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Fluvastatin activates sirtuin 6 to regulate sterol regulatory element-binding proteins and AMP-activated protein kinase in HepG2 cells

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ABSTRACT

Sirtuins, a family of NAD^+-dependent deacetylase enzymes, have been identified as mammalian homologs of yeast silent information regulator 2 (SIR2). Sirtuin 6 (SIRT6) plays important roles in cell homeostasis, DNA damage repair, cancer suppression, and aging. SIRT6 overexpression improves metabolic diseases, such as hypercholesterolemia, cholesterol-related disease, and type 2 diabetes via AMP-activated protein kinase (AMPK) activation. SIRT6 is abundant in the liver and is a crucial target for patients with liver steatosis. Compounds for drug repositioning were screened to identify potential SIRT6 activators, and fluvastatin, a synthetic inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase that reduces cholesterol synthesis, was identified to activate SIRT6. When HepG2 cells were treated with fluvastatin, the expression of SIRT6 and phosphorylation of sterol regulatory element-binding protein (SREBP)-1 and AMPKα, which is regulated by SIRT6, increased. In this study, we examined the mechanism underlying cholesterol regulation by fluvastatin via SREBP-1 and AMPKα pathway and suggested that fluvastatin is an SIRT6 activator that regulates cholesterol homeostasis and fatty liver disease.

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1. Introduction

Sirtuin 1-7 (SIRT1-7) are homologs of yeast NAD^+-dependent SIR2 deacetylase, and have been reported to show similar functions such as genomic stability and ageing. SIRT6 is localized in the nucleus [1] and has been found to be involved in catalytic function, DNA repair, metabolism, cancer, and many cellular pathways [2]. The phenotypes of $SIRT6^{-/-}$ mice are loss of body size and weight, osteopenia, short lifespan, lipodystrophy, and severe metabolic defects. SIR6 is endogenously activated by lamin A in DNA damage and promotes DNA repair; therefore, it is an effective therapeutic strategy for Hutchinson-Gilford progeria syndrome (HGPS) [3]. Interaction of USP10 and SIRT6 suppresses tumor cell growth by p53-mediated suppression of the oncogene c-Myc [4]. SIRT6 is associated with the tumor suppressor p53 and plays a critical role in glucose metabolism via p53 transcription activity and gluconeogenesis [5]. In normal nutrient conditions, SIRT6 inhibits Hif1α-dependent glucose-related gene transcription for glucose homeostasis [6]. In addition, SIRT6 is capable of deacetylating H3K9 and H3K56 to maintain the chromatin structure. H3K9 is the significant regulatory site of chromatin and is deacetylated in telomeres. Mechanistically, SIRT6 directly blocks the expression of insulin/insulin-like growth factor (IGF)-Akt signaling-related genes by deacetylating H3K9 and suppresses c-Jun, a subunit of the transcription factor AP-1. Acetylation of H3K56 leads to various functions such as ribosomal RNA biogenesis, tumor proliferation, migration, and regulation of DNA damage response [7–9].

A recent metabolic study identified the role of SIRT6 in the regulation of lipid synthesis [10] and cell growth in hepatocellular carcinoma (HCC) [11]. The liver is an important organ that maintains energy homeostasis through regulation of cholesterol and glucose biosynthesis [12]. Although SIRT6 plays an important role in the liver, the compound that activates SIRT6 has not yet been identified. Overexpression of SIRT6 in HepG2 cells has been shown to increase phosphorylated AMP-activated protein kinase (AMPK), liver kinase B1 (LKB1), and sterol regulatory element-binding protein (SREBP1) and reduce lipid biosynthesis. The heterotrimeric serine/threonine kinase AMPK plays a crucial role in regulating lipogenesis and is one of the important downstream factor of LKB1. Moreover, LKB1 can activate phosphorylated AMPK at Thr172, resulting in changes in the expression of SREBP1 [13–15]. The activation of AMPK by its upstream kinase LKB1 can serve as a

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cellular energy sensor and promotes the inhibition of hepatic steatosis and SREBP1 [16,17]. In cholesterol homeostasis and fatty acid synthesis, SREBP1 serves as a key regulator and transcriptional factor [18]. High expression of SREBP1 has been found in several human cancers, metabolic diseases such as diabetes, nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH), obesity, and hyperlipidemia. SREBP1 is suppressed by its upstream kinase AMPK [19,20].

In a previous study, it was reported that statins affect the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and are widely used as lipid synthesis lowering drugs that reduce liver steatosis in mice [21]. Among various statins, fluvastatin promoted apoptosis in human HCC and breast cancer cells [22,23]. Thus, fluvastatin can inhibit not only nitric oxide and cholesterol synthesis, but also hepatic steatosis in hepatocytes [24–26]. Fluvastatin exerted its effects via inhibition of the cholesterol biosynthetic transcription factor SREBP1 [27]. Other statins such as mevastatin and atorvastatin, except for fluvastatin, serve as effective drugs for cardiovascular system or neuronal survival through AMPK signaling [28,29]. Although there are several studies reporting that fluvastatin regulates SREBP1 for inhibiting lipid synthesis, there is no research on the effects of fluvastatin on phosphorylation of AMPK, which is upstream target of SREBP. In this study, fluvastatin plays the role of SIRT6 activator and inhibits hepatic lipid synthesis through phosphorylation of AMPK/SREBP-1 signaling pathway in HCC cells.

