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3-Chloro-4,5-dihydroxybenzaldehyde inhibits adipogenesis in 3T3-L1 adipocytes by regulating expression of adipogenic transcription factors and AMPK activation

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ABSTRACT

Obesity is a serious health issue in many industrialized countries. It is a medical condition with excessive levels of fat accumulated in adipocytes. The objective of the present study was to determine the inhibitory effect of 3-chloro-4,5-dihydroxybenzaldehyde (CDB) on adipogenesis in 3T3-L1 adipocyte cells. CDB suppressed the differentiation and decreased lipid accumulation and triglycerides contents in 3T3-L1 adipocytes. Its suppression effect on fat accumulation was mediated via expression of adipogenesis factors (C/EBPα, SREBP-1c, PPARγ, and adiponectin) during adipocyte differentiation in white adipocyte cells. CDB's ability to suppress fat accumulation was increased in a concentration-dependent manner. It inhibited fatty acid synthesis related proteins including FAS, FABP4, leptin, and perilipin. It also increased expression of phosphorylated AMPK in adipocytes cells. These observations suggest that CDB has potential anti-obesity effect with ability to improve metabolic diseases.

1. Introduction

According to World Health Organization, more than 1.9 billion adults of 18 years and older were obese in 2014 [1]. Obesity falls under metabolic syndrome diseases such as type 2 diabetes, hypertension, and cardiovascular disease [2]. Chronic obesity is characterized by excess body fat with harmful effects on the human body such as non-alcoholic fatty liver disease, pulmonary disease, gallbladder disease, osteoarthritis, stroke, coronary heart disease, and cancer [3,4]. Overweight or excessive fat accumulation and adipogenesis are closely associated with obesity and metabolic diseases [5,6]. Adipocyte differentiation is closely associated with the onset of obesity in the human body. A considerable amount of literature has been published that to develop an anti-obesity drug that primarily regulates adipocyte differendation. Fat accumulation in adipocytes are associated with the regulation of adipogenic-specific factors including enhancer binding protein (C/EBP) family, sterol regulatory element-binding protein 1c (SREBP-1c), and peroxisome proliferator activated receptor-γ (PPARγ) [7,8].

Synthetic drugs such as Xenical® (Olistate) and Reductil® (Sibutramine) are commonly used as anti-obesity agents. However, they can cause several side effects such as insomnia, thirst, headache, constipation, and steatorrhea [9,10]. Phenolic compounds are widely used in medicine, cosmetics, and functional foods. They are known to have a variety of biological activities, including anti-oxidant, anti-inflammatory, and hepatoprotective effects [11,12].

Some phenolic compounds such as 2,4,6-Trihydroxybenzaldehyde, flavonoids, ursolic acid, and dieckol are known to have anti-adipogenic effects [13–16].]. Aromatic aldehydes have been reported to possess a wide range of potential bioactive properties including anti-cancer, antibacterial, anti-oxidant, anti-inflammation and immunomodulatory effects [17–22]. However, among the aromatic aldehydes, no reports have yet been published on the biological activities of 3-chloro-4,5-dihydroxybenzaldehyde (CDB). Especially, no reports have yet been published on the effects of CDB upon fat accumulation in adipocytes. The present work, aimed at investigating the inhibitory effects of CDB against adipogenesis in 3T3-L1 preadipocyte cells.

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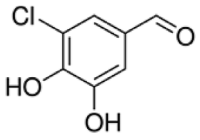

Fig. 1. Chemical structure of 3-chloro-4,5-dihydroxybenzaldehyde (CDB).

2. Materials and methods

2.1. Materials

Dimethyl sulfoxide (DMSO), 3-chloro-4,5-dihydroxybenzaldehyde (purity; ≥ 97%, Fig. 1), and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), bovine serum (BS), phosphate-buffered saline (pH 7.4; PBS), and penicillin-streptomycin (PS) were purchased from Gibco BRL (Grand Island, NY, USA). All chemicals and reagents used were of analytical grade and obtained from commercial sources. Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), insulin, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies to CCAAT/enhancing-binding protein-α (C/EBPα), sterol regulatory element binding protein-1c (SREBP-1c), peroxisome proliferator-activated receptor-γ (PPARγ), and adiponectin were purchased from Cell Signaling Technology (Bedford, MA, USA). Antibodies to perilipin, fatty acid synthase (FAS), fatty acid binding protein (FABP4), leptin, and adenosine monophosphate-activated protein kinase (AMPK) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture

3T3-L1 preadipocytes were obtained from American Type Culture Collection (Rockville, MD, USA). They were cultured in DMEM containing 1% PS and 10% bovine calf serum (Gibco BRL) at 37 °C with 5% CO_2 atmosphere. To induce differentiation, culture media of confluent preadipocytes after two days of culture (designated as day 0) were replaced with MDI differentiation media (DMEM containing 1% PS, 10% FBS, 0.5 mM IBMX, 0.25 μM dexamethasone, and 5 μg/ml insulin) and differentiated for 2 days. These cells were then maintained for another two days in DMEM containing 1% PS, 10% FBS, and 5 μg/ml insulin. Cells were then maintained in post-differentiation medium (DMEM containing 1% PS and 10% FBS). The medium was refreshed every two days. To examine effects of test samples on differentiation of preadipocytes to adipocytes, cells were cultured with MDI medium in the presence of test samples. Differentiation was measured based on the expression of adipogenic markers and appearance of lipid droplets. It was complete on Day 8.

