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Blockade of lipid accumulation by silibinin in adipocytes and zebrafish

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ABSTRACT

Silibinin is a compound present mainly in milk thistle. In this study, we investigated the mechanism by which silibinin suppresses adipogenesis of 3T3-L1 cells, and evaluated the anti-adipogenic effect of silibinin in zebrafish. Silibinin reduced lipid accumulation by downregulating adipogenic factors, such as, peroxisome proliferator-activated receptor γ (PPARγ), CCAAT-enhancer binding protein α (C/EBPα), and fatty acid-binding protein 4 (FABP4). The reduction of these adipogenic protein levels was associated with the regulation of early adipogenic factors, such as, C/EBPβ and Krüppel-like factor 2 (KLF2), and was reflected in downregulation of lipid synthetic enzymes. Silibinin arrested cells in the G_0/G_1 phase of the cell cycle, accompanied by downregulation of cyclins and upregulation of p27, a cell cycle inhibitor. These results correlated with the finding of deactivation of extracellular signal-regulated kinase (ERK) and AKT, a serine/threonine-specific kinase. In addition, silibinin activated AMP-activated protein kinase α (AMPKα) to inhibit fatty acid synthesis. As observed in 3T3-L1 cells, silibinin inhibited lipid accumulation in zebrafish with the reduction of adipogenic factors and triglyceride levels. Our data revealed that silibinin inhibited lipid accumulation in 3T3-L1 cells and zebrafish, and this inhibitory effect was associated with abrogation of early adipogenesis via regulation of cell cycle and AMPKα signaling.

1. Introduction

Silibinin, a natural plant flavonoid, is the main active constituent found in milk thistle (*Silybum marianum*), a plant of the *Asteraceae* family [1]. It is known to have a major role in the hepatoprotective properties of silymarin, the most commonly available crude extract of milk thistle [1]. Based on its well-defined cytoprotective properties, silibinin is utilized as an adjuvant for liver detoxifying agents like Legalon™ or Hepatron™ [2,3]. In addition, silibinin, either alone or as a major component of silymarin, is known to have an anti-neoplastic effect on various types of cancer [4]. A recent study showed that silibinin has an anti-adipogenic effect on 3T3-L1 cells [5], in which expression of Insig1 and Insig2 proteins are increased by silibinin treatment.

Adipogenesis produces cells that store fat in the form of lipids. Obesity is defined by excessive accumulation of body fat, which results from a chronic imbalance of energy homeostasis [6]. It is associated with several metabolic complications, such as diabetes and atherosclerosis [6]. Excessive energy storage is caused by abnormal adipocyte differentiation, which is accompanied by an increase in the number (hyperplasia) and size (hypertrophy) of adipocytes [7]. In cell culture, these processes are stimulated by exposure to hormone cocktails consisting of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) [8]. Such treatment induces re-entry into the cell cycle, and drives hyperplasia due to mitotic clonal expansion (MCE) [9], following the activation of cell proliferative signaling pathways such as the PI3K/AKT and ERK pathways [10]. The hyperplasia triggers changes in expression of specific genes that initiate differentiation into adipocytes. Early adipogenic factors, including C/EBPβ, KLF4, and KLF5, are transcriptionally activated [11], while negative adipogenic factors like KLF2 are repressed [12]. This early phase of the adipogenic program activates the expression of late adipogenic factors, such as, C/EBPα and PPARγ, key transcriptional regulators of adipocyte maturation, including of lipogenesis [11]. Activation of late adipogenic factors is known to promote expression of enzymes involved in triglyceride and fatty acid synthesis [13,14]. Adenosine 5′-monophosphate (AMP)-activated kinase (AMPK) is one of the important signaling molecules in the pathway controlling the lipid biosynthetic process, and it acts as a cellular energy sensor [15]. AMPK inhibits the activation of Acetyl-coA carboxylase (ACC) and sterol regulatory element-binding protein 1-c (SREBP1-c), which are responsible for fatty acid synthesis at the biochemical and transcriptional levels, respectively [16,17].

The genetic pathways employed in zebrafish to promote adipogenesis and lipid accumulation are conserved in mammals [18], and in recent years, the zebrafish has been recognized as a good

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animal model for the study of various diseases. The zebrafish also provides various advantages as a research model: it has a shorter generation time and costs less to maintain than other animal models, and produces abundant, externally fertilized embryos [19]. Furthermore, zebrafish embryos and larvae are transparent, which allows direct visualization of internal tissues and organs [19]. Thus, scientists investigating various research areas, including metabolic diseases, have adopted zebrafish as an *in vivo* model [18].

In this current study, we investigated the anti-adipogenic effects of silibinin in cell lines and in zebrafish. Although the anti-adipogenic effect of silibinin in 3T3-L1 cells has been explored previously, the mechanism by which silibinin inhibits adipogenesis and lipid accumulation still remains unclear. This study reveals that silibinin inhibits adipogenesis by regulating both the cell cycle and early adipogenic signaling. Furthermore, supporting previous studies, this work describes an anti-adipogenic effect of silibinin in zebrafish.