2. Materials and methods

2.1. Cell culture

The human HCC cell line HepG2 (ATCC HB-8065) and human embryonic kidney cell line HEK293 (ATCC CRL-1573) were purchased from American Type Culture Collection (ATCC, Manassas, USA). HepG2 cells were maintained in RPMI-1640 and HEK293 cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum (Welgene, Dae-gu, Korea) and 1% penicillin/streptomycin (Gibco, Gland Island, NY, USA) at 37 °C with 5% CO_2. Fluvastatin was purchased from Tocris Bioscience (Tocris Bioscience; Ellisville, MO) and dissolved in dimethyl sulfoxide (DMSO).

2.2. SIRT6 deacetylase activity assay

The deacetylase activity of SIRT6 was determined with the CycLex SIRT6 Deacetylase Fluorometric Assay Kit, according to the manufacturer's instructions. Briefly, the reaction compound mixture contained 50 μM finally 5 μM, 10 μM fluoro-substrate peptide, 0.8 mM NAD, and SIRT assay buffer giving total volume 50 μl. The mixture was incubated for 1 h at 37 °C. The fluorescence intensity was measured using a microtiter plate fluorometer (Molecular devices, spectra Max i3) with excitation wavelength of 490 ± 10 nm and emission wavelength of 530 ± 10 nm. All tests were performed in triplicate. All compounds were tested at 5 μM concentration. The EC_{50} of fluvastatin was calculated from seven independent concentrations.

2.3. Immunofluorescence staining

HepG2 cells were seeded into 96-well plates (1×10^4 cells/mL) and incubated with 5 μM fluvastatin for 24 h. The cells were fixed with 4% formaldehyde for 10 min at room temperature and washed with 1× phosphate-buffered saline (PBS) three times. HepG2 cells were treated with PBS containing 0.25% Triton X-100 for 10 min at room temperature and washed three times for 5 min. The cells were incubated overnight with diluted anti-SIRT6 primary antibody (1:500; Abcam) with 1% bovine serum albumin (BSA) in PBST at 4 °C. After incubation, HepG2 cells were washed three times in PBS and incubated with the secondary antibody in 1% BSA for 1 h at room temperature in dark. The cells were observed under a Spectra max i3 (Molecular devices).

2.4. Western blot analysis

HepG2 cells were grown to 80% confluence and treated with 5 μM fluvastatin for 24 h. The cells were lysed in PRO-PREP protein extraction solution (iNtRon Biotechnology, Kyungki-Do, Korea) and each sample was resolved by 4–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Bio-rad, Hercules, CA, USA). The membranes were incubated overnight at 4 °C using antibodies against the following proteins: AMPKα, p-AMPKα (Thr172); acetyl-CoA carboxylase (ACC), p-ACC (Ser79); p-SREBP1 (Ser372); LKB1, p-LKB1; Histone H3; Histone H3 (acetyl Lys9); Histone H3 (acetyl Lys56); (Cell Signaling Technology, Danvers, MA, USA); LKB1 and SIRT6 (Abcam, Cambridge, MA, USA); SREBP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and β-actin (Sigma, Saint Louis, Missouri, USA). All membranes were washed in tris buffered saline with Tween-20 (TBST, Welgene) and incubated for 1 h with secondary antibodies (Thermo Fisher, IL, USA). The protein bands were visualized using an enhanced chemiluminescence (ECL) solution (GE Healthcare Life Sciences, U.K.).

2.5. Cholesterol and triglyceride assay

HepG2 cells were grown to 80% confluence in 10-cm culture plate and treated with 5 μM fluvastatin for 24 h. The assay kits were purchased from Cayman (Cayman, USA). The cholesterol and triglyceride measurements were performed as described previously [13].

2.6. Small interfering RNA transfection and quantitative reverse transcriptase-polymerase chain reaction analysis

Small interfering RNA (siRNA) for SIRT6 was purchased from Dharmacon (Lafayette, CO, USA). siRNA for control was purchased from Thermo scientific. HepG2 cells were seeded at a density of 1×10^6 cells/mL in a 6-well culture plate and incubated for 24 h. Transfection of SIRT6 and control siRNA into HepG2 cells was performed using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's instructions. Briefly, 75 pmol siRNA was diluted with 200 μL serum-free opti-MEM (Invitrogen) and incubated with 7.5 μL Lipofectamine RNAiMAX at room temperature for 20 min. This mixture and opti-MEM were added to the 6-well culture plate and incubated for 4–6 h. After incubation, the medium was replaced with culture medium and incubated for 1–2 days. Then, RNA and proteins were extracted for expression analysis.

2.7. Reverse transcriptase polymerase chain reaction (RT-PCR)

The total RNA was isolated using TRIzol reagent (Invitrogen). The following primer sequences were synthesized by Macrogen (Seoul, Korea): SIRT6 (forward, 5′- CCA AGT TCG ACA CCA CCT TT -3′; reverse, 5′- CGG ACG TAC TGC GTC TTA CA -3′), GAPDH (forward, 5′- CGC TCT CTG CTC CTC CTG TT -3′; reverse, 5′- CCA TGG TGT CTG AGC GAT GT -3′). The cDNAs were amplified by PCR under the synthesis conditions: 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C 30 s, and extension at 72 °C for 2 min in a thermal cycler. The PCR samples were examined by electrophoresis on a 2% agarose gel for 25 min. The results were visualized using LAS 500 (GE healthcare).

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