2.3. Cell viability assay

Cytotoxicity of CDB against 3T3-L1 cells was assessed via a colorimetric MTT assay. Briefly, 3T3-L1 preadipocytes grown in 24-well plates were treated with CDB at 37 °C for 48 h. MTT stock solution (100 μl; 2 mg/ml in PBS) was then added to each well to have a total reaction volume of 600 μl. After 4 h of incubation, plates were centrifuged (800 × g, 5 min) and the supernatant was aspirated. Formazan crystals formed in each well were dissolved in 300 μl of DMSO and the absorbance of each well was measured on an ELISA plated reader at wavelength of 540 nm.

2.4. Determination of lipid accumulation by Oil Red O staining

To induce adipogenesis, 3T3-L1 preadipocytes were seeded into 6-well cell culture plates and maintained for two days after reaching confluence. Culture media were then replaced with differentiation medium (DMEM containing 10% FBS, 0.5 mM IBMX, 0.25 μM Dex, and 10 μg/ml insulin) and cells were then treated with test samples. After two days, the differentiation medium was replaced with adipocyte growth medium (DMEM supplemented with 10% FBS and 5 μg/ml insulin) which was refreshed every two days. After adipocyte differentiation, cells were stained with Oil Red O, an indicator of cell lipid content using published method [23] with slight modifications. Briefly, cells were washed with PBS, fixed with 10% buffered formalin, and stained with Oil Red O solution (0.5 g in 100 ml isopropanol) for 60 min. After removing the staining solution, the dye retained in cells was eluted into isopropanol and the optical density was then measured at wavelength of 520 nm.

2.5. Western blot analysis

Cells were lysed in lysis buffer (20 mM Tris, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM Na_3VO_4, 1% NP-40, 10 mg/ml Aprotinin, 10 mg/ml leupeptin, and 1 mM PMSF) for 60 min and then centrifuged at 12,000 rpm for 15 min at 0 °C. Protein concentrations were determined by using BCA™ protein assay kit. Cell lysate containing 40 μg of protein was then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred from gels onto nitrocellulose membranes. These membranes were then blocked with 5% non-fat dry milk in TBST (25 mM Tris-HCl, 137 mM NaCl, 2.65 mM KCl, 0.05% Tween 20, pH 7.4) for 1 h. Primary antibodies at 1:1000 dilution were incubated with membranes at 4 °C overnight. After washing with TBST, these membranes were then incubated with secondary antibodies at 1:3000 dilution. Signals were then developed using an ECL western blotting detection kit and exposed to X-ray films.

2.6. Statistical analysis

Student's t-test and one-way analysis of variance (ANOVA) were used to determine statistical significance of differences between values of various experimental and control groups. Data are expressed as means ± standard errors from at least three independent experiments performed in triplicates. Statistical significance was considered when *P*-value was less than 0.05.

3. Results

3.1. CDB inhibits 3T3-L1 adipocyte differentiation

Differentiated 3T3-L1 cell line has been widely used as an *in vitro* model of white adipocytes for mimicking adipogenesis [24,25]. In the present study, we measured cytotoxicity and inhibitory effects of CDB on adipogenesis in 3T3-L1 cells. Cytotoxicity was measured using MTT assay to determine its non-cytotoxic concentrations. CDB did not significantly affect viability of 3T3-L1 cells at concentrations up to 100 μg/mL after incubation for 24 h (Fig. 2A) or 48 h (Fig. 2B). Intracellular lipid accumulation was monitored by Oil-Red-O staining to determine the effect of CDB on adipogenesis. Results indicated that treatment with CDB inhibited adipocyte differentiation and significantly inhibited lipid accumulation (Fig. 3A). When adipocyte cells were treated with CDB prior to differentiation, dramatic reduction in triglyceride level was observed (Fig. 3B). These results indicate that CDB could reduce the accumulation of triglyceride and fat in 3T3-L1 adipocytes.

3.2. Effects of CDB on expression of adipogenic-specific proteins during differentiation of 3T3-L1 cells

During differentiation for fat accumulation, adipocytes are known to secrete adipogenic-specific proteins, including CCAAT/enhancing-binding protein-α (C/EBPα), sterol regulatory element binding protein-