2. Material and methods

2.1. Materials

Silibinin (at ⩾98% purity) was obtained from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle 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), 3-isobutyl-1-methylxanthine (IBMX), insulin, and Oil red O were purchased from Sigma (St. Louis, MO, USA), as were aprotinin, leupeptin, benzamidine, pepstatin, sodium orthovanadate, phenylmethylsulfonyl fluoride, and phosphatase inhibitor cocktails I and II. Antibodies against PPARγ, C/EBPα, FABP4, C/EBPβ, AKT, phospho-AKT, ERK, phospho-ERK, AMPKα, phospho-AMPKα, ACC, phospho-ACC, phosphor-RB, and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against LPIN1, FASN, cyclinD, cyclin A, cyclin E, CDK2, and p27 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were purchased from Sigma.

2.2. Animal maintenance

All zebrafish experiments were approved by the internal Animal Ethics Committee at Korea University. Wild-type adult zebrafish (*Danio rerio*) were obtained from Chungnam National University (Daejeon, South Korea). Embryos and larvae, obtained by natural mating, were raised in embryo water containing sea salts (60 μg/ml, Sigma) until 5 days post-fertilization (5 dpf). Then, larvae were maintained in 100-mm plates at a density of around 20 larvae per 100 mL, and were fed *ad libitum* with hardboiled egg yolk as a high-fat diet (HFD), in the presence or absence of the indicated compounds (silibinin or curcumin), once a day for 12–15 days (17–20 dpf). Silibinin was treated with two doses (2 and 5 μM), and curcumin (2.5 μM) was used as a positive control.

2.3. Nile red staining, fluorescence imaging, and quantification

Zebrafish larvae, which are maintained in the presence or presence of silibinin (2 and 5 μM) and curcumin (2.5 μM), were used for imaging analysis. The fish were starved for 24 h before Nile red staining to ensure that their digestive tracts were empty. Nile red (Invitrogen N-1142) solution (1.25 mg/ml) was prepared in acetone and stored in the dark at −20 °C. The stock solution was diluted to 50 ng/ml in fish water, and then fish were incubated in the diluted solution for 5–10 min at 28 °C, in the dark, to stain them. Fish were then washed thrice with distilled water and anesthetized with a few drops of a tricaine stock solution (4 mg/ml, pH 7; Sigma). The fish were mounted in 3% methylcellulose, and stained parts of the fish were imaged under a fluorescence dissecting microscope (TE300, Nikon, Philadelphia, PA, USA) equipped with a GFP long-pass filter. Fluorescence images were acquired with a Pixera Penguin 600CL digital camera and the InStudio software (Pixera, Santa clara, CA, USA). Nile red staining was quantified using the ImageJ software (National Institutes of Health, Bethesda, MD).

2.4. Cell culture and harvesting

3T3-L1 preadipocytes obtained from the American Type Culture Collection (ATCC, CL-173, manassas, VA, USA). 3T3-L1 cells were plated and cultured in DMEM medium containing 1.5 g/l sodium bicarbonate, 1% P/S, and 10% BCS at 37 °C in 5% CO_2. Adipocyte differentiation was induced by treating 2-days post-confluent cells with 10% FBS and a hormone mixture (MDI) consisting of 0.5 mM IBMX, 1.0 μM DEX, and 1.67 μM insulin (at day 0). The culture medium was replaced with DMEM supplemented with 1.67 μM insulin and 10% FBS for a further 2 days (at day 2). This medium was then replaced with fresh DMEM containing 10% FBS every other day, until the indicated time points (days 6–12). For the treatments, 2 days post-confluent cells were differentiated in the presence or absence of silibinin (10, 20, and 30 μM).

2.5. XTT assay

Undifferentiated 3T3-L1 preadipocytes, or confluent, differentiating 3T3-L1 cells that were treated with silibinin (0–80 μM), were incubated for 48 h. 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reagent and *N*-methyl dibenzopyrazine methyl sulfate (PMS) were added to the culture, followed by incubation for 2 h at 37 °C. The soluble formazan salt content of the medium was measured at 450 nm and 690 nm. Cell viability was expressed as the percent of viable cells compared to the control.

2.6. Trypan blue assay

Confluent 3T3-L1 cells were differentiated by incubation with MDI for 2 days with or without silibinin (20 and 30 μM). Cells were trypsinized, and washed with PBS buffer. Collected cells were stained with trypan blue dye (0.5%, Sigma–Aldrich) for 3 min. Stained viable cells were counted using hemocytometer under microscope.

2.7. Oil red O staining and lipid quantification

Cells differentiated in the presence or absence of silibinin (10, 20, and 30 μM) and undifferentiated cells were washed with PBS, fixed with 4% formaldehyde, and incubated at 4 °C for 1 h. Next, the fixed cells were washed with PBS and then stained with 0.5% Oil red O in 60% isopropanol overnight at room temperature. The extent of differentiation was observed under a microscope, and cells were photographed after washing. Oil red O dye was eluted with 100% isopropanol, and the absorbance at 490 nm was determined.

2.8. RNA extraction and real time PCR

Differentiated (day 6 or day 8) cells or undifferentiated cells were washed with PBS, as were zebrafish. Then, total RNA was extracted from 3T3-L1 adipocytes, or from 10 to 20 zebrafish at 20 days post fertilization, using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. One