piscataway, NJ, USA). The membranes were blocked in a solution of Tris-buffered saline containing 5% nonfat dry milk. Protein was visualized using the SuperSignal® West Pico chemiluminescent substrate (Pierce Chemical Co.) assay.

Statistical analysis. Data are presented as the means ± SEM. The significance between groups was determined using either an unpaired two-tailed Student's t-test or one-way ANOVA as appropriate. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Astragaloside IV increases basal and insulin-stimulated glucose uptake in C2C12 myotubes. In order to examine the effect of astragaloside IV on glucose uptake in C2C12 myotubes in the presence or absence of insulin, differentiated C2C12 myotubes were serum starved overnight in 0.2% BSA + DMEM, and were then treated with astragaloside IV at concentrations ranging from 6.25 μM to 50 μM for 12 h. Astragaloside IV increased glucose uptake in the C2C12 myotubes in a dose-dependent manner with the most significant increase at 12.5 μM (Fig. 1A). In the basal state, glucose uptake was elevated with increasing concentrations of astragaloside IV up to 50 μM (Fig. 1A); however, in the insulin-stimulated C2C12 myotubes, glucose uptake was significantly increased only at a concentration of 12.5 μM astragaloside IV (Fig. 1A). We then treated the C2C12 myotubes with 12.5 μM astragaloside IV for different periods of time. The C2C12 myotubes showed a significant increase in glucose uptake after 1 h, and the most significant effect was observed at 2 h, and was sustained until 12 h (Fig. 1B). Astragaloside IV increased glucose uptake in C2C12 myotubes in a dose- and time-dependent manner.

Astragaloside IV ameliorates palmitate-induced insulin resistance in C2C12 myotubes. In a previous *in vitro* experi-

ment, insulin-stimulated glucose uptake was decreased in C2C12 myotubes exposed to palmitate for 16 h (30). In the present study, we determined the effect of astragaloside IV on glucose uptake reduced by palmitate in C2C12 myotubes. The C2C12 myotubes were treated with 12.5 μM astragaloside IV and serum starved for 2 h prior to incubation with 0.75 mM palmitate for 16 h. A 16-h incubation period with 0.75 mM palmitate decreased the insulin-stimulated uptake of 2-DG (P<0.05 vs. insulin-stimulated cells incubated with BSA alone) (Fig. 1C). However, pre-treatment with astragaloside IV restored glucose uptake in the palmitate-exposed C2C12 myotubes (P<0.05) (Fig. 1C).

Effect of astragaloside IV on the phosphorylation of IRβ in the insulin-stimulated state. The subcellular localization of IRβ was evaluated by subcellular fractionation followed by western blot analysis. As shown in Fig. 2, astragaloside IV had no significant effect on the protein expression or the phosphorylation of IRβ in the presence or absence of palmitate.

Effect of astragaloside IV on the phosphorylation of IRS1 and AKT in the basal state. The proteins, IRS1 and AKT, play important roles in insulin signaling, and the phosphorylation of IRS1 and AKT may promote glucose uptake in skeletal muscle (31). In this experiment, we found that astragaloside IV significantly enhanced the phosphorylation of IRS1(Y612) as well as AKT (Ser473) and (Thr308) (P<0.05; Fig. 3A).

Effect of astragaloside IV on the phosphorylation of IRS1 and AKT in the insulin-stimulated state. As expected, astragaloside IV significantly increased the insulin-stimulated phosphorylation of IRS1(Y612) and AKT (Ser473) and (Thr308) (P<0.05; Fig. 3B) in the C2C12 myotubes. Palmitate exposure reduced the levels of total protein as well as the phosphorylation of IRS1 and AKT. Pre-treatment with astragaloside IV ameliorated the reduced phosphorylation levels; however, it had no effect on the protein expression of IRS1 and AKT (P<0.05; Fig. 3B).

Effect of astragaloside IV on the mRNA and protein expression of GLUT4. The elevated mRNA and protein expression of GLUT4 is associated with increased cell surface levels of GLUT4 (32). Thus, we examined the mRNA and protein expression of GLUT4 in the C2C12 myotubes in the presence or absence of palmitate. Astragaloside IV had no direct effect on the mRNA and protein expression of GLUT4 in the C2C12 myotubes (Fig. 4A and B; P<0.05). Exposure to palmitate for 16 h caused a marked reduction in the mRNA and protein levels of GLUT4 in the C2C12 myotubes; however, pretreatment with astragaloside IV attenuated the deleterious effect of palmitate (Fig. 4A and B; P<0.05).

