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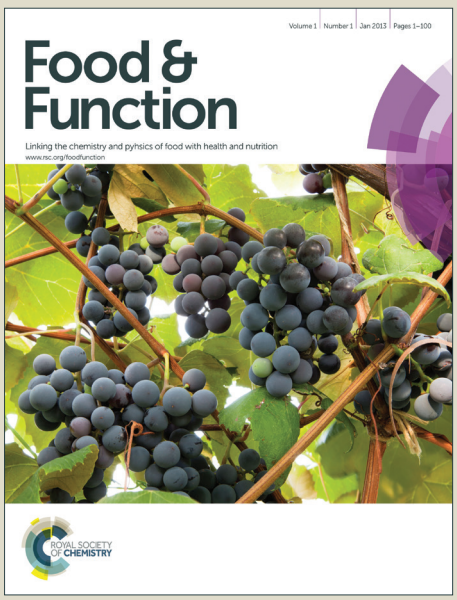

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LBP-4a Improves Insulin Resistance via Translocation and Activation of GLUT4 in OLETF rats

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Abstract

Lycium barbarum polysaccharide (LBP) has been shown to ameliorate insulin resistance (IR), but the identification of compounds from LBP and the mechanisms have not been clarified. In this study, LBP-4a was purified from *Lycium barbarum* by DEAE cellulose and Sephadex G-100 column chromatography, and the effects of LBP-4a on IR were investigated. The results indicated that LBP-4a caused translocation of the glucose transporter isoform 4 (GLUT4) to the cell surface, which in turn stimulated glucose uptake and the effect was sensitive to wortmannin, an inhibitor of phosphoinositol 3-kinase (PI3-K) and SB203580, an inhibitor of p38 mitogen activated protein kinase (p38 MAPK(α, β)). Furthermore, the effects of LBP-4a on p38 MAPK activities were abrogated by pretreatment of rat adipocytes using SB203580. In summary, LBP-4a improved IR via translocation and activation of GLUT4 in OLETF rats, and the activations of PI3-K and p38 MAPK contributed to these effects.

Keywords: *Lycium barbarum* polysaccharide; insulin resistance; adipocytes; GLUT4; p38 MAPK

1. Introduction

Diabetes is a major health problem, which affects 3% worldwide population. Over 90% of patients with diabetes are type 2 diabetes (T2D). Insulin resistance (IR) plays an important role in the pathogenesis of T2D. The long course and serious complications with T2D often results in high morbidity and mortality, and the treatments of diabetes cost vast amounts of resources, including medicines, diets, physical training and etc. across all countries[1]. For patients suffering from diabetes, medicinal therapy is the primary alternative. Recently, there has been increasing interest for development and utilization of antidiabetic plant products due to the possibility of fewer side effects and better economic feasibility, especially for developing countries.

Lycium barbarum, small red berry commonly used for home cooking in China, known as a "fruit for long life" is also traditionally used in Chinese herbal medicine. *Lycium barbarum* polysaccharide (LBP) is found to have hypoglycemic activity and is considered for treating diabetic retinopathy[2]. We have shown the effects of LBP on the improvement of antioxidant ability and DNA damage in T2D rats[3]. We have also demonstrated that LBP-4 could ameliorate IR and protect the kidney in streptozotocin-induced diabetic rats[4].

Insulin-induced intracellular signaling events have been investigated extensively in recent years. Insulin stimulates glucose transport in target cells by translocation of the glucose transporter isoform 4 (GLUT4) from an intracellular storage pool to the plasma membrane[5]. In addition to insulin, multiple other factors are capable of

stimulating GLUT4 translocation to the cell surface and glucose uptake. For example, exercise induces GLUT4 translocation and glucose uptake in skeletal muscle through an insulin-independent pathway[6]. Furthermore, it has been confirmed that the LBP improves IR and the mechanism involves increasing the GLUT4 level of cell-surface in rat skeletal muscle [7]. Insulin binding to its receptor results in receptor autophosphorylation on tyrosine residues and the tyrosine phosphorylation of insulin receptor substrates (IRS-1, IRS-2 and IRS-3) by the insulin receptor tyrosine kinase. The subsequent activation of phosphatidylinositol 3-kinase (PI3-K) is necessary for the recruitment of GLUT4 to the cell surface. In addition, emerging evidence suggests that a second signaling cascade independent of the PI3-K pathway is required for the insulin-dependent translocation of GLUT4[8,9]. Recent studies have suggested that insulin-stimulated GLUT4 translocation in rat skeletal muscle, L6 muscle cells and 3T3-L1 adipocytes[10] are not sufficient to achieve the maximal increase in glucose uptake. Therefore, these studies propose that the intrinsic activity of the translocated GLUT4 is subject to regulation. Unlike GLUT4 translocation, very little is known about the signals controlling GLUT4 catalytic activity and whether or not p38 mitogen activated protein kinase (p38 MAPK) contributes to the stimulation of glucose uptake by LBP-4a in rat adipocytes.

A suitable antidiabetic agent should have actions similar to those of insulin. To address this question, we separated, purified and identified LBP-4a, and assessed its efficacy in improving the IR, and then analyzed the mechanism of this postulated effect by evaluating the activity of LBP-4a in adipocytes isolated from experimental

OLETF rats.

2. Materials and Methods

2.1Materials

The fruits of *Lycium barbarum* were collected in the Ningxia Hui Autonomous Region which was the well-known production area of *Lycium barbarum* in China, and were authenticated at the Agricultural college of Northwest A&F University. A specimen (NO.20110609) was deposited in the herbarium of the Botany Department.

Bovine serum albumin (BSA) and phenylemthylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, Mo, USA). Standard reagents for SDS-PAGE and immunoblotting were purchased from Bio-Rad (Mississauga, ON, Canada). Phosphospecific antibody to p38 MAPK was purchased from New England Biolabs (Mississauga, ON, Canada). Activating transcription factor (ATF)-2 fusion protein was purchased from New England Biolabs (Beverly, MA). Enhanced chemiluminescence reagent [g-32P] ATP, protein A-Sepharose, and IgG conjugated to horseradish-peroxidase were acquired from Amersham Pharmacia Biotech (Baie d'Urfe´, QC, Canada). SB203580 was purchased from Calbiochem (La Jolla, CA, USA). Polyclonal antibodies to p38 MAPKα, p38 MAPKβ, were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 2-Deoxy-D-[3H] glucose and enhanced chemiluminescence detection reagent were purchased from Amersham Biosciences (Little Chalfont, UK).

