Food and Chemical Toxicology 64 (2014) 217–224

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Cucurbitacin B and cucurbitacin I suppress adipocyte differentiation through inhibition of STAT3 signaling

Cho-Rong Seo [a,1], Dong Kwon Yang [b,1], No-Joon Song [a], Ui Jeong Yun [c], A-Ryeong Gwon [c], Dong-Gyu Jo [c], Jae Youl Cho [d], Keejung Yoon [d], Jee-Yin Ahn [e], Chu Won Nho [f], Woo Jin Park [b], Seung Yul Yang [g], Kye Won Park [a,*]

[a] Department of Food Science and Biotechnology, College of Biotechnology and Bioengineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[b] College of Life Sciences, Gwangju Institute of Science and Technology, Gwangju 500-712, Republic of Korea
[c] School of Pharmacy, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[d] Department of Genetic Engineering, College of Biotechnology and Bioengineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[e] Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon 440-746, Republic of Korea
[f] Functional Food Center, Korea Institute of Science and Technology, Gangneung Institute, Gangwon-do 210-340, Republic of Korea
[g] Department of Horticulture, Sunchon National University, 413 Jungangno, Suncheon, Jeonnam 540-742, Republic of Korea

ARTICLE INFO

Article history:
Received 26 October 2013
Accepted 27 November 2013
Available online 3 December 2013

Keywords:
Cucurbitacin B
Adipocyte differentiation
STAT3
PPARγ
Cucurbitacin I

ABSTRACT

Cucurbitacin B, a member of the cucurbitaceae family, can act as a STAT3 signaling inhibitor to regulate the growth of hepatocellular carcinoma. STAT3 signaling has been shown to inhibit adipocyte differentiation through C/EBPα and PPARγ. Based on these studies, we hypothesized that cucurbitacin B would prevent PPARγ mediated adipocyte differentiation through STAT3 signaling. To test this hypothesis, mesenchymal C3H10T1/2 and 3T3-L1 preadipocyte cells were treated with a sub-cytotoxic concentration of cucurbitacin B. Cucurbitacin B treatment inhibits lipid accumulation and expression of adipocyte markers including PPARγ and its target genes in a dose-dependent manner. Cucurbitacin B treatment impairs STAT3 signaling as manifested by reduced phosphorylation of STAT3 and suppression of STAT3 target gene expression in preadipocytes. The anti-adipogenic effects of cucurbitacin B are significantly blunted in cells with STAT3 silenced by introducing small interfering RNA. Finally, our data show that cucurbitacin I, another cucurbitacin family member, also inhibits adipocyte differentiation by suppressing STAT3 signaling. Together, our data suggest the possibility of utilizing cucurbitacins as a new strategy to treat metabolic diseases and implicate STAT3 as a new target for the development of functional foods and drugs.

1. Introduction

The global epidemic of obesity is characterized by accumulation of excess fat in the body (Kopelman, 2000). Excess fat in obese individuals can be generated from excess calories by lipogenesis and stored in the body's metabolic tissues resulting in metabolic diseases (Kahn and Flier, 2000; Spiegelman and Flier, 2001).

Bioactive compounds isolated from edible foods and herbal extracts are extensively studied as alternatives and tools for identification of new molecular targets for anti-obesity (Hasani-Ranjbar et al., 2009). Resveratrol found in grapes, red wine, and nuts increases lipolysis, lowers body fat, and mimics calorie restriction (Baur et al., 2006; Howitz et al., 2003). Recent investigation on resveratrol also highlighted Sirtuin 1 (Sirt1) (Picard et al., 2004) and phosphodiesterase 4 (PDE4) (Park et al., 2012) as potential molecular targets for metabolic diseases. Sirt1 senses the nutritional status of metabolic tissues and acts as a metabolic master regulator (Feige et al., 2008). Competitive inhibition of cAMP-degrading phosphodiesterase by resveratrol activates AMP-activated protein kinase, resulting in protection against metabolic diseases associated with aging (Park et al., 2012). Thus, studies on signaling pathways targeted by bioactive molecules can illuminate new players in metabolism and alternative future strategies for obesity-related metabolic diseases.

Signal transducer and activator of transcription 3 (STAT3) signaling has been shown to play roles in cancer progression, inflammation, stem cell self-renewal, and differentiation (Debnath et al., 2012). STAT3 is phosphorylated and activated by cytokines and growth factors binding to the epidermal growth factor, fibroblast growth factor receptors, hepatocyte growth factor receptors, platelet growth factor receptor, and vascular endothelial growth factor receptor (Grivennikov and Karin, 2010). In contrast, the

* Corresponding author. Tel.: +82 31 290 7804; fax: +82 31 290 7882.
E-mail address: kwpark@skku.edu (K.W. Park).
[1] These authors contributed equally.

