Allium Hookeri Extract Enhances Glucose Uptake through GLUT4 Up-regulation in 3T3-L1 Cells

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Diabetes mellitus is associated with insulin resistance, which leads to down-regulation of insulin signaling and the decreased glucose uptake. Adipocytes are sensitive to insulin, and closely implicated in insulin resistance and diabetes. Insulin stimulates differentiation of preadipocytes to adipocytes, and increases glucose transport. Allium species have been used as traditional medicine and health-promoting foods. Allium hookeri (A. hookeri) is reported to improve the pancreatic β-cell damage and exhibit pancreatic anti-inflammatory activity in streptozotocin-induced diabetic rats. We investigated whether A. hookeri extract (AHE) may stimulate glucose uptake in adipocytes through increasing insulin sensitivity. AHE enhanced fat accumulation, a differentiation biomarker, under the partial induction of differentiation by insulin. PPARγ, a transcription factor highly expressed in adipocytes, promotes adipocyte differentiation and insulin sensitivity. AHE increased the differentiation of preadipocytes through up-regulation of PPARγ. The activation of PPARγ increases the GLUT4 expression during adipocyte differentiation. GLUT4 is responsible for glucose uptake into the adipocytes. AHE increased the expression of GLUT4 in adipocytes, and subsequently enhanced the insulin-stimulated glucose uptake. These results suggest that AHE promotes adipocyte differentiation through activation of PPARγ, and leads to enhance glucose uptake in adipocytes along with GLUT4 up-regulation. Thus, AHE may be effective for the insulin-sensitizing and anti-diabetic activities.

Key words: Allium hookeri, adipocyte differentiation, glucose uptake, PPARγ, 3T3-L1 cells

Introduction

Adipocytes have been studied as a potential target for diabetes mellitus as well as obesity [9, 14]. Dysfunctional adipocytes induce insulin resistance and inflammation [2]. Insulin is an essential regulator for stimulating the adipocyte differentiation which is induced through transcription factors such as peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α [1, 5, 10]. PPARγ plays the major role in the adipogenic transcriptional cascade, and activates the expression of various genes involved in glucose and lipid metabolism [5, 13, 20]. PPARγ activates expression of glucose transporter 4 (GLUT4) during adipogenesis which is responsible for glucose uptake into adipocytes [13, 20].

Allium species have been used as traditional medicine and health-promoting foods [3, 4, 11]. Allium hookeri (A. hookeri), a member of Liliaceae family, is widely found in Sri Lanka, India, China and Bhutan, and has anti-oxidant and anti-inflammatory activities [3, 7, 12]. Water extract of A. hookeri protected oxidative stress-mediated inflammatory responses and pancreatic β-cell damage in streptozotocin-induced diabetic rats [12]. In addition, A. hookeri leaf or root decreased blood glucose level, and increased plasma insulin level in type 2 diabetic mice [8].

In this study, we investigated whether A. hookeri extract may modulate adipocyte differentiation under sub-optimal concentrations of insulin, and elucidated its mechanism for increasing insulin sensitivity.

Materials and Methods

Materials

3T3-L1 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco’s Modified Eagle’s Medium (DMEM), bovine calf serum (BCS) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Insulin and bovine serum...
albumin (BSA) were obtained from Roche Diagnostics (Mannheim, Germany). Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX) and Oil Red O were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2-deoxy-D-[1-3H(N)] glucose was obtained from American Radiolabeled Chemicals, Inc. (Saint Louis, MO, USA). Antibodies against PPARγ, GLUT4 and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

**Preparation of AHE**

All parts of *A. hookeri* were harvested before it was growing 30 cm by height, and then dried. Ten kg of *A. hookeri* was extracted with 27 liters of hot water overnight. The AHE was sterilized with 0.2 μm Millipore filter kit (Merck Millipore; Darmstadt, Germany). The AHE at the concentrations of 10-50 mg/ml was directly used for further study.