2.2 Preparation of Polysaccharide Fractions (LBP-4a) from Lycium barbarum

Isolation, purification and identification of LBP-4a were based on our previous

published work[4]. Briefly, the dried fruit samples were refluxed three times to remove lipids with chloroform: methanol solvent (2:1) (v/v). After filtering, the residues were air-dried and then refluxed again with 80% ethanol. The residues were extracted three times in hot water (90℃) and filtered. The combined filtrate was precipitated with 95% ethanol, 100% ethanol and acetone, respectively. After filtering and centrifuging, the precipitate was collected and vacuum-dried, giving crude polysaccharides (yield was 3.25 %±0.14%, and the purity of LBP was 95.8 %±2.0%). Crude LBP was eluted and isolated on a DEAE cellulose column (ø25 mm×350 mm) with distilled water and 0.05-0.5 mol/L NaCl. The collected four fractions were dialyzed, centrifuged, and freeze-dried. For gel permeation chromatography, samples were dissolved in 20 ml of buffer, then applied to a Sephadex G-100 column (ø26 mm×400 mm), and eluted with 0.05 mol/L NaCl at a flow rate of 0.5 ml/min. The fractions were collected using an elution pattern and concentrated in an evaporator at 60℃. The concentrate was dialyzed in distilled water for 72 h, and then freeze-dried.

2.3 Characterization of LBP-4a

The structure of LBP-4a was detected by ultraviolet (UV) and infrared spectroscopy (IR). The high-performance liquid chromatography (HPLC) system was used to determine the molecular weight (MW) of LBP-4a, and this was compared at the retention time of a pullulan standard. Paper chromatography was carried out on filter paper sheets using an n-butanol: pyridine: water (6:4:3) solvent. Detection was developed using chromogenic agent and aniline phthalate spray.

2.4 Experimental Animals and Treatments

Otsuka Long-Evans Tokushima Fatty (OLETF) rats are a newly developed model of human NIDDM. It has been reported that insulin-induced intracellular signaling was decreased in OLETF rats. Insulin-induced intracellular signaling events have been investigated extensively in recent years. Eighteen male OLETF rats were provided by the Tokushima Research Institute (Otsuka Pharmaceutical Tokushima, Japan). All investigations were carried out in accordance with the "Guiding Principles in the Care and Use of Animals" and the University of HeiLongJiang August First Land Reclamation Protocol for Animal Studies. Rats were fed standard rat chow, including 5% fat. At the age of 26 weeks, OLETF rats were randomly assigned to three groups of six rats each: control group, LBP-4a treated group and insulin treated group. In the LBP-4a group, rats were treated by intragastric administration with LBP-4a (10 mg/kg·d, the optimal dose chosen according to our previous study) dissolved in normal saline and control group rats received normal saline for four weeks. The insulin group rats were given 1.25 mU /one animal insulin intraperitoneally (*i.p.*).

2.5 Glucose Tolerance Test

All experimental rats were fasted overnight. Glucose (2 g/kg body weight) was injected intraperitoneally, and blood was collected from the tail vein at different time points (0, 30, 60 and 120 min after glucose loading, respectively). Blood glucose was measured using the One-Touch Fast Take glucose meter (Jingdu,Japan).

2.6 Hyperinsulinemic-euglycemic Clamp

Briefly, unrestrained conscious model rats were allowed to rest for 40 min before the initial blood sample (300 μl) was obtained. For hyperinsulinemic-euglycemic clamp, a continuous intravenous infusion of insulin was then started at the rate of 4 mU/kg

/min and continued for 2 h. The arterial blood-glucose concentration was clamped using a variable-rate glucose infusion. Control rats were infused with saline for the same period of time, and no exogenous glucose was necessary to maintain euglycemia. Tracer injection (2-Deoxy-[^{3}H] D-glucose) was administered 20 min before the end of the clamp to determine glucose uptake.

2.7 2-Deoxy-[^{3}H]D-glucose Uptake

Epididymal adipocytes were isolated by collagenase type 2/DNAse digestion (6 and 0.2 mg/ml, respectively). After digestion, the reaction mixture was filtered trough a 250-µm nylon mesh. The adipocytes were washed three times with Krebs-Ringer HEPES buffer (KRHB) containing 1% (wt/vol) fatty acid-free BSA and then twice with KRHB containing 4% BSA. After final washing, the floating adipocytes were resuspended in KRHB plus 4% BSA and aliquoted for all experimental conditions. The cells were grown in six-well plates. To determine whether PI3K and p38 MAPK were involved in the signaling pathways that were potentially used by LBP-4a. Cells were incubated for 20 min with or without 1 µmol/L wortmannin and 10 µmol/L SB203580 followed by incubation with 13.9 mU /mL insulin for 30 min in the continued presence of the inhibitors. 2-Deoxy-^{3}H-D-glucose (2-DG) uptake was determined over a 5-min period. Nonspecific uptake was determined in the presence of 10 µmol/L cytochalasin-B and was subtracted from the total uptake. The uptake of 2-DG was terminated by rapidly aspirating off the radioactive incubation medium and washing the cells three times in ice-cold phosphate-buffered saline.

2.8 Preparation of Total Membrane Fraction from Epididymal Adipose Tissue

Overnight-fasted rats were injected either with saline or insulin (8 U /kg) for 4 min. All experimental rats were anesthetized with pentobarbital sodium (50 mg/kg body weight, i.p.). Total membrane fraction of epididymal adipose tissue was prepared according to the method described by Ruan et al.[11] with minor modifications. Epididymal adipose tissues isolated from all experimental rats were pooled and homogenized in buffer A [10 mmol/L Tris-HCl, 1 mmol/L EDTA, 250 mmol/L sucrose and 0.1 mmol/L PMSF, pH 7.4] using a polytron homogenizer. The homogenate was centrifuged at 1,700 rpm for 10 min at 4 ℃ and the resulting supernatant was centrifuged at 8,600 rpm for 10 min at 4℃. The supernatant was centrifuged at 185,000 rpm for 60 min at 4℃, and then the pellet was dissolved in buffer B (10 mmol/L Tris–HCl, 1 mmol/L EDTA, pH7.4) and stored at -70℃.