0278-6915/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2013.11.040

transforming growth factor-β receptor pathway inhibits STAT3 signaling at least in part by suppressing STAT3 phosphorylation (Starsichova et al., 2010). Once STAT3 is phosphorylated, it induces the transcription of genes involved in cell cycle progression, angiogenesis, and apoptosis. Knockdown of STAT3 using STAT3 small interfering RNA (siRNA) resulted in induction of Fas and Fas-Associated protein with Death Domain (FADD) expression, indicating that STAT3 also represses the expression of certain genes (Clarkson et al., 2006). Interestingly, a recent study showed that STAT3 mediates adipogenesis through activation of CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) expression (Zhang et al., 2011). Therefore, modulation of STAT3 levels can be a useful strategy against metabolic diseases in addition to various STAT3-associated diseases (Debnath et al., 2012).

STAT3 signaling plays roles in adipocyte differentiation through inhibition of critical adipogenic transcription factors, C/EBPα and PPARγ (Zhang et al., 2011). Cucurbitacin family members found in pumpkins, cucumbers, and melons have been shown to act as STAT3 inhibitors in certain cancer cells (Chambliss and Jones, 1966; Chan et al., 2010; Sun et al., 2010; van Kester et al., 2008). However, the role of cucurbitacin family members in adipocyte differentiation has not been investigated (Chen et al., 2005). Based on these observations, we hypothesized that Cucurbitacin B would impair adipogenesis through suppression of STAT3 signaling. To test this, the commonly used mesenchymal C3H10T1/2 and 3T3-L1 preadipocyte cells were differentiated into adipocytes and treated with sub-cytotoxic concentrations of Cucurbitacin B. In this study, we demonstrate a new role of cucurbitacin B and cucurbitacin I in adipogenesis and further determine the underlying mechanism of how these cucurbitacins exert inhibitory effects in adipocyte differentiation.

2. Materials and methods

2.1. Cell culture

Cucurbitacins and the STAT3 inhibitor WP-1066 were purchased from Sigma Aldrich (St. Louis, MO) and dissolved in dimethyl sulphoxide (DMSO) (Sigma, St. Louis, MO). Mouse 3T3-L1 and C3H10T1/2 cell lines were purchased from the American Type Culture Collection (Rockville, MD). C3H10T1/2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and antibiotics (penicillin and streptomycin, Hyclone), and were incubated at 37 °C under 5% CO_2. 3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% fetal calf serum (Hyclone). Mouse embryonic fibroblasts (MEFs) were freshly isolated from E13.5-E14.5 embryos. For adipocyte differentiation, cells were seeded at 5×10^4/ml in 6-well tissue culture plates, and confluent cells were incubated for 2 days in DMEM supplemented with 10% FBS, 1 μM dexamethasone (Sigma, St. Louis, MO), 0.5 mM isobutyl-1-methylxanthine (Sigma), and 5 μg/ml insulin (Sigma). Cells were refreshed with DMEM containing 10% FBS and 5 μg/ml insulin every 2 days. GW7845 (20 nM, a PPARγ ligand, kindly provided by the Tontonoz Lab) was further supplemented for the adipocyte differentiation of C3H10T1/2 cells and MEFs. After differentiation, cells were fixed with 4% paraformaldehyde in PBS at room temperature for 4 h, and stained with 0.5% Oil Red O (Sigma) in a mixture of isopropanol and distilled water at a 3:2 ratio for 45 min. Cells were washed with water, and photographed under a microscope. To quantify the intracellular triglyceride content, stained cells from at least two independent experiments were resolved with isopropanol and measured with a spectrophotometer at 520 nm.

2.2. Cell viability assays

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louise, MO) assay. 3T3-L1 cells were seeded at 1.5×10^4 cells per well in 96-well plates and incubated in culture medium until 70–80% confluence. After the cells reached confluence, cells were treated with 100, 200, 300 nM of cucurbitacin B in triplicate. After 24, 48, and 72 h, MTT (5 mg/mL in PBS) was added and cells were incubated at 37 °C for an additional 4 h. The formazan crystals were dissolved in 200 μL DMSO and absorbance was measured at 520 nm using a microplate reader.