Astragaloside IV enhances the basal and insulin-stimulated translocation of GLUT4, and partly attenuates the palmitate-induced decrease in the insulin-stimulated translocation of GLUT4. The subcellular localization of GLUT4 was evaluated by subcellular fractionation followed by western blot analysis. As shown in Fig. 4C, the surface levels of basal GLUT4 were increased by astragaloside IV (P<0.05), whereas basal GLUT4 surface levels were not detected in the palmitate-exposed group. Insulin stimulation resulted in a
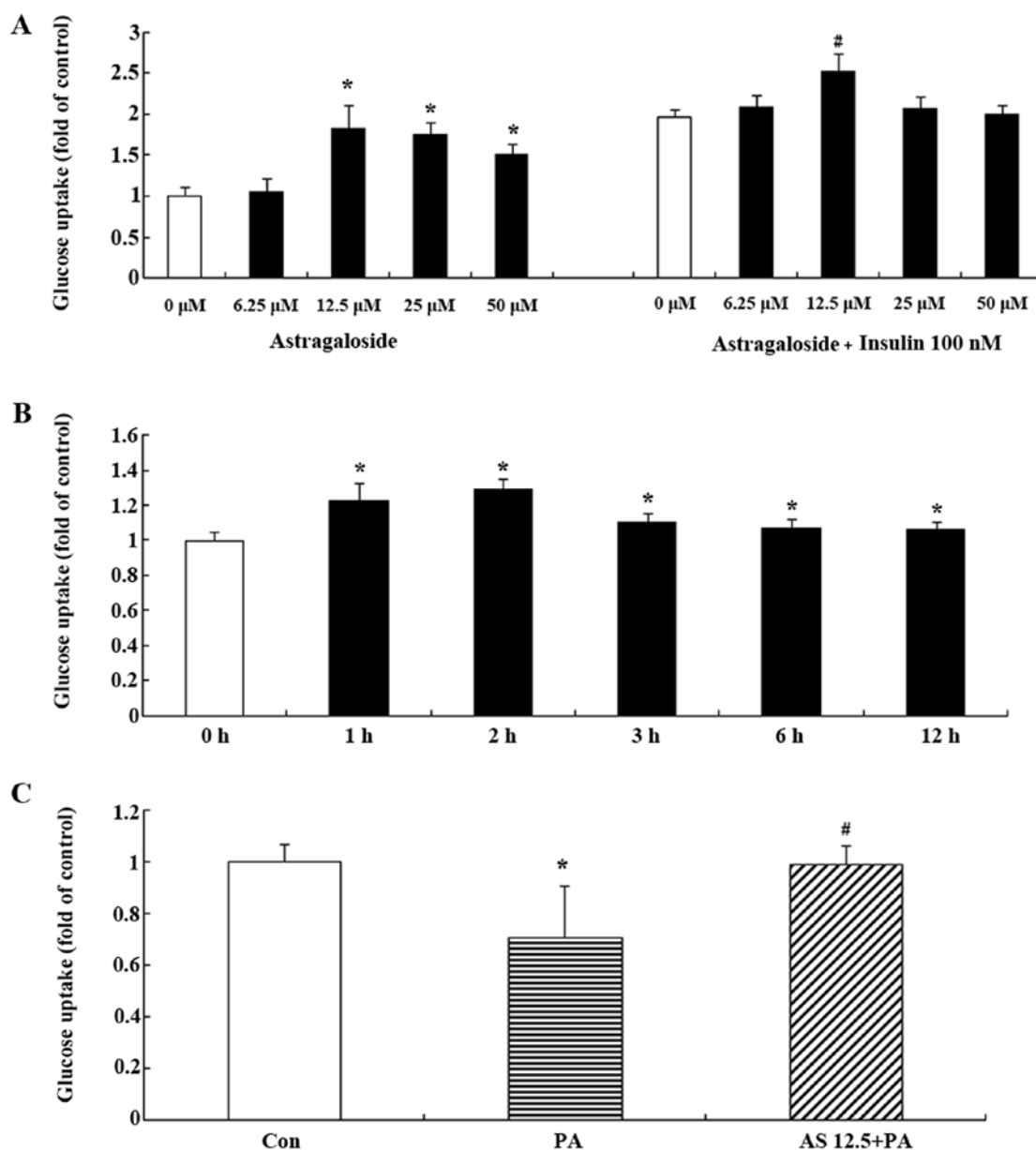
Figure 1. Dose- and time-dependent effect of astragaloside IV on radiolabeled 2-deoxyglucose (2DG) uptake in C2C12 myotubes. C2C12 myoblasts were cultured and differentiated into myotubes as described in the Materials and methods. (A) Myotubes were incubated with or without astragaloside IV at the indicated concentrations for 12 h, in the presence or absence of insulin stimulation (100 nM) for 30 min. Thereafter, [³H] 2DG uptake was determined as described in the Materials and methods. (B) Myotubes were treated with 12.5 μM Astragaloside IV for the indicated periods of time. Data are presented as the means ± SEM. *P<0.05 between the indicated treatment and the corresponding corresponding control. (C) Astragaloside IV ameliorated palmitate-induced insulin resistance in C2C12 myotubes. C2C12 myotubes were treated with 12.5 μM astragaloside IV for 2 h, exposed to 0.75 mM BSA-complexed palmitate or BSA for 16 h, and finally incubated with 100 nM insulin for 30 min. #P<0.05 between the astragaloside IV + palmitate (PA) + insulin group and the PA + insulin group. *P<0.05 between the control (Con) + insulin group and the PA + insulin group. AS, astragaloside IV.

