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In vivo and in vitro application of black soybean peptides in the amelioration of endoplasmic reticulum stress and improvement of insulin resistance

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ABSTRACT

Aims: Hepatic endoplasmic reticulum (ER) stress plays a key role in the development of obesity-induced insulin resistance. This study evaluated the effects of peptides from black soybean (BSP) on ER stress and insulin signaling in vitro and in vivo.
Main methods: Using C2C12 myotubes or HepG2 cells, we evaluated the effects of BSP on the expression of proteins involved in insulin signaling and in the ER stress response in insulin-sensitive or insulin-resistant cells. BSP was given orally to *db*/*db* mice for 5 weeks to investigate its antidiabetic effects in vivo and the underlying mechanisms.
Key findings: BSP increased GLUT4 translocation and glucose transport in myotubes and stimulated Akt-mediated glycogen synthase kinase-3β (GSK-3β) and Foxo1 phosphorylation in HepG2 cells. BSP significantly restored the suppression of insulin-mediated Akt phosphorylation in insulin-resistant cells. BSP significantly inhibited the activation of ER stress-responsive proteins by thapsigargin. BSP also significantly reduced blood glucose and improved glucose tolerance in *db*/*db* mice. The serum lipid profile (triglyceride and high-density lipoprotein concentrations) improved concomitantly with the BSP-induced downregulation of hepatic fatty acid synthase expression in *db*/*db* mice. Consistent with the results observed in HepG2 cells, BSP downregulated the elevated hepatic ER stress response in diabetic mice concomitantly with an increased expression of phospho-Foxo1.
Significance: A peptide mixture, BSP, showed beneficial effects through multiple mechanisms involving the suppression of hepatic ER stress and restoration of insulin resistance, suggesting that it has potential as an antidiabetic agent.

Introduction

The growing morbidity of the disease cluster known as the metabolic syndrome has become a major threat to human health. Over the past decade, many studies have shown that obesity is associated with the activation of cellular stress signaling and inflammatory pathways associated with insulin resistance (Hirosumi et al. 2002; Uysal et al. 1997; Yuan et al. 2001).

The origin of the cellular stress that accompanies obesity is thought to be associated with disruption of homeostasis within the endoplasmic reticulum (ER). Because hepatic regulation of glucose homeostasis is the major factor controlling plasma glucose concentrations, the induction of hepatic ER stress in obesity induces insulin resistance. It has been reported that chronic obesity-induced metabolic stress disrupts ER function, which acts as a molecular sensor of the stress and induces insulin resistance (Nakatani et al. 2005; Özcan et al. 2004). In addition, molecular or chemical chaperones reduce ER stress and improve insulin resistance in type 2 diabetes (Ozawa et al. 2005; Özcan et al. 2006). Therefore, pharmacological agents that reduce ER stress with minimal adverse effects might become novel therapeutics for treating type 2 diabetes.

Nutritional intervention studies performed in animals and humans have shown that a soybean-based diet has beneficial health effects (Anderson et al. 1995; Bhathena and Velasquez 2002; Reynolds et al. 2006; Trujillo et al. 2005). Among the ingredients in a soybean diet, isoflavones are associated with improvements in type 2 diabetes and hyperlipidemia (Bhathena and Velasquez 2002; Cederroth et al. 2008; Crouse et al. 1999). On the other hand, several studies have shown that soy protein or soy peptide molecules also have biological effects on metabolic disorders (Kohno et al. 2006; Nishi et al. 2003; Song et al.

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2005). However, because of the unknown interactions among ingredients in a soy diet, it is difficult to conclude whether soy protein itself has beneficial effects on metabolic disorders (Sacks et al. 2006). Until now, the therapeutic potential of peptides derived from soybean protein isolates without isoflavones against diabetes was unknown.

We have previously reported that novel peptide mixtures derived from black soybean (BSP) inhibit the development of diet-induced obesity in mice by activating leptin-like signaling and AMP-dependent protein kinase (AMPK) (Jang et al. 2008). AMPK has multiple biological effects, including the regulation of intracellular glucose transport (Kola et al. 2006; Long and Zierath 2006). However, in our earlier studies, we did not investigate whether BSP affects insulin signaling, insulin resistance, or the molecular mechanisms involved in glucose control. Here, we examined whether BSP's antiobesity effect is involved in the amelioration of insulin resistance and hyperglycemia. We found that BSP increased Akt-mediated forkhead transcription factor, Foxo1, and glycogen synthase kinase-3β (GSK-3β) phosphorylation in insulin-sensitive and insulin-resistant cells and increased intracellular glucose transport in myotubes. BSP also has antidiabetic effects and inhibits the elevation of plasma triglyceride concentrations in diabetic (*db*/*db*) mice through multiple mechanisms, including the inhibition of hepatic ER stress. Our results suggest that BSP activates the insulin-signaling pathway and ameliorates ER stress, making it a potential therapeutic peptide for type 2 diabetes.