2.9 Preparation of Subcellular Fractionation from Adipocyte

After the isolated adipocytes from epididymal fat pads were incubated in the absence or presence of 1.39 mU /mL insulin in oxygen for 20 min at 37℃, the adipocytes were homogenized in Buffer C (250 mmol/L sucrose, 20 mmol/L Hepes, 1 mmol/L EDTA, pH 7.4), supplemented with 1 mmol/L PMSF, 1 mmol/L leupeptin, 1 mmol/L aprotinin, and 1 mmol/L pepstatin. Adipocyte homogenates were centrifuged at 5,000 rpm for 5 min to remove the fat cake. The homogenates were spun at 19,000 rpm for 20 min. The pellets were resuspended in 3 ml Buffer C without 250 mmol/L sucrose, layered on 6 ml sucrose cushion (38% sucrose in Buffer C) and then spun at 100,000 rpm for 60 min. The membranes recovered on top of the sucrose cushion were resuspended in Buffer B and spun at 40,000 rpm for 20 min. The pellet was

designated as plasma membranes (PM). The supernatant of the 19,000 rpm spin was centrifuged at 41,000 rpm for 20 min to pellet high density microsomes (HDM). The supernatant of the 41,000 rpm spin was spun at 180,000 rpm for 75 min to pellet low density microsomes (LDM). The whole procedure was carried out at 4℃. Purity of the membrane fractions was checked by 5'-nucleotidase and galactosyltransferase as markers of PM and LDM, respectively. The protein concentrations were measured with a protein assay kit (Bio-Rad Protein Assay, Bio-Rad, USA). PM and LDM (20 μg) were separated in 10% SDS-PAGE and transferred to PVDF in 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol. After transfer, the membrane was blocked in 5% nonfat milk. The first antibodies used for the detection of GLUT4 were generated against the COOH-terminus deduced from transporter. The results were then visualized with horseradish peroxidase-conjugated secondary antibodies. After washing, protein bands were visualized by using an enhanced chemiluminescence (ECL) system. Densitometric analysis of the western blots was performed by using a GS-670 Imaging Densitometer (BioRad) and Molecular Analyst Software (version 1.3). The relative values of the samples were determined by giving an arbitrary value of 1.0 to the control group.

2.10 p38 MAPK Phosphorylation

Briefly, adipocytes were lysed with 150 μl concentrated 2×Laemmli sample buffer supplemented with 1 mmol/L dithiothreitol (DTT), 1 mmol/L Na_3VO_4, 100 nmol/L okadaic acid, protease inhibitors (1 mmol/L benzamidine, 10 μmol/L E-64, 1 μmol/L leupeptin, 1 μmol/L pepstatin A, and 0.2 mmol/L phenylmethylsulfonyl fluoride), and

7.5% β-mercaptoethanol. The lysates were transferred to Eppendorf tubes, vortexed for 1 min, passed 5 times through a 25-gauge syringe, and heated for 15 min at 65℃. Samples were centrifuged for 5 min (1,000 rpm), and then 40 μl (50 μg protein) of the supernatant was resolved by 10% SDS-PAGE, electrotransferred onto PVDF membranes, and immunoblotted for phospho-p38 MAPK (1:1,000 dilution of primary antibodies). The bands of the proteins were visualized as mentioned above.

2.11 In Vitro p38 MAPK Activity Assay

Protein kinase activity was measured as described previously[12] with modifications. Anti-p38 MAPKα or anti-p38 MAPKβ antibodies (2 μg per condition) were adsorbed to protein A-Sepharose beads, by incubating overnight at 4℃ under constant rotation. p38 MAPKα、β were immunoprecipitated from 250 μg of total protein for 2-3 h with the preabsorbed Sepharose beads. Immunocomplexes were isolated and washed four times with 1 ml wash buffer (25 mmol/L HEPES, pH 7.8, 10% glycerol [vol/vol] 1% Triton X-100 [vol/vol], 0.1% bovine serum albumin, and 1 mol/L NaCl) supplemented with 1 mmol/L Na_3VO_4, 1 mmol/L DTT, 1 mmol/L PMSF, and 10 nmol/L okadaic acid and twice with 1 ml kinase buffer (50 mmol/L Tris/HCl, pH 7.5, and 10 mmol/L $MgCl_2$) supplemented with 1 mmol/L Na_3VO_4 and 10 nmol/L okadaic acid. Immunocomplexes were then incubated for 30 min at 30℃ with 30 μl reaction mixture (kinase buffer containing 5 μmol/L ATP, 2 μCi [γ-^{32}P]ATP, and 2 μg ATF-2 fusion protein per condition) on a platform shaker. Reaction was stopped by adding 30 μl of 2×Laemmli sample buffer and heating for 30 min at 37℃. Samples were centrifuged for 5 min (1,000 rpm) then 40 μl of the supernatant was resolved by 13%

SDS-PAGE and electrotransferred onto PVDF membranes. The bands of the proteins were visualized as mentioned above.

2.12 Statistical Analysis

All the results were expressed as the mean ± S.D. *P*-values of less than 0.05 were considered to be significant. Statistical analysis was performed by one-way analysis of variance (ANOVA). Two way ANOVA was used to compare the data between control, insulin or LBP-4a treated rats in the Glucose Tolerance Test. All the grouped data were statistically evaluated with SPSS 13.0 software. At least three times experiments were repeated and three replicates in each experiment in the in vitro studies.

3. Results

3.1 Isolation, purification and characterization of LBP-4a

Water-soluble polysaccharides were obtained by water extraction and ethanol precipitation, then subjected to DEAE-cellulose ion exchange chromatography with NaCl elution resulting in LBP-1, LBP-2, LBP-3 and LBP-4 peaks (10.0, 8.0, 14.0 and 16.0%, respectively) (Fig. 1A). According to the sugar content of various subfraction and the hypoglycemic effects on the rat models in preliminary experiments, we determined that LBP-4 was the most effective component. Therefore, we applied further separation and purification of LBP-4. The LBP-4 fraction (100 mg) was further separated into LBP-4a and LBP-4b (1.07 and 0.57%, respectively) by gel permeation chromatography (Fig. 1B). According to sugar analysis using the sulfuric acid-phenol method and Bradford method, the sugar content of LBP-4a was

92.2%±1.3%, and the protein content was 3.8%.