marked increase in the GLUT4 levels at the cell surface in the C2C12 myotubes (Fig. 4C). Exposure to palmitate decreased the insulin-stimulated translocation of GLUT4, and this effect was partly antagonized by astragaloside IV (P<0.05; Fig. 4C).

Astragaloside IV suppresses the activation of the IKK/IκBα pathway. It is well known that the IKK/IκBα/NF-κB pathway plays an important role in the regulation of inflammatory factors, and IKK and IκBα are key proteins in this pathway. In the basal state, NF-κB binds to its inhibitor IκBα to form an inactivated complex in the cytosol. Exposure to palmitate initiates the IKK/IκBα/NF-κB cascade, thus activating IKK,

which then phosphorylates IκBα, causing the release of IκBα from NF-κB. NF-κB then translocates to the nucleus where it promotes the expression of various inflammatory factors (45). In order to determine the effect of astragaloside IV on the IKK/IκBα/NF-κB cascade, we detected the levels of p-IKK and IκBα using western blot analysis. The four-day differentiated C2C12 myotubes were incubated with astragaloside IV (12.5 μM, 2 h) prior to stimulation with and without 0.75 mM palmitate for 1.5 h. As shown in Fig. 5A, palmitate activated IKK (as demonstrated by the increased level of p-IKK), which lead to the degradation of IκBα (as demonstrated by the decreased level of IκBα) (Fig. 5B). Pre-treatment with astragaloside IV for
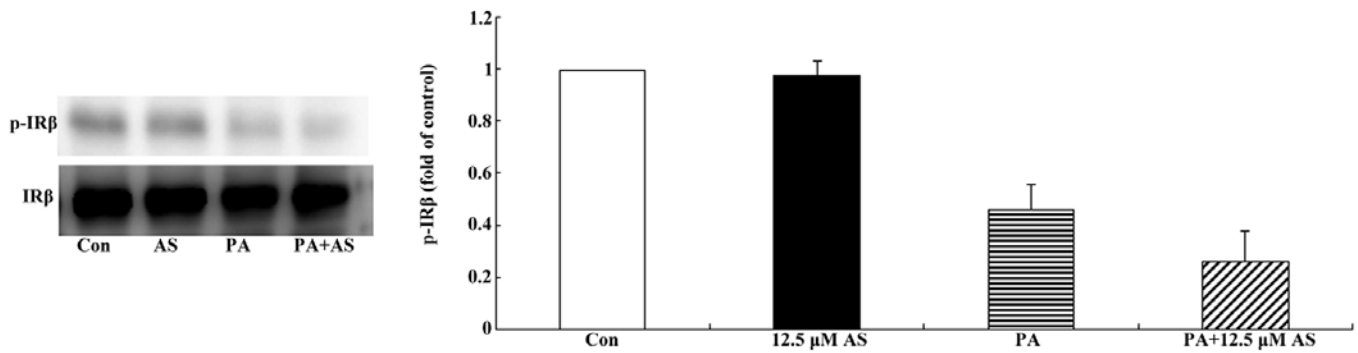