**Cell culture and adipocyte differentiation**

3T3-L1 preadipocytes were cultured and differentiated into adipocytes using a modified protocol [15]. Briefly, 3T3-L1 preadipocytes originating from Swiss mouse embryos were cultured in DMEM containing 10% BCS at 37°C in a 5% CO₂ incubator. To induce differentiation, 2 day post-confluent preadipocytes were cultured for 2 days in differentiation medium containing 0.1 μg/ml insulin, 0.5 mM IBMX, 1 μM dexamethasone and 10% FBS. The medium was then changed to DMEM containing 0.1 μg/ml insulin and 10% FBS, and cells were cultured for a further 2 days. Subsequently, cells were cultured in DMEM supplemented with 10% FBS for an additional 2 days.

**Oil Red O staining**

After induction of adipocyte differentiation, cells were washed with phosphate-buffered saline (PBS), fixed at room temperature with 10% formalin for 1 hr, stained at room temperature with Oil Red O for 1 hr, and washed three times with distilled water. For quantitative analysis, Oil Red O dye was dissolved in isopropanol and the optical density was measured at 490 nm using a microplate reader (Molecular Devices, LLC; Sunnyvale, CA, USA).

**Glucose uptake assay**

Adipocytes were incubated in DMEM containing 0.2% BSA for 4 hr and washed two times with Krebs-Ringer Hepes buffer (KRH buffer; 136 mM NaCl, 4.7 mM KCl, 1.25 mM CaCl₂, 1.25 mM MgSO₄, 20 mM Hepes, pH 7.4). The cells were incubated in KRH buffer with 10 ng/ml insulin at 37°C for 15 min. Glucose uptake was initiated by addition of 0.5 μg/ml 2-deoxy-D-[1-3H(N)] glucose as the final concentration in KRH buffer. After 10 min, the cells were quickly washed twice with ice-cold KRH buffer to terminate the reaction. The cells were lysed with 0.5 N NaOH, and the radioactivity was counted using a liquid scintillation analyzer (PerkinElmer, Inc.; Waltham, MA, USA).

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**Fig. 1. Enhancing effect of AHE on adipocyte differentiation.** Adipocyte differentiation was induced with a mixture of insulin (0.1 μg/ml), IBMX (0.5 mM) and dexamethasone (1 μM). 3T3-L1 cells were treated with AHE (0, 10, 30 or 50 mg/ml) every other day during the first 4 days of differentiation. (A) On day 6, cells were stained with Oil Red O, and visualized under a light microscope (×100). (B) Intensities of Oil Red O staining were quantified. Results are expressed as mean ± SD of triplicate experiments. Statistical significance: **p<0.01.
Western blot analysis

3T3-L1 cells were collected and lysed in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM dithiothreitol, and a protease inhibitor cocktail tablet (Roche Diagnostics; Mannheim, Germany). The total protein concentration of the lysates was determined with the BCA Protein Assay Reagent (Pierce). Proteins in the lysates were separated on a 10% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (GE Healthcare Life Sciences; Piscataway, NJ, USA). Membranes were blocked in 5% BSA overnight at 4°C and then incubated overnight at 4°C with the following primary antibodies: PPARγ, GLUT4 and β-actin. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies overnight at 4°C. The bands were visualized with enhanced chemiluminescence (Amersham Pharmacia Biotech; Buckinghamshire, UK) and exposed to X-ray film (Eastman Kodak; Rochester, NY, USA).

Statistical analysis

All values are presented as mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance with Newman-Keuls multiple comparison test. \( P < 0.05 \) was considered statistically significant.

Results and Discussion

AHE enhanced adipocyte differentiation in 3T3-L1 cells

Insulin stimulates differentiation of preadipocytes to adipocytes, and increases glucose transport and lipid synthesis [1, 17]. Adipocytes are sensitive to insulin and implicated in insulin resistance [16]. In this study, we examined whether AHE modulates the differentiation of 3T3-L1 preadipocytes. Differentiation of preadipocytes was induced by 0.1 μg/ml insulin plus 0.5 mM IBMX and 1 μM dexamethasone, and the cells were simultaneously treated with AHE (0, 10, 30 or 50 mg/ml) during the first 4 days of differentiation. AHE promoted adipocyte differentiation under sub-optimal concentrations of insulin (Fig. 1). The fat content of adipocytes treated with 10, 30 or 50 mg/ml AHE was increased by approximately 0.6-, 1.8- or 2.4-fold, respectively, compared with controls. These results suggest that AHE may promote the adipocyte differentiation by increasing insulin sensitivity.