LBP-4a was identified to be a homogeneous polysaccharide component, which showed a single symmetrical peak following Sephadex G-100 gel chromatography (Fig. 1C). The MW of LBP-4a was 33,867 Da and retention time was 8.257 min by HPLC (Fig. 1D). The MW of LBP-4a was determined based on a standard curve of dextran molecular weights. The linear regression equation of y=-0.408x+8.6753 with a correlation coefficient (R2) of 0.9951 was obtained. In addition, the monosaccharide composition of LBP-4a was analyzed by paper chromatography and revealed the presence of six spots, corresponding to galactose, glucose, rhamnose, arabinose, mannose, and xylose respectively. LBP-4a had two absorption peaks at 199 and 260 nm in the UV spectrum (Fig. 1 E), indicating the presence of polysaccharide and protein. As shown in Fig.1F, according to IR spectrum, the purified LBP-4a displayed a broadly stretched, intense peak at 3,428 cm^{-1} characteristic of hydroxyl group and a weak C-H peak at around 2,915 cm^{-1}. The relatively strong absorption peak at around 1,710 cm^{-1} indicated the carbonyl group. The absorbance of polysaccharides in the range 1,000-1,200 cm^{-1} was the C-O-C and C-O-H link band positions. The backbone of sugar residues chain in LBP-4a contained 1→6 indican bonds according to periodate oxidation. The results of β-elimination reaction indicated that the chain of polysaccharides and protein were connected by O-linked chemical bond[13].

3.2 Effects of LBP-4a on Body Weight, Glucose Infusion Rate (GIR), and Plasma Levels of Glucose and Insulin in OLETF Rats

As shown in Table 1, there was no significant difference in body weight between

control and insulin groups from 26 to 30 weeks of age. LBP-4a administration tended to decrease body weight, although it did not reach the level of statistical significance in OLETF rats. GIR was significantly higher in the insulin treatment group and the LBP-4a group than the control group; however, there was no significant difference between the effects of insulin and LBP-4a. The concentrations of plasma glucose and insulin were significantly lower in the groups of insulin and LBP-4a than in the control group.

3.3 Glucose Tolerance Test

To assess the potential role of LBP-4a in improving IR, we performed the glucose tolerance test. In OLETF rats, blood-glucose levels reached a peak at 30min after glucose administration, and then the glucose levels started to decline, but they continued to be high after 120 min. As shown in Fig 2(A), LBP-4a and insulin resulted in fewer hyperglycemic effects from 30 min and onwards after glucose loading in model rats. At the end of 120 min, the blood glucose reached to near normal levels. The above results indicated that LBP-4a could improve abnormal glucose tolerance in OLETF rats.

3.4 Effects of LBP-4a Administration on 2-deoxyglucose Uptake in Adipocytes from OLETF Rats

As shown in Fig.2(B), LBP-4a effectively stimulated 2-DG uptake into rat adipocytes ($P<0.01$) to a level comparable with that elicited by insulin. To determine whether the effects of LBP-4a on glucose transport required PI3K and p38 MAPK activities, wortmannin (1μmol/l) and SB203580 (10μmol/l) were added before insulin treatment,

313 respectively. The results showed that wortmannin and SB203580 inhibited
314 insulin-induced glucose uptake in LBP-4a-treated adipocytes (42±3% over
315 non-wortmannin, $P<0.01$; 30±1% over non-SB203580, $P<0.01$).

316 ***3.5 Effects of LBP-4a on GLUT4 Protein Levels in Adipocytes***

317 To study the mechanism of LBP-4a on IR, western blot analysis of GLUT4 protein
318 levels in adipocytes was carried out. GLUT4 protein level did not significantly differ
319 among the control (Con), insulin (Ins) and LBP-4a groups (Fig.3(A)).

320 ***3.6 Effects of LBP-4a on GLUT4 Translocation***

321 The aforementioned results suggested that LBP-4a-treated causes the translocation or
322 an increase in the intrinsic activity of GLUT4 glucose transporters in OLETF rats. To
323 further test this concept, we performed GLUT4 translocation assays on membrane
324 fractions from epididymal adipocytes (Fig.3 (B)). In the LBP-4a-treated epididymal
325 adipocytes, there was an increase of GLUT4 in the PM and a corresponding decrease
326 of GLUT4 in the LDM. The results indicated that LBP-4a-treatment caused the
327 transporter translocation to the PM. The level of GLUT4 protein present in the PM of
328 the LBP-4a treated cells was much higher than that of the insulin-treated cells ($P<$
329 0.05).

330 ***3.7 LBP-4a Stimulates p38 MAPK Phosphorylation and Kinase Activity***

331 We next investigated whether the LBP-4a-induced GLUT4 activity was accompanied
332 by p38 activity. Activation of p38 MAPK requires phosphorylation on tyrosine and
333 threonine residues. Insulin reportedly increases the phosphorylation of p38 MAPK in
334 rat skeletal muscle and 3T3-L1 adipocytes[14]. It is unknown whether insulin-mimetic

335 agents, such as LBP-4a, also cause an increase in p38 MAPK phosphorylation. Figure
336 4A and B showed that LBP-4a led to phosphorylation level of p38
337 MAPK(1.8±0.3-fold, $P<0.01$) was increased compared with control.

338 ***3.8 Activation of LBP-4a on both p38 MAPKα and p38 MAPKβ Isoforms***

339 Figure 4 showed that LBP-4a stimulated the phosphorylation of p38 MAPK. p38
340 MAPK consists of four isoforms. Here we determined which p38 MAPK isoforms
341 were activated by LBP-4a and insulin in isolated epididymal adipocytes**.** Although rat
342 adipocytes expressed three isoforms of the enzyme (α, β and γ), immunoprecipitating
343 antibodies were available only for α and β. Each of these proteins was
344 immunoprecipitated using isoform-specific antibodies, and the ability of the
345 immuno-isolated enzymes to phosphorylate ATF-2 was determined. The results
346 demonstrated that LBP-4a-treatment caused a 1.9±0.8-fold increase in p38 MAPKα
347 activity ($P<0.01$) and a 1.8±0.4-fold increase in p38 MAPKβ activity ($P<0.01$).
348 Similarly, insulin caused a 2.1±0.6 -fold increase in p38 MAPKα activity and a 2.4
349 ±0.5-fold increase in p38 MAPKβ activity (Fig. 5).

