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Ameliorative effect of panaxynol on the reduction in high-molecular-weight adiponectin secretion from 3T3-L1 adipocytes treated with palmitic acids

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ABSTRACT

Reduced plasma levels of the high-molecular weight (HMW) form of adiponectin, rather than total adiponectin levels, have been shown to be closely associated with various metabolic diseases including insulin resistance, type 2 diabetes, and cardiovascular disease. Therefore, we sought to explore active, naturally occurring compounds that promote the recovery of HMW adiponectin secretion suppressed by palmitic acid in our model. A total of 90 crude drug extracts were screened for the ability to augment HMW adiponectin secretion from 3T3-L1 adipocytes treated with palmitic acid. Panaxynol was isolated from *Saposhnikovia divaricata* as an active compound with HMW adiponectin promoting properties. Peroxisome proliferator-activated receptor-γ (PPARγ) agonists are reported to increase the secretion of HMW adiponectin, although the effects of panaxynol were found to be independent of PPARγ activation. When the underlying mechanisms were further examined, panaxynol was found to inhibit the palmitic-acid-induced downregulation of forkhead box O1 (FoxO1) protein, and the anti-lipotoxic effects were abolished by a FoxO1 inhibitor. Furthermore, CCAAT/enhancer-binding protein-α (C/EBPα) mRNA levels were also increased by panaxynol. Reactive oxygen species have critical roles in the reduction in HMW adiponection secretion by palmitic acid; however, panaxynol reduced this increase in reactive oxygen species generation, followed by reductions in markers of endoplasmic reticulum stress and inflammation. Taken together, these findings suggest that panaxynol ameliorates the impaired HMW adiponection secretion in adipocytes treated with palmitic acid by restoring FoxO1 expression, owing to inhibition of reactive oxygen species generation, in a PPARγ-independent manner.

1. Introduction

Adiponectin, an adipokine predominantly secreted by white adipose tissue, is critical to insulin sensitivity and energy homeostasis regulation and protective from almost all major obesity-associated diseases, including hypertension, atherosclerosis, and steatohepatitis (Maeda et al., 1996; Achari and Jain, 2017). Adiponectin is found in cells and plasma in three major forms: trimers, hexamers, and high-molecular weight (HMW) 12- to 18-mers (Pajvani et al., 2003). Among them, the HMW form plays important roles in the regulation of insulin signaling, and is closely associated with peripheral insulin sensitivity (Fisher et al., 2005). In patients with obese or type 2 diabetes, plasma levels of HMW adiponectin are decreased (Basu et al., 2007; Arita et al., 1999) and a reduction in HMW adiponectin levels, rather than total adiponectin levels, contributes to the etiology of obesity-associated diseases (Pajvani et al., 2004). Impaired HMW adiponectin secretion is ascribed mainly to a reduction in adiponectin production and multimerization. Transcription of the adiponectin gene is regulated by several transcription factors including peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer-binding protein α (C/EBPα), and forkhead box O1 (FoxO1) (Qiao et al., 2005; Qiao and Shao, 2006). In addition, the cellular formation of HMW adiponectin is also regulated by post-translational modifications. Endoplasmic reticulum (ER)-membrane-associated oxidoreductase 1-Lα (Ero1-Lα) (Qiang et al., 2007), its associated protein ERp44 (Wang et al., 2007), and disulfide bond A oxidoreductase-like protein (DsbA-L) (Liu et al., 2008) are involved in the multimerization and secretion of adiponectin, furthermore DsbA-L and Ero1-Lα are known to be upregulated by PPARγ activation (Liu and Liu, 2012).

A recent study revealed that plasma free fatty acid levels are significantly higher in patients with obesity or type 2 diabetes, and saturated fatty acids such as palmitic acid are causative mediators of chronic low-grade inflammation and insulin resistance (Funaki, 2009; Stahlman et al., 2012). Palmitic acids released from hypertrophied

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adipose tissue have been reported to promote the generation of inflammatory adipokines (Shi et al., 2006), and cause mitochondria dysfunction and reactive oxygen species generation, leading to ER stress (Jeon et al., 2012). Ultimately, as a result of lipotoxicity (Shehzad et al., 2012), phosphorylation of PPARγ by cyclin-dependent kinase 5 or signal-regulated kinase inactivates PPARγ transactivation (Choi et al., 2010; Kaplan et al., 2010), resulting in a reduction in both the transcription and multimerization of adiponectin.

Accordingly, a rationale exists for exploring the potential of therapeutic or preventive agents that increase adiponectin levels, and especially the synthesis and secretion of HMW adiponectin, in the setting of lipotoxicity (Liu and Liu, 2010). In this context, considering that PPARγ regulates the transcription and post-translational modification of adiponectin, PPARγ agonists appear to be suitable candidate agents. However, unfortunately, the PPARγ agonist thiazolidinediones (TZDs) have adverse effects such as weight gain, edema, congestive heart failure, and bone resorption. Thus, enhancing plasma HMW adiponectin levels, without the use of TZDs, might provide a safer, yet effective approach for the treatment of obesity-associated diseases. In this study, we sought to explore the effectiveness of naturally occurring compounds in reversing impaired HMW adiponectin secretion.