Figure 2. Effect of astragaloside IV on the phosphorylation of insulin receptor β (IRβ) in insulin-stimulated C2C12 myotubes. The C2C12 myotubes were treated with astragaloside IV for 2 h prior to incubation with or without 0.75 mM palmitate for 16 h, and then incubated with 100 nM insulin for 30 min. Astragaloside IV had no effect on the protein expression of IRβ or the phosphorylation of IRβ, which was reduced by exposure to palmitate. PA, palmitate; AS, astragaloside IV.
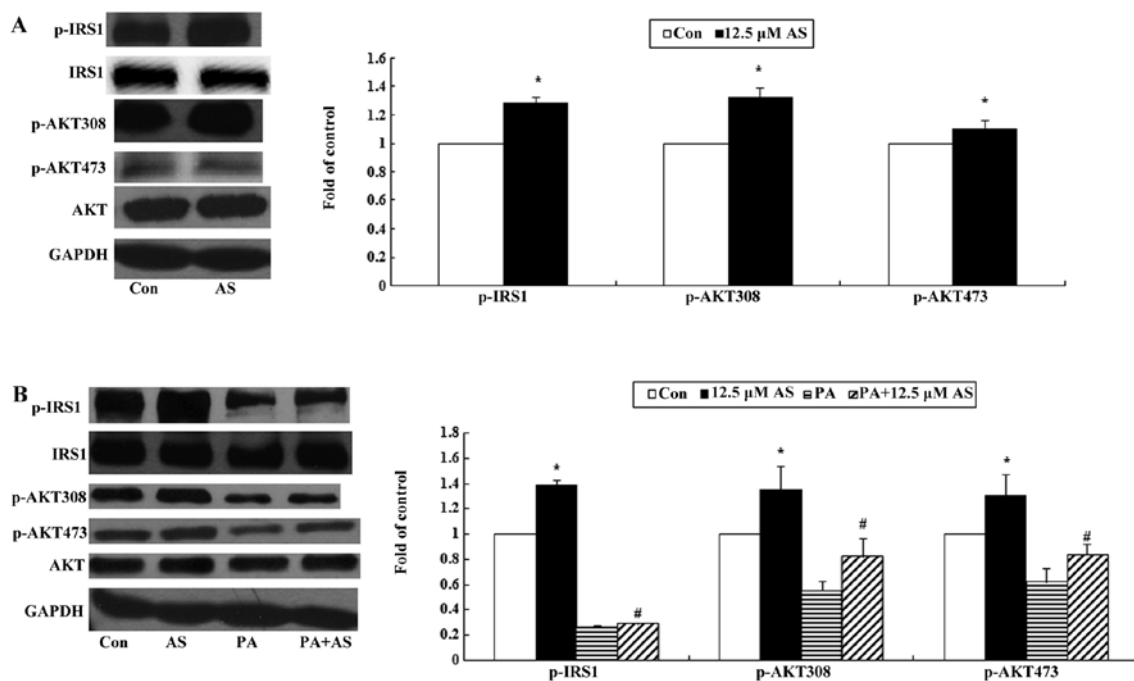

Figure 3. (A) Astragaloside IV increased the phosphorylation of insulin receptor substrate 1 (IRS1) and protein kinase B (AKT) in C2C12 myotubes in the basal state. The C2C12 myotubes were treated with or without 12.5 μM astragaloside IV for 2 h. *P<0.05 between the indicated treatment and the corresponding corresponding control. (B) Astragaloside IV increased the phosphorylation of IRS1 and AKT in insulin-stimulated C2C12 myotubes in the presence or absence of palmitate. The C2C12 myotubes were treated with or without astragaloside IV for 2 h prior to incubation with or without 0.75 mM palmitate for 16 h, and then incubated with 100 nM insulin for 30 min. *P<0.05 between the astragaloside IV treatment group and corresponding control; #P<0.05 between the PA group and the PA + 12.5 μM AS group. PA, palmitate; AS, astragaloside IV; Con, control.

2 h suppressed the activation of IKK and the degradation of IκBα (Fig. 5; P<0.05); this is indicates the inhibitory effect of astragaloside IV on the IKK/IκBα/NF-κB cascade.

