4’-Hydroxyflavanone suppresses activation of sterol regulatory element-binding proteins and de novo lipid synthesis

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ABSTRACT

Sterol regulatory element-binding proteins (SREBPs) are major transcription factors that regulate the expression of genes involved in fatty acid and cholesterol biosynthesis. Here we show that 4’-hydroxyflavanone (4’-HF) impairs the fatty acid synthase promoter activity and reduces the activation of SREBPs and their target gene expression in human hepatoma Huh-7 cells. Moreover, 4’-HF suppresses de novo fatty acid and cholesterol synthesis. This study identifies 4’-HF as an inhibitor of SREBP maturation and lipid synthesis, and provides evidence that 4’-HF may have major potential as a pharmaceutical preparation against hepatic steatosis and dyslipidemia.

1. Introduction

The prevalence of hepatic steatosis is increasing rapidly in developed countries [1]. Obesity and insulin resistance are the most prevailing risk factors for hepatic steatosis which is caused by an imbalance between triglyceride acquisition and removal. In the absence of efficacious therapeutic options, hepatic steatosis causes progressive cirrhosis and liver cancer. Hence, new drugs to treat hepatic steatosis need to be developed.

Sterol regulatory element-binding proteins (SREBPs) are a family of transcription factors comprising SREBP-1a, SREBP-1c, and SREBP-2, which regulate the transcription of genes related to fatty acid and cholesterol biosynthesis [2]. SREBPs are synthesized as precursor proteins that are inserted into the ER membrane and processed to liberate the N-terminal halves, which function as transcription factors in the nucleus. Insulin increases hepatic lipogenesis which is largely mediated by the activation of SREBP-1c at the levels of transcription and proteolytic processing [3–5]. In the liver of many insulin-resistant mouse models, insulin fails to reduce gluconeogenesis though it still promotes lipogenesis accompanied by elevated levels of SREBP-1c [6]. The dysregulation of SREBP-1c has been implicated in the pathogenesis of hepatic steatosis and dyslipidemia [7,8].

In this study, to find small molecules that inhibit SREBP activity, we established Huh-7 cells that stably express a luciferase gene under the control of the human fatty acid synthase (FAS) promoter, which contains SREBP-binding elements. Using these cells, we found that 4’-HF, a synthetic analogue of flavanone, attenuates SREBP activities. We also found that 4’-HF inhibits proteolytic processing of SREBPs, and these inhibitions are followed by the suppression of de novo fatty acid and cholesterol synthesis.

2. Materials and methods

2.1. Reagents

Flavanone, 25-hydroxycholesterol (25-HC), fluvastatin, and lipoprotein-deficient serum (LPDS) were purchased from Sigma. 2’-Hydroxyflavanone and 4’-hydroxyflavanone were purchased from extrasynthese. DMEM were purchased from Wako.

2.2. Cell culture

Huh-7 cells were maintained in medium A (DMEM supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin containing 10% FBS). Huh-7/FAS-Luc cells were maintained in medium A containing 2 µg/mL blasticidin S. The cells were incubated at 37 °C under a 5% CO₂ atmosphere.

2.3. Plasmid construct

A luciferase reporter plasmid, pFAS-Luc, was constructed by cloning BglII–HindIII PCR fragments coding the 5’-untranslated
region of the human FAS gene into the same restriction sites of the pGL4.10 vector (Promega). pFAS-Luc contains the FAS promoter from -987 to +121.

2.4. Stable transfection of Huh-7 cells with the FAS promoter-containing luciferase reporter plasmid

Huh-7 cells were plated in 60-mm dishes at a density of 8 × 10^5 cells/dish and cultured with medium A for 20 h. They were subsequently transfected with 7.62 μg of pGL4-FAS, a reporter plasmid containing the FAS promoter, and 0.38 μg of pMAM2-BSD, an expression plasmid for blasticidin S deaminase [9], by lipofectamine 2000 according to the manufacturer’s instructions. Twenty-four hours after transfection, the cells were plated in 100-mm dishes and cultured with medium A containing 8 μg/mL blasticidin S. The medium was changed every 2nd day until well-defined colonies were evident. Colonies were isolated with cloning cylinders in the presence of 2 μg/mL blasticidin S. The resulting stable cell lines were cultured with medium B [DMEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, a 12.5 μM concentration of a HMG CoA reductase inhibitor (fluvastatin), and 50 μM sodium mevalonate containing 5% LPDS] under sterol-depleted conditions for 16 h. They were then sequentially cultured in the absence or presence of 1 μg/mL 25-HC for 24 h. The cell line that showed the greatest suppression of luciferase activity by 25-HC was selected and designated Huh-7/FAS-Luc.