Materials and methods

Materials

Isoflavone-free BSP was obtained from Nong Shim Co., Ltd (Seoul, South Korea), as described previously (Jang et al. 2008). Primary antibodies against Akt, phospho-Akt(Ser^{473}), eukaryotic initiation factor 2 alpha (eIF-2α), phospho-eIF-2α(Ser^{51}), c-jun NH_2-terminal kinase (JNK), phospho-c-jun(Ser^{63}), Foxo1, phospho-Foxo1(Ser^{256}), p38 mitogen-activated protein kinase (MAPK) and phospho-p38 MAPK (Thr^{180}) (all from Cell Signaling Technology, Beverly, MA); insulin receptor substrate 1 (IRS-1) (Upstate Biotechnology, Lake Placid, NY); insulin, GSK-3β, phospho-GSK-3β, glucose transporter 4 (GLUT4), 78 kDa glucose-regulated/binding immunoglobulin protein (Grp-78) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) were purchased commercially. All other chemicals or solvents were of the highest grade and were purchased commercially.

Induction of insulin resistance or ER stress in HepG2 cells

Human hepatoma HepG2 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and glucose (5.5 mM) in a humid atmosphere containing 5% CO_2 at 37 °C. To induce insulin resistance to a high concentration of glucose (30 mM) in HepG2 cells, growth medium with normal glucose (5.5 mM) was switched to a hyperglycemic medium for 24 h (Zang et al. 2004). After the induction of insulin resistance, the cells were treated with insulin (10 nM, Sigma-Aldrich, St. Louis, MO) for 10 min. To induce ER stress, HepG2 cells grown in medium with normal glucose were treated with thapsigargin (1 μM, Sigma-Aldrich) for 4 h.

Measurement of 2-deoxyglucose transport

Proliferating C2C12 myoblasts (American Type Culture Collection, Manassas, VA) at 80% confluency were induced to differentiate into myotubes by switching the serum-enriched medium to differentiation medium (98% DMEM and 2% (v/v) horse serum), as described previously (Jang et al. 2008). Confluent cells differentiated in 60 mm dishes were incubated with serum-free media for 3 h and treated with glucose-free Krebs-phosphate buffer (20 mM HEPES, pH 7.4, 136 mM NaCl, 4.7 mM KCl, 1 mM $CaCl_2$, 0.95 mM NaH_2PO_4, 1 mM $MgSO_4$, 4.05 mM Na_2HPO_4 and 0.5% bovine serum albumin) containing insulin (100 nM) or BSP for 30 min. Cells were washed in warm glucose-free Krebs-phosphate buffer and incubated for 30 min at 37 °C in the same buffer containing 0.5 μCi/ml of ^{3}H-2DG (Amersham Radioactive Chemicals, UK) and 100 μM 2-deoxy-d-glucose (Sigma-Aldrich). Nonspecific glucose transport was measured in the presence of cytochalasin B (10 μM, Sigma-Aldrich), an inhibitor of the glucose transporter. The nonspecific uptake was subtracted from the total uptake. Cells were washed four times with ice-cold buffer on ice and solubilized in 0.1 N NaOH. An aliquot of each extract was neutralized for the estimation of radioactivity in a scintillation counter.

Analysis of GLUT4 translocation in subcellular membranes

To measure the plasma membrane translocation of GLUT4, confluent myotubes grown in 150-mm culture plates were treated with insulin (100 nM), metformin (1 mM, Sigma-Aldrich) or BSP for 30 min. Plasma membranes from myotubes were prepared using the methods described previously (Clancy and Czech 1990). Briefly, cells were scraped into 24 ml of ice-cold Buffer A (250 mM sucrose, 20 mM HEPES, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and were homogenized immediately with a Teflon pestle. Aliquots of homogenates were centrifuged at 250,000×*g* for 1.5 h to collect the total membranous proteins, which were then resuspended in Buffer B (20 mM HEPES, 1 mM EDTA, pH 7.4). The remaining homogenate was pelleted by centrifugation (16,000×*g* for 20 min), and the pellets were resuspended in 3 ml of Buffer B. The samples were applied to a sucrose cushion (1.12 M sucrose in Buffer B) and centrifuged at 100,000×*g* for 1 h. The plasma membranes were removed from the top of the sucrose cushion, resuspended in Buffer B and centrifuged at 30,000×*g* for 30 min. The plasma membranes were resuspended in Buffer B to about 5 mg protein/ml. The total and plasma membranous fractions were subjected to SDS-PAGE after measuring the protein content using a BCA protein assay kit (Pierce, Rockford, IL).

Immunoblotting

To prepare whole-cell lysates, cells were washed twice with ice-cold PBS containing Na_3VO_4 (1 mM), scraped into ice-cold lysis buffer (50 mM Tris–HCl, pH 7.2, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 0.1% SDS, and protease inhibitors), and sonicated to homogenize the cell suspension. The collected tissues of mice were homogenized in lysis buffer using a Teflon homogenizer. The cell debris of the homogenates was removed by centrifugation at 1000×*g* for 10 min, and aliquots of protein extracts (15–20 μg) were subjected to western blot analysis with an appropriate antibody. Immunoreactive bands were visualized by an enhanced chemiluminescence detection system (Pierce) and were analyzed using the image analysis software Bio 1D.

Animal experiments

The care of animals was conducted according to the Guide for Care and Use of Laboratory Animals of the National Institutes of Health of Korea. The study protocol was approved by the Animal Care and Handling Committee of Inha University, Incheon, South Korea. Seven-week-old male C57BL/KsJ *db*/*db* and age-matched lean *db*/$^+$ mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed individually in a temperature-controlled (22±2 °C) and humidity-controlled (45%–55%) room under a 12 h light (07:00–19:00)–12 h dark cycle with free access to food and water, as described previously (Jang et al. 2008; Ko et al. 2008). After 1 week of acclimation, mice were fed a chow diet and water for 5 weeks. During the experimental