Astragaloside IV decreases the mRNA expression of MCP-1, IL-6 and TNFα. *In vitro*, the exposure of skeletal muscle cells to palmitate and subsequent incubation also induced the expression of proinflammatory factors such as IL-6 and TNFα. Following a 4-h incubation with 0.75 mM palmitate, the C2C12 myotubes displayed significantly elevated mRNA expression of MCP-1, IL-6 and TNFα (Fig. 6). Similar to the inhibitory effect of astragaloside IV on the IKK/IκBα/NF-κB cascade, pre-treatment with astragaloside IV for 2 h suppressed

the palmitate-induced increase in the mRNA expression of MCP-1, IL-6 and TNFα (P<0.05) (Fig. 6).

Effect of astragaloside IV on the mRNA expression of TLR4. TLR4, a receptor of palmitate, is involved in FFA-induced insulin resistance. Through binding to TLR4, palmitate increases the expression of inflammatory factors by activating the IKK/IκBα/NF-κB cascade, with subsequent feedback regulation of the mRNA expression of TLR4 (45). The C2C12 myotubes incubated with 0.75 mM palmitate for 16 h exhibited markedly increased mRNA expression of TLR4 (Fig. 6). Pre-treatment with astragaloside IV downregulated the mRNA level of TLR4 induced by palmitate (P<0.05) (Fig. 6).
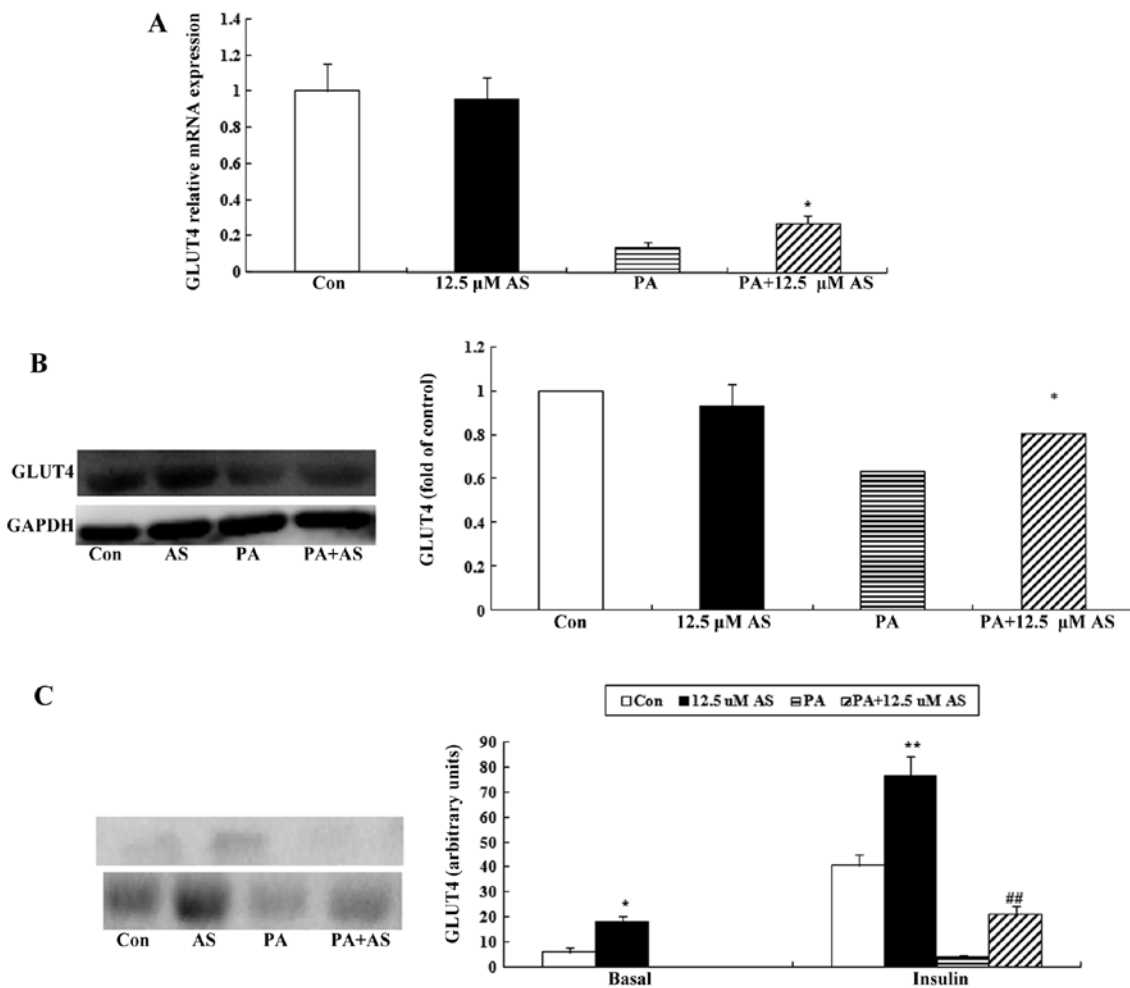
Figure 4. (A and B) Astragaloside IV attenuates the palmitate-induced decrease in the mRNA and protein levels of glucose transporter 4 (GLUT4). The C2C12 myotubes were treated with astragaloside IV for 2 h prior to incubation with or without 0.75 mM palmitate for 16 h. *P<0.05 between the PA group and the PA + 12.5 μM AS group. (C) Astragaloside IV enhances the basal and insulin-stimulated translocation of GLUT4, and partly attenuates the palmitate-induced decrease in the insulin-stimulated translocation of GLUT4. The C2C12 myotubes were treated with astragaloside IV for 2 h prior to incubation with or without 0.75 mM palmitate for 16 h, and then incubated with or without 100 nM insulin. The plasma membranes were separated as described in the Materials and methods and subsequently western blot analysis of the proteins was performed. *,**P<0.05 between the astragaloside IV treatment group and corresponding control; ##P<0.05 between the PA group and the PA +12.5 μM AS group. PA, palmitate; AS, astragaloside IV; Con, control.

