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Kazinol B from *Broussonetia kazinoki* improves insulin sensitivity via Akt and AMPK activation in 3T3-L1 adipocytes

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ABSTRACT

In this study, we evaluated the insulin-sensitizing effect of flavans purified from *Broussonetia kazinoki* Siebold (BK) on 3T3-L1 adipocytes. Among the tested compounds, kazinol B enhanced intracellular lipid accumulation, gene expression of proliferator-activated receptorγ (PPARγ) and CCAAT/enhancer binding protein-alpha (C/EBPα), and consistently induced PPARγ transcriptional activation. To further investigate the insulin-sensitizing effect of kazinol B, we measured glucose analogue uptake by fully differentiated adipocytes and myotubes. Kazinol B increased 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) uptake by cells by upregulating the gene expression and translocation of glucose transporter 4 (GLUT-4) into the plasma membrane in adipocytes. Kazinol B stimulated the gene expression and secretion of adiponectin, which is associated with a low risk of types 1 and 2 diabetes mellitus. We also suggested the mechanism of the antidiabetic effect of kazinol B by assaying Akt and AMP-activated protein kinase (AMPK) phosphorylation. In conclusion, kazinol B isolated from BK improved insulin sensitivity by enhancing glucose uptake via the insulin-Akt signaling pathway and AMPK activation. These results suggest that kazinol B might be a therapeutic candidate for diabetes mellitus.

1. Introduction

Over 29 million people suffer from diabetes mellitus (DM) in United States, and type 2 DM accounts for >90% of DM cases. Type 2 DM patients have a defect in insulin utilization in metabolic organs and tissues, causing resistance to insulin. Insulin resistance leads to a high blood glucose level accompanied by high blood pressure, high cholesterol or triglyceride levels. It is thus associated with chronic diseases such as obesity, hypertension, atherosclerosis, liver failure, and certain cancers. Glucose transport into metabolic organs and tissues is required to maintain a normal blood glucose level in DM patients, and is an important strategy for diabetes treatment.

The insulin-Akt/PKB (protein kinase B) and AMP-activated protein kinase (AMPK) signaling pathways have been reported to mediate insulin utilization. In adipocytes, insulin-mediated glucose uptake is dependent on GLUT4 expression and translocation [1].

Activation of the insulin and AMPK signaling pathways cooperatively triggers translocation of glucose transporter 4 (GLUT4) to the plasma membrane and subsequently increases glucose uptake by adipocytes. In fasting-induced insulin-resistant mammals, AMPK-evoked translocation of GLUT4 contributes to insulin signaling [2]. Metformin, an antidiabetic drug, regulates GLUT4 translocation by regulating Cbl and Cbl-associated protein (CAP) signals via AMPK in 3T3-L1 adipocytes [3]. Plant-derived flavonoids have been reported to modulate GLUT2 expression or GLUT4 translocation via phosphoinositide 3-kinase (PI3K)/Akt in various cell and animal models [4].

Paper mulberry, *Broussonetia kazinoki* Siebold (BK), exhibits various pharmacological activities, including inhibition of atopic dermatitis-like response [5], an anticancer effect via inhibition of angiogenesis [6] and production of reactive oxygen species [7], an anti-inflammatory effect [8], depigmenting effect [9] and stimulation of myoblast differentiation [10]. Interestingly, an extract of BK showed antidiabetic and antihyperglycemic potency in diabetic rats [11]. Also, Shibano et al. reported alkaloids as inhibitors of glycosidase, implying their therapeutic potential for diabetes [12]. We reported that a flavan compound from BK, kazinol U, protected pancreatic β-cells against cytokine-induced toxicity [13], but the insulin-sensitizing potential of compounds from BK has not been studied to date.

In a previous study, we reported kazinol B, an isoprenylated flavan, to be an inhibitor of nitric oxide production in LPS-induced macrophages [8]. As chronic inflammation is associated with the pathogenesis

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of obesity and insulin resistance, the anti-inflammatory potency of kazinol B might be useful for the treatment of diabetes. In this study, we evaluated the antidiabetic potential of kazinol B in mouse 3T3-L1 preadipocytes. Kazinol B promoted glucose uptake by adipocytes and myotubes. Furthermore, we found that Akt and AMPK activation are responsible for the insulin-sensitizing effect of kazinol B.

2. Materials and methods

2.1. Purification of kazinol B from BK

Root of *B. kazinoki* was collected from Goesan city, Chungbuk province, Korea in 2014, and a voucher specimen (No. SPH 14001) was deposited in the herbarium of Sookmyung Women's University. Air-dried root bark (0.5 kg) was extracted with methanol and evaporated to dryness. The extracts were dissolved in water and successively partitioned with n-hexane, ethyl acetate and chloroform. Kazinol B (13 mg) was purified from the ethyl acetate soluble fraction (25 g) as described previously [14]. The purity and structure were confirmed by ^{1}H NMR spectroscopy.