2. Materials and methods

2.1. Chemicals and reagents

3-Isobutyl-1-methylxanthine (IBMX), insulin, Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO). Non-essential amino acids, dexamethasone (DEX), and palmitic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum (FBS) and calf serum (CS) were purchased from Nichirei Biosciences Inc. (Tokyo, Japan) and SAFC Bioscience Inc. (Lenexa, KS), respectively. Rosiglitazone (PPARγ agonist) was obtained from Alexia Biochemicals (San Diego, CA). GW9662 (PPARγ antagonist) was obtained from Sigma-Aldrich (St. Louis, MO). AS1842856 (FoxO1 inhibitor) and CDP-Star were from Merck and Tropix, respectively. Anti-adiponectin antibody, anti-PPARγ antibody, and anti-β-actin antibody were purchased from Thermo Scientific, Santa Cruz Biotechnology Inc., and BIO Vision, respectively. Alkaline phosphatase (AP)-conjugated anti-rabbit IgG, anti-FoxO1 antibody, and anti-phospho-FoxO1 antibodies were obtained from Cell Signaling Technology, Inc (Dnanvers, MA). *Saposhnikovia divaricata* Schischk. and other crude drugs were purchased from Tsumura & Co. Ltd. (Tokyo, Japan). A specimen (No. 020210002) was deposited in the Herbarium of the Laboratory of Natural Resources, School of Pharmacy, Aichi Gakuin University. All test chemicals were dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) and stored at −80 °C until use.

2.2. Preparation of methanol extracts of crude drugs

Ninety crude drugs (Tsumura & Co., Tokyo, Japan), frequently used for preparation of Kampo prescriptions (therapeutic drugs in Japanese traditional medicine) were selected to prepare methanol extracts as follows. Crude drugs (10 g) were extracted with methanol (100 ml × 3) for 24 h at room temperature and the extracts were filtered and concentrated in vacuo. The resulting residues were dissolved in DMSO.

2.3. Cell culture

Mouse 3T3-L1 fibroblasts and human embryonic kidney (HEK) 293 cells were provided by the RIKEN BioResource Center (Tsukuba, Japan) through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology. 3T3-L1 cells were grown to confluency in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5% CO_2. At 2 days after reaching confluency, the cells were exposed to differentiation medium containing 10% FBS, 50 ng/ml insulin, 0.25 μM dexamethasone (DEX), and 0.5 mM IBMX for 48 h. The cells were further incubated in post-differentiation medium containing 10% FBS and 50 ng/ml insulin for 4 days by changing with fresh medium every other day. At the day 6, the cells were treated with 300 μM palmitic acid dissolved in 0.1% BSA solution for 1 day. After washing the cells thoroughly with PBS, panaxynol or the methanol extracts of crude drugs dissolved in DMSO were added to the cell culture, and incubated for 2 days. The culture medium was harvested and secreted adiponectin was determined by western blot analysis.

2.4. Western blot analysis

The culture medium was subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) using 2–15% gradient gel under non-reducing and non-heat-denaturing condition to detect the three oligomeric forms of adiponectin. Separated

proteins were transferred onto a polyvinylidene fluoride membrane (Millipore, MA, U.S.A.). The membrane was blocked in a solution of 5% powdered-skim milk in Tris-buffered saline (TBS) for 1 h and then incubated with anti-adiponectin antibody (1:1000 dilution) in TBS containing 1% powdered skim milk and 0.05% Tween-20 overnight at 4 °C. The membrane was washed three times with wash buffer (0.05% Tween-20 in TBS) and then incubated with AP-conjugated anti-rabbit immunoglobulin G (IgG) antibody in TBS containing 1% powdered skim milk and 0.05% Tween-20 for 1 h at room temperature. The membrane was washed three times with wash buffer, and antibody-bound proteins were detected using CDP-Star™ (Tropix) as the substrate for AP.

2.5. Quantitative RT-PCR

Total RNA was isolated from the cultured cells using RNAiso Plus (Takara Bio Inc., Ohtsu, Japan). First-strand cDNA was synthesized from 250 ng of total RNA using ReverTra Ace qPCR Master Mix with DNA remover (Toyobo, Japan) according to the manufacturer's instructions. The obtained cDNA was subjected to PCR amplification using the Takara-bio TP800 Thermal Cycler Dice® real-time system (Takara Bio, Inc.) with SYBR Green PCR Master Mix. The primers were 5′-ACAAGGCCGTTCTCTTCACC-3′ (Adiponectin, sense), 5′-CCAGATG GAGGAGCACAGAG-3′ (Adiponectin, antisense), 5′-GATGGAAGACCAC TCGCAT-3′ (PPARγ, sense), 5′-AACCATTGGGTCAGCTCTT-3′ (PPARγ, antisense), 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ (β-actin, sense), 5′-ATGGAGCCACCGATC CACA-3′ (β-actin, antisense), 5′-TGGATCGG AACCAAATGAGA-3′ (MCP-1, sense), 5′-AGTGCTTGAGGTGGTTG TGG-3′ (MCP-1, antisense), 5′-CCAGACCCTCACACTCA GATC-3′ (TNFα, sense), 5′-CACTTGGTGGTTTGCTACGAC-3′ (TNFα, antisense), 5′-ACTACTCTTGACCCTGCGT-3′ (CHOP, sense), 5′-CTCTGACTGGAAT CTGGAG-3′ (CHOP, antisense), 5′-AGCAACGAGTACCGGGTACG-3′ (C/EBPα, sense), 5′-TGTTTGG CTTTATCTCGGCTC-3′ (C/EBPα, antisense). Levels of mRNA expression were subsequently normalized relative to β-actin mRNA levels and calculated according to the delta–delta Ct method. Data are represented as means ± S.D. of three determinants from a representative of three independent experiments, which showed similar results.

2.6. Luciferase reporter gene assay

The PPARγ transcription activity was determined using luciferase reporter assays as follows. HEK293 cells were maintained in MEM containing 10% FBS, non-essential amino acids, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified atmosphere of 5% of CO_2 in air. HEK293 cells were seeded in 24-well culture plates at 1.5×10^5 cells/well and transfected using a standard calcium phosphate precipitation method with 100 ng of pCMX-mPPARγ expression