Discussion

The classic pathway of insulin signaling involving the trans-location of GLUT4 (33,34) to the cell surface is triggered by the autophosphorylation of the insulin receptor on multiple tyrosine residues following insulin binding. This results in the tyrosine phosphorylation of a family of IRS proteins and the activation of a complex network of downstream molecules, including phosphatidylinositol 3-kinase (PI3K) and the serine/threonine kinase AKT. Skeletal muscle is the most important tissue involved in insulin-stimulated glucose disposal, and insulin resistance in skeletal muscle is a major defect in most obese phenotypes (35).

The present study demonstrated that astragaloside IV regulates glucose uptake and delineates the proximal signaling events mediating this response. We demonstrated that astragaloside IV increased basal glucose uptake in the C2C12 myotubes in a dose- and time-dependent manner; the highest rate of glucose uptake was observed at a concentration of 12.5 μM of astragaloside IV and a treatment period of 2 h produced the most significant effect on glucose uptake. Astragaloside IV also increased insulin-stimulated glucose uptake, which revealed that it is capable of enhancing insulin sensitivity in C2C12 myotubes. We found that astragaloside IV increased glucose uptake in the C2C12 myotubes through the phosphorylation of IRS1 and AKT in the basal state. Astragaloside IV also increased the insulin-stimulated phosphorylation of IRS1 and AKT, thereby displaying a synergistic interaction with insulin. We also examined the phosphorylation of IRβ and found that astragaloside IV did not activate IRβ, which suggests that astragaloside IV activated IRS1 and AKT independently of IRβ.