350 Addition of 10 μmol/L SB203580 directly to the immunoprecipitates inhibited
351 the activities of p38 MAPKα and p38 MAPKβ (SB203580+LBP-4a 0.7±0.1 -fold
352 vs.control, SB203580+insulin 0.8±0.2 -fold vs.control; SB203580+LBP-4a
353 0.6±0.1-fold vs.control, SB203580+ insulin0.7±0.2 -fold vs.control), confirming that
354 the kinase activity measured was due to p38 MAPK.

355 ## 4. Discusstion

356 It was reported that many edible plant polysaccharides could reduce the high

357 blood-glucose level[15]. Some constituents of *Lycium barbarum* fruits had been
358 chemically investigated, especially *Lycium barbarum* polysaccharide (LBP)
359 components. Five polysaccharides (LBP1-LBP5) were isolated and structurally
360 elucidated[16,17]. In this study, we evaluated the constituent of LBP-4a. We found that
361 the administration of LBP-4a to OLETF rats significantly reduced blood-glucose and
362 insulin levels and increased glucose infusion rate (GIR). The results of glucose
363 tolerance test indicated that LBP-4a could improve abnormal glucose tolerance in
364 OLETF rats. The above results suggested that LBP-4a had glucose-moderating action
365 and could ameliorate oral glucose tolerance and improve IR.

366 Being a rate-limiting step in glucose metabolism, the expression and function of
367 the GLUT4 had been extensively studied and found to be tightly regulated at protein
368 levels[18]. We assessed the effect of LBP-4a on GLUT4 levels in epididymal
369 adipocytes obtained from OLETF rats. The results of the present study showed that
370 GLUT4 protein level did not significantly differ among the Con, Ins and LBP-4a
371 groups (Figure 3A); however, LBP-4a increased surface exposure of GLUT4 in
372 adipocytes from OLETF rats, and Figure 3B showed a greater GLUT4 translocation
373 effect of LBP4a than that of insulin. This seemed to be contradictory to the findings of
374 Figure 2B and Table 1, which showed a lesser 2-DG uptake and GIR in the LBP-4a
375 group. This demonstrated that both LBP-4a and insulin could induce GLUT4
376 translocation to the cell surface in adipocytes, but the effect of glucose control was
377 different. It remained to be studied further whether there were other factors being
378 involved. In this study, we needed to further discuss other major mechanisms of

379 LBP-4a in improving glucose control. It was now apparent that increased plasma
380 membrane glucose transporter content was insufficient to fully account for the
381 insulin-stimulated elevation in glucose uptake. It had been proposed that
382 insulin-stimulated glucose transport might include changes in the intrinsic activity of
383 GLUTs[19]. Similarly, in cultured cells, glucose uptake could be stimulated by protein
384 synthesis inhibitors, such as anisomycin, without any increase in cell surface glucose
385 transporters[20]. In addition, there were reports that glucose uptake could be reduced
386 despite normal GLUT4 translocation [21]. These studies suggested that the intrinsic
387 activity of cell surface glucose transporters might be regulated by insulin and other
388 agents that stimulated glucose uptake. We had known that four inhibitors of p38
389 MAPK reduced insulin-stimulated glucose uptake without altering the translocation of
390 glucose transporters[22]. These results suggested that p38 MAPK might contribute to
391 the regulation of the intrinsic activity of glucose transporters. It was worth paying
392 more attention to whether LBP-4a could modulate the intrinsic activity of cell surface
393 glucose transporters.

394 LBP-4a stimulated 2-deoxyglucose uptake in isolated adipocytes. The stimulation
395 by LBP-4a was partly blocked by wortmannin, an inhibitor of PI3-K, and SB203580,
396 an inhibitor of p38 MAPK. In the present study, we examined the effect of LBP-4a
397 and insulin on the phosphorylation and activation of different isoforms of p38 MAPK
398 in adipocytes of OLETF rats. LBP-4a increased the kinase activities of the p38
399 MAPKα and β isoforms. Finally, we explored the effect of SB203580 on GLUT4
400 transporter activity. The results suggested that the enhanced glucose uptake by

LBP-4a in adipocytes might result from some main intracellular mediators of the insulin signaling pathway, specifically PI3-K and p38 MAPK. In adipocytes, the inhibition of LBP-4a-stimulated glucose uptake caused by wortmannin was similar to that of insulin stimulation. The p38 MAPK inhibitor, SB203580, led to a similar situation. It was possible that the p38 MAPK was activated to phosphorylated p38 MAPK and then p-p38 phosphorylated and increased the intrinsic activity of GLUT-4.

All of the above findings supported the hypothesis that stimulation of glucose transport consisted of at least two contributory mechanisms: translocation of GLUT4 to the plasma membrane and stimulation of their intrinsic activity. We directly demonstrated for the first time that LBP-4a improved IR through increasing the translocation and activity of GLUT4 in adipocytes.

5. Conclusion

LBP-4a improved IR via translocation and activation of GLUT4 in OLETF rats, and PI3-K and p38 MAPK activities contributed to these effects.

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Conflict of Interest statement

The authors declare that they have no conflict of interest.

