Biochemical and Biophysical Research Communications 469 (2016) 646–653

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Cholecalciferol inhibits lipid accumulation by regulating early adipogenesis in cultured adipocytes and zebrafish

Joo Hyoun Kim, Smee Kang, Yu Na Jung, Hyeon-Son Choi*

Department of Food Science and Technology, Seoul Women's University, 621 Hwarang-ro, Nowon-gu, Seoul 139-774, South Korea

ARTICLE INFO

Article history:
Received 7 December 2015
Accepted 12 December 2015
Available online 17 December 2015

Keywords:
Cholecalciferol
3T3-L1
Zebrafish
Adipogenesis
AMPKα
Cell cycle arrest

ABSTRACT

Cholecalciferol (CCF) is a common dietary supplement as a precursor of active vitamin D. In the present study, the effect of CCF on lipid accumulation was investigated in adipocyte cells and zebrafish models. CCF effectively inhibited lipid accumulation in both experimental models; this effect was attributed to the CCF-mediated regulation of early adipogenic factors. CCF down-regulated the expressions of CCAAT-enhancer-binding protein-β (C/EBPβ), C/EBPδ, Krueppel-like factor (KLF) 4, and KLF5, while KLF2, a negative adipogenic regulator, was increased by CCF treatment. CCF inhibited cell cycle progression of adipocytes through down-regulation of cyclin A and cyclinD; p-Rb was suppressed by CCF, but p27 was up-regulated with CCF treatment. This CCF-mediated inhibition of cell cycle progression is highly correlated to the inhibitions of extracellular signal-regulated kinase (ERK), serine threonine-specific kinase (AKT), and mammalian target of rapamycin (mTOR). Furthermore, CCF-induced inactivation of acetyl-CoA carboxylase (ACC), a fatty acid synthetic enzyme, with the activation of AMP-activated protein kinase α (AMPKα) was also observed. Consistent with the observations in adipocytes, CCF effectively inhibited lipid accumulation with the down-regulation of adipogenic factors in zebrafish. The present study indicates that CCF showed anti-adipogenic effect in adipocytes and zebrafish, and its inhibitory effect was involved in the regulation of early adipogenic events including cell cycle arrest and activation of AMPKα signaling.

1. Introduction

Cholecalciferol (CCF or vitamin D_3) is a precursor of active vitamin D (1,25- hydroxyvitamin D) [1]. CCF is endogenously synthesized from 7-dehydrocholesterol under the epidermal layer of skin via ultraviolet B (UVB) exposure from sunlight. It is then converted to 25-hydroxy CCF by hepatic 25-hydroxylase and is further hydroxylated by renal 1-a-hydroylase to produce 1,25-hydroxyvitamin D, a biologically active form [2]. CCF is also the most commonly-consumed dietary source of vitamin D. Dietary CCF taken up by enterocytes is incorporated into chylomicrons and thereby reaches the liver and kidneys via the blood stream for further hydroxylation [2].

Accumulated studies reveal the existence of strong links between vitamin D and obesity [3,4]. The study of vitamin D on obesity or adipogenesis was mostly performed using 1,25-hydroxyvitamin D. A recent study reported that 1,25-hydroxyvitamin D levels were lower in obese people, compared to normal weight individuals [4]. Kong and Li showed that the inhibition of adipogenesis by 1,25-hydroxyvitamin D is associated with the blockade of adipogenic factors, such as peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein-alpha (C/EBPα) [3]. They also suggested that 1,25-hydroxyvitamin D-induced stabilization of cellular VDR is linked to the inhibition of adipogenesis. Besides, the levels of vitamin D have been associated with various diseases such as inflammation and cancer [5,6].

Excessive adipogenesis results from an imbalance in energy homeostasis, and thereby causes obesity, which is associated with other metabolic complications such as diabetes, atherosclerosis, and hypertension [7]. Accordingly, the proper control of the process of adipogenesis is important for reducing the risk of metabolic diseases. Cultured adipogenic processes occur in post-confluent cells with re-progression of the cell cycle and a dramatic increase in cell number, called mitotic clonal expansion (MCE) [8]. This increase in cell number (hyperplasia) is accompanied by the activation of cell signaling pathways, such as PI3K/AKT and ERK pathways

* Corresponding author.
E-mail address: hschoi@swu.ac.kr (H.-S. Choi).

http://dx.doi.org/10.1016/j.bbrc.2015.12.049
0006-291X/

[9]. Other signaling molecules affecting lipid biosynthesis include AMPK and mTOR [10,11]. Along with these signaling events, various genes necessary to trigger the differentiation are transcriptionally activated; early adipogenic factors, including the consecutively activated C/EBPβ, KLF4, and KLF5, as well as the up-regulated C/EBPα and PPARγ [12].

The synthesis of CCF via exposure to sunlight is needed to satisfy the proper vitamin D requirements of the body. However, since many factors such as sunburn, skin aging, and change of skin color weaken this method of cutaneous synthesis of vitamin D, people have been increasingly interested in dietary sources of vitamin D [13]. CCF is naturally found in many dietary sources, including fish liver oils, oily fish, egg yolk, and wild mushrooms, even if the content varies [14]. Although the effects of 1,25-hydroxyvitamin D on adipogenesis have been studied, there is limited information about the effects of its precursor, CCF. Accordingly, the present study investigated the effect of CCF on adipogenesis in both cultured adipocytes and a zebrafish model, showing that it modulates the early stage of adipogenesis; the CCF-modulated signaling in this process reveals different aspects from findings reported in the previous studies of 1,25-hydroxyvitamin D.