Randle *et al* (36), described the glucose fatty-acid cycle, which is a metabolic pathway linking fat and carbohydrate metabolism. *In vivo*, fatty acid metabolism not only provides ATP, but also decreases glucose consumption. Elevated fatty acid uptake always follows increased fatty acid oxidation, which leads to reduced glucose consumption and insulin tolerance. Abnormal fatty acid metabolism exists in obese and diabetic patients due to elevated basal lipolysis and the impaired ability of insulin to mediate the conversion of serum
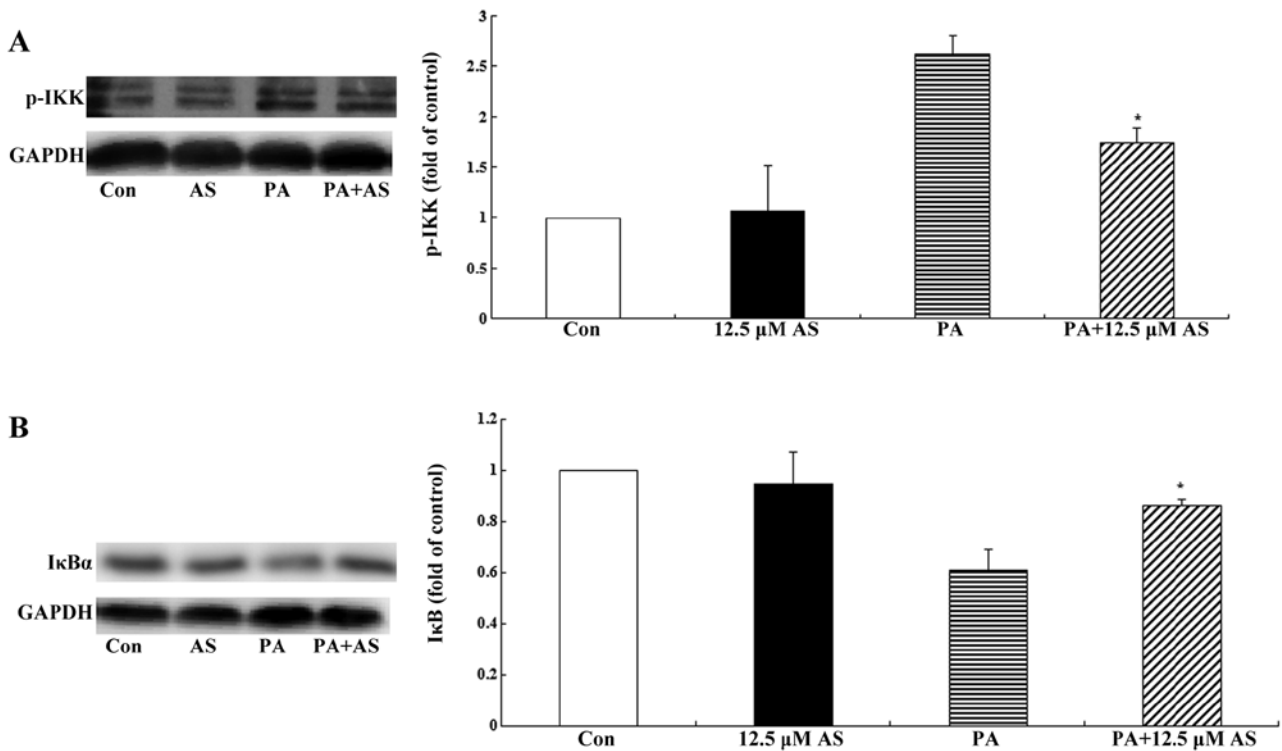
Figure 5. (A) Astragaloside IV suppresses the palmitate-induced increase in the phosphorylation of inhibitory κB kinase (IKK). The C2C12 myotubes were treated with astragaloside IV for 2 h prior to incubation with or without 0.75 mM palmitate for 1.5 h. *P<0.05 between the PA group and the PA + 12.5 μM AS group. (B) Astragaloside IV inhibited the palmitate-induced degradation of IκBα. The C2C12 myotubes were treated with astragaloside IV for 2 h prior to incubation with or without 0.75 mM palmitate for 1.5 h. *P<0.05 between the PA group and the PA + 12.5 μM AS group. PA, palmitate; AS, astragaloside IV; Con, control.
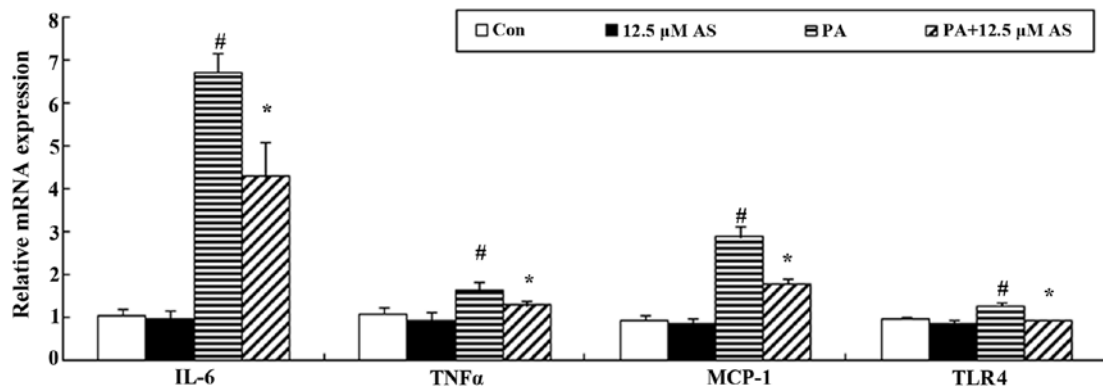

Figure 6. Astragaloside IV suppresses the palmitate-induced increase in the mRNA expression of interleukin-6 (IL-6), tumor necrosis factor α (TNFα), monocyte chemotactic protein-1 (MCP-1) and Toll-like receptor 4 (TLR4). The C2C12 myotubes were treated with astragaloside IV for 2 h prior to incubation with or without 0.75 mM palmitate for 4 h. *P<0.05 between the PA group and the PA + 12.5 μM AS group. #P<0.05 between the control group and the palmitate group. PA, palmitate; AS, astragaloside IV; Con, control.