2.5. Luciferase assays using an Huh-7/FAS-Luc cell line

Huh-7/FAS-Luc cells were plated in 12-well plates at a density of 1 × 10^5 cells/well, cultured with medium A for 20 h, and then the cells were switched to medium C (medium A supplemented with 12.5 μM fluvastatin and 50 μM sodium mevalonate) for 16 h. After incubation for another 24 h in the absence or presence of 100 μM of the test compound, luciferase activity and protein content in the cell extracts were measured as described previously [10]. Normalized luciferase values were determined by dividing the luciferase activity by the protein contents in the cell extracts.

2.6. Real-time PCR

Huh-7 cells were plated in 6-well plates at a density of 5 × 10^5 cells/well and cultured with medium A for 20 h. The cells were then switched to medium B for 16 h. After incubation for another 24 h in the absence or presence of 100 μM 4′-HF, total RNA was extracted from Huh-7 cells using Isogen (Wako) according to the manufacturer’s instructions. cDNA was synthesized and amplified from 2 μg of total RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time PCR (Taqman probe and SYBR green) analysis was performed on an Applied Biosystems StepOnePlus instrument. Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. The TaqMan ID number for genes analyzed are as follows: FAS, Hs00188012_m1; Stearoyl-CoA Desaturase 1 (SCD1), Hs00748952_s1; and GAPDH, 4352934. The sequences of the primers for HMG-CoA synthase are 5′-TACCATGTCAGGGGTACGTC-3′ and 5′-GGTTGACAGGAGGCTCAGTC-3′, those for HMG-CoA Reductase are 5′-TACCATGTCAGGGGTACGTC-3′ and 5′-AAGCTTAGACACAACTCATC-3′, and those for acyl-CoA synthetase long-chain family member 3 (ACSL3) are 5′-CCCCGTGAAACTTGTCTGGT-3′ and 5′-TCCGCTGTTAATGTTTTAAA-3′.

2.7. Antibodies

Monoclonal anti-SREBP-1 (2A4) and anti-SREBP-2 (1C6) antibodies were obtained from Santa Cruz. Monoclonal anti-β-actin (AC-15) antibody was purchased from Sigma. The anti-SREBP-2 polyclonal antibody (RS004) has been described previously [11]. Peroxidase-conjugated affinity-purified goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories.

2.8. Immunoblotting

Huh-7 cells were plated in 6-well plates at a density of 5 × 10^5 cells/well and cultured with medium A for 20 h. The cells were then switched to medium B in the absence or presence of 100 μM 4′-HF for 3 or 6 h. The cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM sodium chloride, 0.1% SDS, 0.5% deoxycholate, and 1% Triton X-100) supplemented with protease inhibitors. The lysates were subjected to SDS–PAGE, transferred onto a PVDF membrane, and probed with the antibodies indicated in the figure legends. Immunoreactive proteins were visualized using ECL (GE Healthcare) or Immobilon (Millipore) Western blotting detection reagents. The signals on the membrane were detected by a LAS-3000 Luminoimager (Fujifilm).