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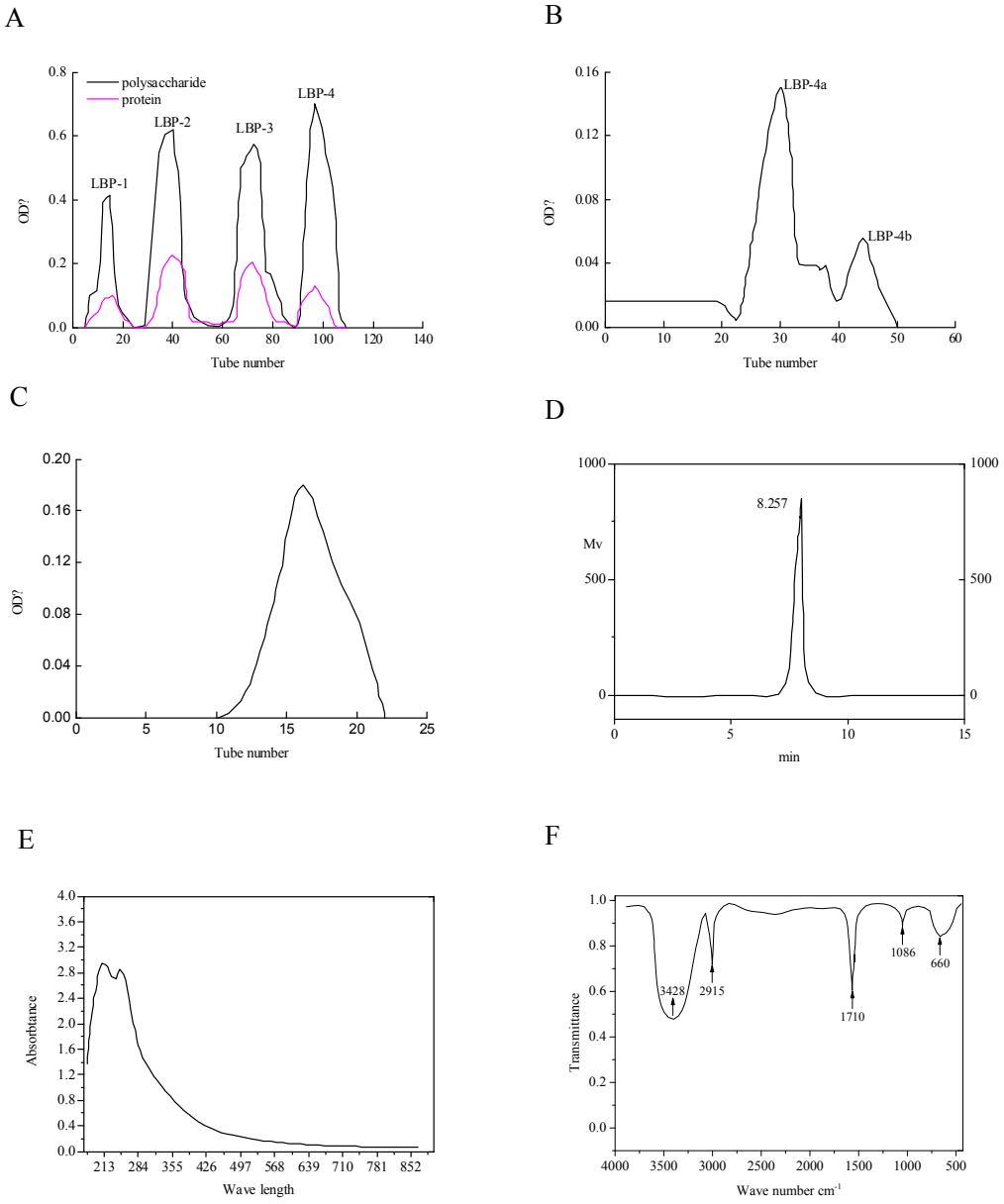
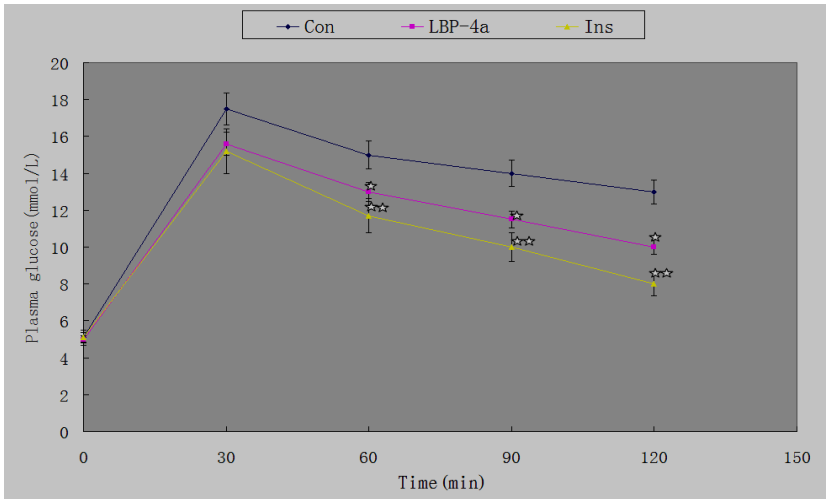


Fig. 1. Isolation, purification and characterization of LBP-4a. A: Chromatographic isolation of LBP on DEAE-cellucose (OH^-) column. B: Chromatographic isolation of LBP-4 on Sephadex G-100 column. C: Chromatography of LBP-4a on Sephadex G-100 column. D: High-performance gel filtration chromatograms of molecular weight in LBP-4a .E: The UV spectra of LBP-4a. F: The IR spectra of LBP-4a.

534 **A**



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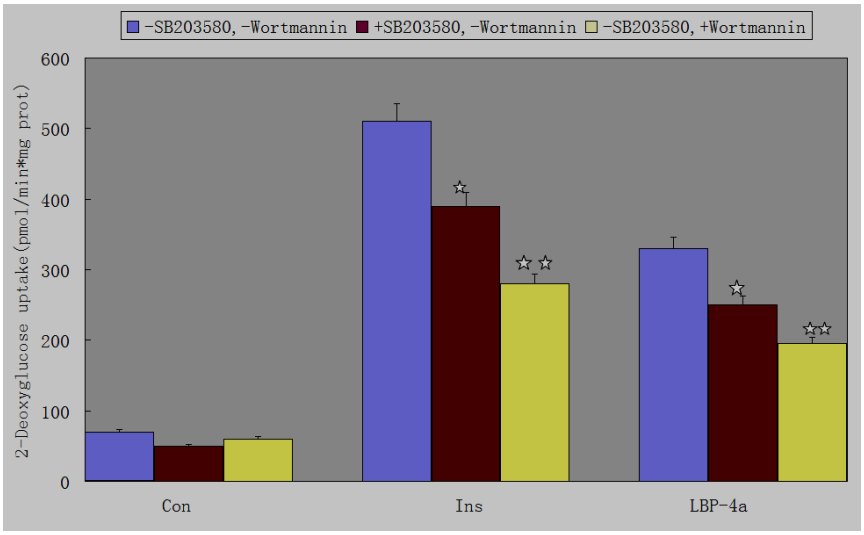
536 **B**