2.3. Expression analysis

Total RNA was isolated from 3T3-L1 cells using TRIzol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA (cDNA) was synthesized from 0.5 μg of total RNA using the AMV Reverse Transcription System kit (Promega, Madison, WI) with random primers. After cDNA synthesis, the final 25 μl volume of the amplification mixture containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), primers, and cDNA was subjected to 40 amplification cycles of polymerase chain reaction (PCR) using a Thermal Cycler Dice (Takara, Shiga, Japan). Expression was normalized to 36B4. All real-time PCRs were performed at least twice. △cycle threshold (CT) was used to calculate the differences between the target CT value and the control (36B4) for each sample: △CT = CT(target)-CT(control). The relative expression level was calculated using $2^{-\Delta CT}$. The oligonucleotide primer (Integrated DNA Technologies, San Diego, CA) sequences used for PCR were as follows: peroxisome proliferator-activated receptor γ (PPARγ), PPARγ F, 5′-CCATTCTGGCCCACCAAC-3′ and PPARγ R, 5′-AATGCGAGTGGTCTTCCATCA-3′; adipocyte binding protein 2 (aP2), aP2 F, 5′-CACCGCAGACGACAGGAAG-3′ and aP2 R, 5′- GCACCTGCACCAGGGC-3′; cluster of differentiation 36 (CD36), CD36 F, 5′-GGCCAAGCTATTGCGACAT-3′ and CD36 R, 5′-CAGATCCGAACACAGCGTAGA-3′; CCAAT-enhancer-binding proteins (C/EBPα), C/EBPα F, 5′-GCGGGCAAAGCCAAGAA-3′ and C/EBPα R, 5′-GCGTTCCCGCCGTACC-3′; adiponectin F, 5′-CCGGAACCCCTG GCAG-3′ and adiponectin R, 5′-CTGAACGCTGAGCGATACACA-3′; acidic ribosomal phosphoprotein P0 (36B4), 36B4 F, 5′-AGATGCAGCAGATCCGCAT-3′ and 36B4 R, 5′-GTTCTTGCCCATCAGCACC-3′.

Western blotting was performed as previously described (Song et al., 2013). Cells were harvested in 200 μl of sample buffer and heated at 100 °C for 10 min. The proteins were separated by 10% SDS–PAGE and electrophoretically transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Membranes were blocked with blocking buffer (5% skim milk in TBS, 0.1% Tween 20) for 30 min at room temperature and treated with STAT3, phosphorylated STAT3 (P-STAT3) (sc-7196; Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin antibodies (A5316; Sigma), followed by incubation with horseradish peroxidase-conjugated secondary antibody (Zymed Laboratories, San Francisco, CA). Antibody binding was detected on X-ray film using Enhanced Chemiluminescence Western Blotting Detection Reagent (Amersham Biosciences).

2.4. STAT3 inhibition studies

Scramble control, signal transducer and activator of transcription 3 (STAT3)-specific oligos were synthesized by Genolution Pharmaceuticals, Inc. (Seoul, Korea). Two independent small interfering RNAs (siRNAs) were used to silence STAT3 expression. The sense sequences of STAT3-specific siRNA are as follows: Stat3 #1: 5′-GAGU UGAAUUAUCAGCUUAUU-3′; Stat3 #2: 5′-CAUCAAUCCUGUGGUAUAAUU-3′. The sense sequence of control nonspecific scramble RNA is 5′-CCUCGUGCCGUUCCAUCAGG UAGUU-3′. Cells plated at a density of 1×10^5 cells per well in a 6-well plate were transfected with 50 nmol of scrambled RNA, STAT3-specific siRNA using RNAiMAX (Invitrogen), as previously described (Song et al., 2013; Zhang et al., 2011). Cells were treated with siRNA for 8 h, and the medium was exchanged. After 48 h, cells were processed using differentiation protocols. Transfection was carried out in duplicated wells and repeated three times. For WP-1066 (STAT3 inhibitor, Calbiochem, Gibbstown, NJ) treatment experiments, C3H10T1/2 cells were treated with 10 μM in the presence of Cu B or Cu I and then allowed to differentiate to adipocytes for 5 days.

2.5. Statistical analysis

Data are presented as the means ± SEM. Differences in gene expression and lipid accumulation were analyzed using a two-tailed unpaired Student's *t*-test or using analysis of variance followed by Student–Newman–Keuls tests. Statistical significance was defined as $P < 0.05$. All computations were performed using statistical analysis software (PASW Statistics 17).

3. Results

3.1. Cucurbitacin B inhibits lipid accumulation during adipocyte differentiation

Since cucurbitacin family members including cucurbitacin B have previously been shown to have cytotoxic effects in certain cancer cells (Lee et al., 2010; Thoennissen et al., 2009), the potential cytotoxic effect of cucurbitacin B in preadipocytes was evaluated (Fig. 1). Treatment of 3T3-L1 cells with 100–300 nM cucurbitacin B for 24 h did not significantly affect cell viability (Fig. 1B). Similarly, cucurbitacin B treatment for 48 and 72 h did not decrease cell numbers (Fig. 1C and D). Thus, we used cucurbitacin B at 100–300 nM to assess its effects in adipocyte differentiation.