2.9. Determination of cholesterol and FA synthesis rates

Huh-7 cells were plated in 12-well plates at a density of 2 × 10^5 cells/well and cultured with medium A for 20 h. The cells were then switched to medium D (DMEM supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin containing 5% LPDS) for 16 h. After incubation for another 18 h in the absence or presence of 100 μM 4′-HF, the cells were placed in fresh medium supplemented with [14C]acetate (1.6 μCi/mL) and incubated for 6 h. The cells were washed, scraped, and collected by centrifugation. The pelletted cells were mixed with 1 mL 8 N potassium hydroxide and 1 mL ethanol per well. The mixture was heated at 100 °C for 2 h and extracted twice with 2 mL of petroleum ether (cholesterol extract). Two milliliters of the lower aqueous layer was initially mixed with 1 mL of 12 N hydrochloric acid and then extracted twice with 3 mL of petroleum ether (FA extract) [12]. The lipid extracts were resolved by thin-layer chromatography (Silica gel 60, Merck) and quantified with a BAS2000 image analysis system (Fujifilm). Normalized cholesterol and FA synthesis rates were determined by dividing the signals of [14C]-cholesterol and [14C]-FA by the amounts of total cellular protein determined by the BCA protein assay (Pierce).

2.10. Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed using StatView software. Student’s t-test was used to compare pairs of groups. Differences were considered significant at p < 0.05.

3. Results

3.1. 4′-HF suppresses the promoter activity of the FAS gene

To identify compounds that suppress SREBP activity, we established a stable cell line that expresses a luciferase reporter gene under the control of a FAS promoter that is driven by SREBP. Human hepatoma Huh-7 cells were transfected with pFAS-Luc together with the expression plasmid for blasticidin S deaminase, and then incubated in a medium containing blasticidin S. The clones resistant to blasticidin S were isolated, expanded, and designated the Huh-7/FAS-Luc cells. SREBP activity can be monitored by luciferase levels using this cell line, since luciferase activities of Huh-7/FAS-Luc cells were diminished by treatment with...
25-HC which reduces proteolytic activation of SREBP (Fig. 1B). In the course of identifying compounds that suppress SREBP activity, we identified 4′-HF as a potent inhibitor of FAS promoter activity (Fig. 1B). Interestingly, flavanone and other flavanone derivatives including 2′-HF and 3′-HF did not decrease, but rather slightly increased luciferase activity in Huh-7/FAS-Luc cells (Fig. 1B).

### 3.2. 4′-HF inhibits SREBP processing

To investigate the effect of 4′-HF on SREBP proteolytic processing, Huh-7 cells were treated with 4′-HF for 3 or 6 h. SREBP-1 was detected by an antibody recognizing its N-terminus [SREBP-1(N)], and SREBP-2 was detected by antibodies recognizing its N-terminus [SREBP-2(N)] as well as C-terminus [SREBP-2(C)]. Consistent with a previous report [13], SREBP processing was reduced by 25-HC, as estimated by the decreased amounts of its mature and cleaved forms (Fig. 2). 4′-HF suppressed SREBP processing within 3 h of its application and this inhibitory effect continued up to 6 h (Fig. 2). It should be noted that the amount of the mature form of SREBP-2 [SREBP-2(N)] was reduced by 4′-HF to the same degree as by 25-HC, whereas the reduction of the cleaved form of SREBP-2 [SREBP-2(C)] differed between 4′-HF and 25-HC.

![Figure 2: 4′-HF suppresses the processing of SREBP-1 and SREBP-2.](image)

**Fig. 2.** 4′-HF suppresses the processing of SREBP-1 and SREBP-2. Whole cell extracts were subjected to SDS/PAGE and immunoblotting (IB) with anti-SREBP-1(N) (2A4), anti-SREBP-2(N) (R004), anti-SREBP-2(C) (1C6), or anti-β-actin antibodies. The same results were obtained in more than three separate experiments.

### 3.3. 4′-HF suppresses the expression of SREBP target genes and de novo fatty acid and cholesterol synthesis

To examine whether 4′-HF causes a decrease in the endogenous mRNA levels of SREBP targets, Huh-7 cells were treated with 4′-HF for 24 h. Quantitative real-time PCR analyses revealed that 4′-HF treatment caused a significant decrease in the mRNA levels of SREBP targets including HMG-CoA synthase, HMG-CoA reductase, FAS, and SCD1 but not ACSL3 which is not an SREBP target (Fig. 3). Consistent with these changes in gene expression, 4′-HF treatment significantly decreased de novo fatty acid and cholesterol synthesis (Fig. 4).