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539 **Fig.2. Effects of LBP-4a treatments on glucose levels in OLETF rats and SB203580 and wortmannin on**
540 **glucose uptake in isolated adipocytes (A)Effect of LBP-4a treatments on glucose levels in OLETF rats. A**
541 **blood sample was collected by tail bleeding, and respective treatments were administered. Blood samples**
542 **were extracted at the times indicated in the figure. Glucose levels were quantified by a glucometer. The data**
543 **are the mean± S.D. of six animals. $^{\star}P<0.05$ and $^{\star\star}P<0.01$ vs. control. (B)LBP-4a increased glucose**
544 **uptake and the effects of SB203580 and wortmannin on LBP-4a-stimulated glucose uptake in isolated**
545 **adipocytes. Cells were incubated for 20 min with or without 1 µmol/l wortmannin and 10 µmol/l of**
546 **SB203580 before stimulation with insulin (13.9 mU /mL) for 30 min. 2-Deoxyglucose glucose uptake was**
547 **determined over a 5-min period. Results are the mean ± S.D. at least three different experiments, n=3.$^{\star}P <$**
548 **0.05, $^{\star\star}P < 0.01$ compared with non-SB203580 and non- wortmannin respectively.**

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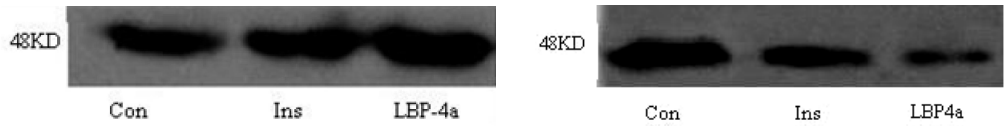
A

B PM LDM



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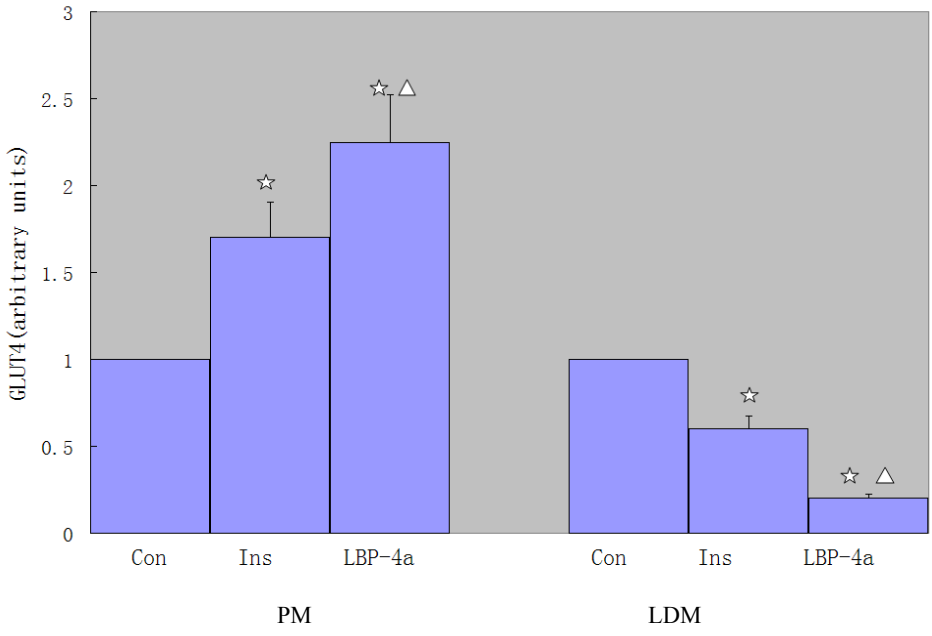
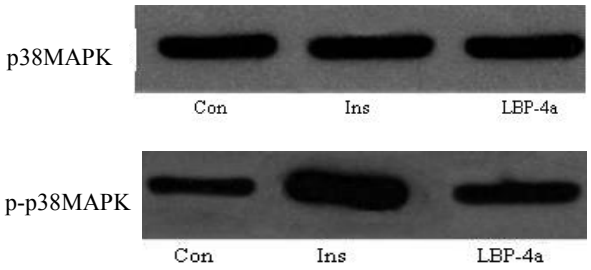


Fig.3. Effect of LBP-4a on GLUT4 in epididymal adipocytes of OLETF rats. (A) The amounts of GLUT4 in the total membranes isolated from epididymal adipocytes of OLETF rats.(B) A representative western blot of GLUT4 in PM and LDM fractions of isolated epididymal adipocytes from LBP-4a-treated and untreated OLETF rats in the absence or presence of 1.39 mU /mL insulin. Membrane proteins (20 μg) were loaded into each lane. (C) Quantification of GLUT4 in PM and LDM by densitometer scanning within the linear range and quantitated using the computer software. The quantitated values represent the mean± S.D. at least three different experiments, n=3. All values were expressed relative to control GLUT4, which was assigned a value of 1. ☆$P < 0.05$ comparing the levels to control. △$P < 0.05$ comparing the levels to insulin.