2.2. Cell culture and preadipocyte differentiation

Mouse 3T3-L1 preadipocytes (obtained from the American Type Culture Collection, Manassas, VA) were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (WelGENE, Daegu, Korea) supplemented with 10% newborn calf serum (Gibco BRL Life Technology, Grand Island, NY, USA) in a 5% CO_2 atmosphere. Two days after cells reached confluence (differentiation day 0), the medium was replaced with medium containing MDI mixture (1 μg/ml isobutyl-methylxanthine, 1 μM dexamethasone, and 1 μg/ml insulin) (Sigma, St. Louis, MO). After two days (differentiation day 2), cells were supplemented with insulin-containing DMEM. During differentiation, cells were maintained by replenishing with new insulin-containing medium every 2 days.

C2C12 myoblasts (kindly provided by Prof. GU Bae, Sookmyung Women's University, Seoul, Korea) were cultured in growth medium containing 15% FBS. Cells at near confluence were cultured in differentiation medium (DMEM containing 2% horse serum) until myotube formation was observed (normally at 2–3 days of differentiation).

2.3. MTT assay and ORO staining of lipid drop formation in 3T3-L1 cells

To assess the effect of kazinol B on preadipocyte viability, 3T3-L1 cells were plated in 96-well plates and incubated for 72 h. The cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Sigma, St. Louis, MO) solution at 37 °C. After 3 h, the MTT solution was removed and 100 μl of DMSO were added to extract MTT formazan crystals. The absorbance at 570 nm was measured using a GloMax®-Multi Microplate Multimode Reader (Promega, Madison, WI).

The accumulated lipid content of differentiated 3T3-L1 cells was evaluated by Oil Red O (ORO) (Sigma, St. Louis, MO) and images were obtained on day 8 (D8) of differentiation. Differentiated adipocytes were washed with phosphate-buffered saline (PBS) and fixed in 10% formalin, and then stained with 0.5% ORO in 60% isopropanol. To quantify intracellular lipid storage, ORO inside cells was extracted with 4% Nonidet P-40 (NP-40) in isopropanol. The absorbance of the extract solution was measured at 520 nm using a microplate reader. Intracellular lipid accumulation in 3T3-L1 cells was photographed using an inverted phase-contrast microscope (TH4, Olympus, Tokyo, Japan).

2.4. Glucose uptake

For glucose uptake assay, fully differentiated 3T3-L1 adipocytes and C2C12 myoblasts were pre-incubated for 12 h in serum-free and low-glucose DMEM, followed by treatment with kazinol B for 24 h. After 1 h stimulation with insulin, 20 μM of the fluorescent glucose analogue 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG) (Invitrogen, Carlsbad, CA, USA) was added, and the cultures were incubated for a further 1 h. Fluorescence retained in the cell monolayer was measured using a microplate reader set at an excitation wavelength of 465 nm and an emission wavelength of 540 nm.

2.5. Adiponectin secretion

Adiponectin secretion was evaluated according to the manufacturer's instructions (Mouse Adiponectin/Acrp30 DuoSet, R & D Systems, Minneapolis, MN, USA). 3T3-L1 preadipocytes were differentiated in MDI medium containing kazinol B or rosiglitazone. Conditioned medium was collected at D5 and the adiponectin concentration was measured.

2.6. PPAR reporter assay

CV-1 cells were transiently transfected with plasmid mixture containing PPARγ expression vector and tk-PPRE-luciferase (Luc) vector for 6 h, and then treated with kazinol B for 24 h. The β-galactosidase reporter gene is used as a control for transfection efficiency in this reporter assay system. The luciferase activity in cell lysates was measured using a luciferase assay system (Promega, Madison, WI) and β-gal activities were measured as the absorbance at 410 nm using a microplate reader. All constructs were kindly provided by Dr. Ronald M. Evans at The Salk Institute (La Jolla, CA).

2.7. RNA extraction and quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR)

Differentiated adipocytes with MDI medium containing kazinol B were lysed with TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) for RNA preparation at D5. The cDNA was amplified from total RNA to estimate gene expression level during adipocyte differentiation by RT-qPCR. PCR reactions were performed using SYBR® Green PCR Master Mix and an Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA, USA). mRNA levels were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as an internal control. Primers used are shown in Table 1.

2.8. Western blot analysis and preparation of plasma membrane fraction

Western blot analysis was performed to determine the effect of kazinol B on PPARγ, C/EBPα and GLUT4 protein levels and phosphorylation of Akt and AMPK in 3T3-L1 cells. 3T3-L1 preadipocytes were differentiated in MDI medium in the presence or absence of kazinol B as described above. At D5, cells were collected and resuspended in lysis buffer (25 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail (Calbiochem, Darmstadt, Germany)). Protein was electrophoresed in SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was probed with primary

Table 1
Oligonucleotide primer sequences used for the RT-qPCR analysis.

Gene name	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
ARγ2	AACTCTGGGAGATTCTCCTGTTGA	GAAGTGCTCATAGGCAGTGCAT
C/EBPα	TGCTGGAGTTGACCAGTAC	AAACCATCCTCTGGGTCTCC
Adiponectin	TGTAGGATTGTCAGTGGATCTG	GCTCTTCAGTTGTAGTAACGTCATC
GLUT4	GGGTCCTTACGTCTTCCTTCT	CCTCTGGTTTCAGGCACTTT
GAPDH	TGCACCACCAACTGCTTAG	GGCATGGACTGTGGTC TGAG

PPARγ2, peroxisome proliferator activated receptor subtype gamma 2; C/EBPα, CCAAT/enhancer binding protein-alpha; GLUT4, glucose transporter subtype 4; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.