### 4. Discussion

The present study demonstrates that 4′-HF suppresses the promoter activity of the FAS gene in Huh-7/FAS-Luc cells. Biochemical analyses further reveal that decreased SREBP processing in response to 4′-HF treatment suppresses endogenous SREBP target gene expression and de novo fatty acid and cholesterol synthesis in Huh-7 cells.

SREBP proteolytic processing is tightly regulated by the interaction between two ER membrane proteins, the SREBP cleavage-activating protein (SCAP), and the insulin-inducing gene (Insig). When cells are depleted of sterols, SCAP escorts SREBPs from the ER to the Golgi. Thereafter, SREBPs are processed by two proteases, site-1 protease and site-2 protease, within the Golgi. Once the ER membrane cholesterol content increases, SCAP binds to cholesterol, induces conformational changes, binds to Insig, and thereby remains on the ER membrane [14]. To date, several small molecules other than cholesterol have been reported to inhibit SREBP processing. Betulin, a naturally occurring pentacyclic triterpene, and oxysterols act by binding to SCAP and Insig, respectively, and consequently stimulate the interaction between SCAP and Insig [13,15]. Polyunsaturated fatty acids and the diarylthiazole derivative fatostatin are known to impair SREBP processing [16,17]. However, the mechanism by which these molecules inhibit SREBP processing is currently unknown. In the present study, we
demonstrated that 4′-HF inhibits SREBP processing. Interestingly, the decline in the cleaved form of SREBP-2 [SREBP-2(C)] by 4′-HF was weak in comparison with that of the mature form of SREBP-2 [SREBP-2(N)]. These results suggest the possibility that 4′-HF stabilizes the cleaved form of SREBP-2 in addition to inhibiting SREBP processing. Given that 4′-HF hinders processing of both SREBP-1 and SREBP-2 to the same degree, it may be important for common factor(s) such as SCAP or Insig that are involved in SREBP processing. It is also probable that 4′-HF modulates certain intracellular protein kinase signaling pathways, which in turn affect SREBP processing. A plausible candidate is AMPK because it has been reported to phosphorylate SREBPs and attenuate SREBP processing [18]. However, at present, it is largely unknown whether 4′-HF influences any intracellular protein kinase activities. Further studies are required to elucidate a link between 4′-HF and SREBP processing.

We found that 4′-HF, but not flavanone or the flavanone derivatives 2′-HF and 3′-HF, suppresses SREBP activity. These results indicate that the presence of a 4′-hydroxyl group of flavanone is essential for this suppressive effect. It has also been reported that 4′-HF exhibits several biological activities such as decreasing collagen synthesis in human fibroblasts [19] and vasorelaxing effects in isolated rat aortic ring [20]. However, the 4′-hydroxyl group of flavanone is unlikely to be essential for these activities since flavanone and 6′-HF also exhibit a similar effect on collagen synthesis and vasorelaxing activity, respectively [19,20]. These findings imply that 4′-HF exerts these biological effects via several distinct pathways.

We established Huh-7/FAS-Luc cells using the promoter region of the human FAS gene (~987 to +121) that contains a number of DNA sequence motifs specific for LXR, Sp1, and NF-Y [21]. Thus, it is possible that 4′-HF attenuates FAS gene expression by impairing any of these transcription factors in addition to SREBPs. However, since 4′-HF did not affect the expression of the ACSL3 gene, which is a LXR target gene [22], altered LXR activity is unlikely to be involved in a 4′-HF-dependent decrease in FAS gene expression.

In conclusion, using our novel assay system with Huh-7/FAS-Luc cells, we identified 4′-HF as an inhibitor of SREBP processing and de novo fatty acid and cholesterol synthesis. Further studies are necessary to determine the molecular mechanism of 4′-HF action. This study provides evidence that 4′-HF may have major potential as a pharmaceutical preparation against hepatic steatosis and dyslipidemia.

**Acknowledgements**

The authors would like to thank Enago (www.enago.jp) for the English language review. This work was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**References**