A



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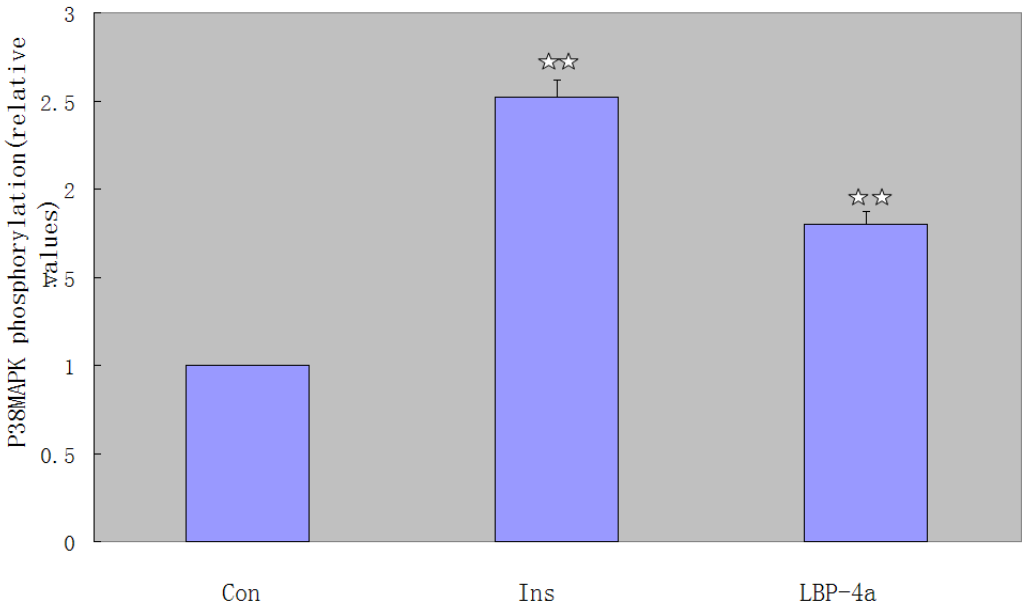

Fig.4. p38 MAPK phosphorylation by LBP-4a. A: Representative immunoblot. The immunoblot shown was stripped of bound antibodies and then reprobed for p38 MAPK. The specific phosphorylation site of p38MAPK was Thr180/Tyr182. B: Immunoblots were scanned within the linear range and quantitated using the computer software. The quantitated values represent the mean± S.D. at least three different experiments, n=3. All values were expressed relative to control p38 MAPK phosphorylation, which was assigned a value of 1. ☆☆*P* < 0.01 compared with control.

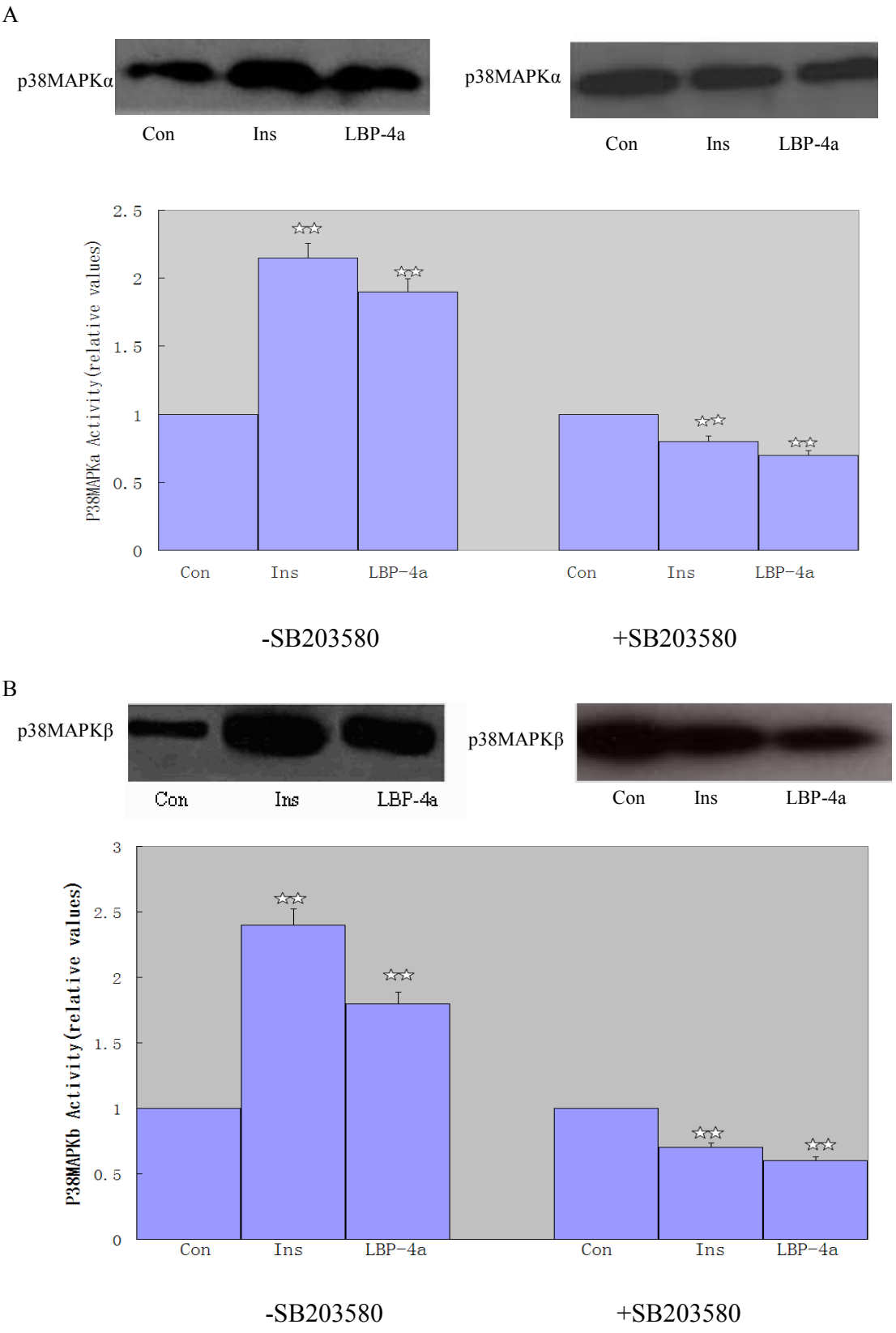

Fig.5. Activation of p38 MAPKα and β isoforms by LBP-4a. p38 MAPK α and β were immunoprecipitated, and kinase activity was determined by an in vitro kinase assay using ATF-2 as substrate. The quantitated values represent the mean± S.D. of three experiments. All values were expressed relative to control, which was assigned a value of 1. ☆☆*P* < 0.01 compared with control.

LBP-4a

PI3-Kinase P38 MAPK

GLUT4translocation GLUT4 activation

Stimulation of glucose uptake

Improving IR

Stimulation of glucose transport by LBP-4a consisted of two events: PI3-Kinase-dependent translocation and p38 MAPK-dependent stimulation intrinsic activity of GLUT4.