FFA into triglyceride (37). Insulin-stimulated glucose transport significantly decreased in skeletal muscle obtained from patients with type 2 diabetes (38) and obesity (39). The translocation of GLUT4 was reduced by 90% in the skeletal muscle of type 2 diabetic patients (40). In this study, astragaloside IV enhanced the basal and insulin-stimulated translocation of GLUT4. The C2C12 myotubes exposed to palmitate exhibited a marked decrease in the mRNA and protein expression of GLUT4 as well as a decrease in the translocation of GLUT4, whereas pre-treatment with astragaloside IV partly attenuated the deleterious effects of palmitate.

Our results indicated that palmitate inhibited the activation of IRS1 and AKT in the C2C12 myotubes, reduced the insulin-stimulated translocation of GLUT4, and finally decreased glucose uptake. Pre-treatment with astragaloside IV ameliorated the palmitate-induced decrease in the phosphorylation of IRS1 and AKT, and partly attenuated the palmitate-induced decrease in the insulin-stimulated translocation of GLUT4. Astragaloside IV displayed a significant effect on insulin resistance induced by palmitate in skeletal muscle.

Previous research has demonstrated that type 2 diabetes and obesity are associated with significant increases in

inflammatory factors (41), and macrophage infiltration in adipose tissue (42,43), which demonstrates the close association between the immune system and insulin resistance (42,44-46). The IKK/IκBα pathway is closely associated with insulin resistance (45,47); it links inflammation to insulin resistance. TLRs are innate immune receptors; the immune response to invading microorganisms is mediated through the activation of TLRs (48). Increasing evidence has demonstrated that saturated fatty acids activate TLR4 signaling which activates the NF-κB pathway (49,50), and play roles in insulin resistance (47,51). Reyna *et al* (52) recognized the importance of TLR4 after examining insulin resistant skeletal muscle obtained from obese and type 2 diabetic subjects. Mice lacking TLR4 have been shown to be partially protected against fatty acid-induced insulin resistance in skeletal muscle (49,53,54). The activation of the IKK/IκBα pathway increases the expression of IL-6 and TNFα, which reduces insulin resistance in skeletal muscle (48,54). IL-6 may be the most critical factor involved in the modulation of insulin resistance (55,56); a previous study found that the concentration of IL-6 was 2-3-fold higher in obese subjects without type 2 diabetes in comparison with lean subjects (55). However, Sell *et al* (57) suggested that MCP-1 was the most important among a number of cytokines, and that physiological concentrations of MCP-1 may lead to insulin resistance.

Our results confirmed that palmitate may bind to TLR4, thus activating IKK, which then phosphorylates IκBα, causing the release of IκBα from NF-κB. NF-κB then translocates to the nucleus where it promotes the expression of inflammatory factors, such as MCP-1, IL-6 and TNFα. These inflammatory factors inhibit the activation of insulin pathway proteins, finally decreasing insulin sensitivity. The pre-treatment of the palmitate-exposed C2C12 myotubes with astragaloside IV decreased the mRNA expression of TLR4, suppressed the activation of IKK and the degradation of IκBα, reduced NF-κB activation (data not shown), and thus reduced the mRNA expression of MCP-1, IL-6 and TNFα, consequently reducing the insulin resistance induced by palmitate. These findings are limited by the absence of data regarding NF-κB due to a lack of funding and we plan to investigate this aspect further in the future.

In conclusion, astragaloside IV increased glucose uptake in C2C12 myotubes through the IRS1/AKT pathway. Moreover, astragaloside IV suppressed the the palmitate-induced activation of the IKK/IκBα pathway and reduced the secretion of inflammatory factors. These results may explain, at least in part, the antidiabetic and insulin-sensitizing effects of astragaloside IV and *A. membranaceus*.

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