AHE increased PPARγ expression in adipocytes

PPARγ is highly expressed in adipocytes, and promotes adipocyte differentiation and insulin sensitivity [5, 13, 18]. AHE treatment significantly enhanced PPARγ expression in adipocytes in a concentration-dependent manner (Fig. 2). The expression of PPARγ in adipocytes treated with AHE at 10, 30 or 50 mg/ml was increased by approximately 0.7-, 1.5- or 2.4-fold, respectively, compared with control culture without AHE. These results suggest that AHE increases the differentiation of 3T3-L1 preadipocytes by activating PPARγ.

AHE enhanced insulin-stimulated glucose uptake in adipocytes

PPARγ increases the GLUT4 expression during adipocyte differentiation which is followed by enhancing glucose uptake [13, 20]. We examined whether AHE enhances the glucose uptake in adipocytes consistent with the increased adipocyte differentiation by AHE. The glucose uptake was measured in adipocytes exposed to AHE during adipo-
AHE significantly enhanced insulin-stimulated glucose uptake in 3T3-L1 adipocytes (Fig. 3A). In particular, 50 mg/ml AHE increased the insulin-stimulated glucose uptake in adipocytes by approximately 56% compared with control culture without AHE. Similarly, A. hookeri significantly reduced blood glucose level, and increased plasma insulin level in the type 2 diabetic mice [8]. GLUT4 is a major insulin-responsive glucose transporter, which is responsible for glucose uptake into the adipocytes [1, 6, 19]. AHE significantly increased the GLUT4 expression in adipocytes (Fig. 3B). The expression of GLUT4 in adipocytes treated with AHE at 10, 30 or 50 mg/ml was increased by approximately 0.6-, 2.0- or 2.3 folds, respectively, compared with controls. These results indicate that AHE may enhance insulin-mediated glucose uptake in adipocytes by increasing GLUT4 expression during adipogenesis.

In conclusion, AHE promotes adipocyte differentiation through activation of PPARγ, and leads to enhance glucose uptake in adipocytes along with GLUT4 up-regulation (Fig. 4). Thus, AHE may be effective for the insulin-sensitizing and anti-diabetic activities.

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초록: GLUT4 상향조절을 통한 *Allium hookeri* 추출물의 3T3-L1 세포 내 포도당 흡수 증진 효과

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삼채 (*Allium species*)는 전통적인 약재나 건강 증진 식품으로 사용되어 왔다. 특히, *Allium hookeri* (*A. hookeri*)는 제 2형 당뇨병 모델 마우스에서 혈당 감소 효과가 보고되었다. 본 연구에서는 *A. hookeri* 추출물이 3T3-L1 세포에서 인슐린 민감성을 증진시키는지 시험하였다. 3T3-L1 지방세포분화가 불완전하게 유도되는 저농도의 인슐린 조건에서, *A. hookeri* 추출물은 세포 내 지방 함량을 증가시키고, 분화 유도 전사인자인 PPARγ의 발현을 상승시켰다. 또한, *A. hookeri* 추출물은 포도당 수송체 4(GLUT4)의 발현을 증가시킴으로써 세포 내 포도당 흡수 (glucose uptake)를 향상시켰다. 이러한 결과들은 *A. hookeri* 추출물이 인슐린 민감성을 증진시켜 PPARγ와 GLUT4를 활성화 하고, 세포 내 포도당 흡수를 촉진한다는 사실을 보여준다. 따라서, *A. hookeri* 추출물은 당뇨병의 예방 및 치료에 임상적으로 응용될 수 있을 것으로 생각된다.