2. Materials and methods

2.1. Materials

Cholecalciferol (≥98% purity) was obtained from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), bovine calf serum (BCS), fetal bovine serum (FBS), penicillin-streptomycin (P/S), phosphate-buffered saline (PBS), and trypsin-EDTA were purchased from Gibco (Gaithersburg, MD, USA). Dexamethasone (DEX), IBMX, insulin, and Oil red O were purchased from Sigma. Antibodies against PPARγ, C/EBPα, aP2, C/EBPβ, Akt, p-Akt, ERK, p-ERK, AMPKα, p-AMPKα, ACC, p-ACC, mTOR, p-mTOR, and β-actin were purchased from Cell Signaling Technologies (Danvers, MA, USA). Antibodies against LPAATϕ, LIPIN1, DGAT1, Cyclin D, Cyclin A, p-Rb, Cdk2, and p27 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Aprotinin, leupeptin, benzamidine, pepstatin, sodium orthovanadate, phenylmethylsulfonyl fluoride, and phosphatase inhibitor cocktails I and II and all other chemicals were purchased from Sigma.

2.2. XTT assay

3T3-L1 preadipocytes or confluent cells were treated with CCF (0–100 μM) and incubated for 48 h. XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) and *N*-methyl dibenzopyrazine methyl sulfate (PMS) were added to the culture, and further incubated for 2 h at 37 °C. The dissolved formazan salt level of the medium was measured at 450 nm and 690 nm.

2.3. Cell culture and sample treatment

3T3-L1 cells (American Type Culture Collection CL-173; ATCC, Manassas, VA, USA) were plated and cultured in DMEM medium containing 3.7 g/L sodium bicarbonate, 1% P/S, and 10% BCS at 37 °C in 5% CO_2. Adipocyte differentiation was induced by treating 2-day post-confluent cells with 10% FBS and a hormone cocktail (MDI) consisting of 0.5 mM IBMX, 1.0 μM DEX, and 1.67 μM insulin (day 0). CCF was dissolved in dimethyl sulfoxide (DMSO) at a stock solution of 100 mM, and was diluted to 5, 10, and 25 μM with DMSO. These samples or vehicle (DMSO) were added to the media at volume percent concentration of 0.25%. Differentiated cells were harvested at day 6 or 8 for the analyses.

2.4. Trypan blue assay

Confluent preadipocytes were incubated with MDI for 2 d for differentiation, in the presence or absence of cholecalciferol. Cells were trypsinized and harvested after two washes with PBS. Trypan blue dye (0.5%, Sigma) was added to stain the cells. Stained viable cells were observed and counted under a microscope.

2.5. Oil red O staining and lipid quantification

Differentiated 3T3-L1 cells in the presence or absence of CCF were washed with PBS, fixed with 4% formaldehyde at 4 °C for 1 h. The fixed cells were then washed with PBS, stained with 0.5% Oil red O in 60:40 (v/v) isopropanol/H_2O for 2 h at room temperature. Oil red O dye was eluted with 100% isopropanol, and its absorbance was determined at 490 nm.

2.6. RNA extraction and real time PCR

Zebrafish [17–20 days post fertilization (dpf)], undifferentiated or differentiated (day 6 or 8) adipocytes were used to extract RNA. The animals or cells were washed with PBS, and total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. One microgram of total RNA was used to synthesize cDNA with a Maxime RT PreMix KIT (iNtRON Biotechnology, Inc., Seongnam, Korea). cDNA was amplified using the specific primers listed in Table 1S. cDNA was diluted and mixed with SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA), containing 100 ng/mL PCR primers. Reactions were carried out in triplicate for each pair of primers, using the Step One real time PCR system (Applied Biosystems).

2.7. Western blot

Protein extracts (30 or 50 μg) from differentiated (day 6–8) or undifferentiated cells treated with or without CCF were subjected to SDS-PAGE, and immunoblotting was performed with indicated antibodies. Protein bands on the membranes were visualized by enhanced chemiluminescence, using Fluoro Chem FC2 imaging system (Alpha Innotech, Santa Clara, CA, USA).

2.8. Quantification of triglycerides

Accumulated TGs in cells were measured using a total TG assay kit (Zen-Bio, Inc., Research Triangle Park, NC, USA), according to the manufacturer's protocol.

2.9. Analysis of cell cycle progression

Post-confluent preadipocytes were treated with a hormone cocktail in the presence or absence of CCF (20 or 30 μM) for 24 h. Harvested cells were fixed with 70% ethanol for 2 h at 4 °C, washed with PBS, and centrifuged at 500 ×g for 2 min. The resulting pellet was mixed with 40 μg/mL propidium iodine solution containing 1 mg/mL RNase A, to stain DNA at 37 °C for 30 min. The cell cycle progression of sample groups (10,000 cells per experiment) was analyzed using a BD FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, CA) according to the manufacturer's protocols.

2.10. Zebrafish maintenance

Wild-type adult zebrafish (*Danio rerio*) were acquired from Chungnam National University (Daejeon, Korea). Embryos and larvae were obtained by natural coupling and raised in embryo water comprising sea salts (60 mg/mL, Sigma) until 5 dpf. Larvae