Transcription of cytochrome P450 46A1 in NIH3T3 cells is negatively regulated by FBS

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Running title: FBS suppresses *Cyp46A1* transcription

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Abbreviations: Cytochrome P450 46A1, Cyp46A1; Cytochrome P450 3A11, Cyp3A11; Cytochrome P450 27A1, Cyp27A1; cholesterol 25-hydroxylase, Ch25h; CTP: phosphoethanolamine cytidylyltransferase, Pcyt2; Dulbecco's modified Eagle's medium, DMEM; fetal bovine serum, FBS; HMG-CoA reductase, Hmgcr; hydroxycholesterol, HC; 24S-hyroxycholesterol, 24S-HC; 25-hydroxycholesterol, 25-HC; 27-hyroxycholesterol, 27-HC; insulin-like growth factor, IGF; IGF-binding protein, IGFBP; insulin-induced-genes, Insigs; insulin receptor A, IR-A; insulin receptor B, IR-B; liquid chromatography/tandem mass spectrometry, LC-MS/MS; selected reaction monitoring, SRM; sterol regulation element binding protein, SREBP; cleavage-activating protein, SCAP; thin-layer chromatography, TLC; Triple Time of Flight 6600 mass spectrometer, TripleTOF MS/MS; nano liquid chromatography, nanoLC.

Abstract

Extracellular administration of side-chain oxysterols, such as 24S- hydroxycholesterol (24S-HC), 27- hydroxycholesterol (27-HC) and 25-hydroxycholesterol (25-HC), to cells suppresses *Hmgcr* and *Pcyt2* mRNA levels. Oxysterols are also enzymatically produced from cholesterol by Cytochrome P450 46A1 (Cyp46A1), Cyp27A1, Cyp3A11 and cholesterol 25-hydroxylase (Ch25h) in cells. As we performed previous experiments mainly with NIH3T3 cells, we analyzed which type of these oxysterol producing enzymes were expressed in NIH3T3 cells. In NIH3T3 cells, only Cyp46A1 was expressed. When Cyp46A1 was overexpressed in NIH3T3 cells, intrinsic oxysterols were increased as 24S-HC > 25-HC > 27-HC, and d-cholesterol was metabolized to 24S-HC. We are therefore interested in the mechanism regulating the production of endogenous oxysterols in cells by Cyp46A1. Here, we showed that the mRNA and relative protein levels and enzymatic activity of Cyp46A1, and the amount of 24S-HC, 25-HC and 27-HC in NIH3T3 cells, significantly increased under serum starved conditions, and these increases are suppressed by FBS supplementation. To identify this factor in FBS, the aqueous phase obtained by Bligh & Dyer method significantly suppressed *Cyp46A1* mRNA levels, and was fractionated by HPLC. The inhibiting fractions were analyzed by nanoLC and TripleTOF MS/MS and then insulin like factor-II (IGF-II) was identified. *Cyp46A1* mRNA levels in serum-starved cells were significantly suppressed and *Hmgcr* mRNA levels were significantly enhanced by the addition of IGF-II, IGF-I and insulin in NIH3T3 cells, and endogenous oxysterol levels were decreased. These results suggest that mRNA and protein levels of *Cyp46A1* are regulated by factors such as IGF-II in FBS.

1. Introduction

Cholesterol is one of the most abundant lipids in mammalian cells, comprising up to 25% of plasma membrane lipids [1] and playing a key role in membrane integrity and fluidity, thus impacting various aspects of cellular physiology. The pathway for cholesterol biosynthesis from acetyl coenzyme A is active in all nucleated cells [2]. Metabolites downstream from cholesterol include oxysterols (oxygenated cholesterol), bile acids, and steroid hormones [3,4].

Cholesterol homeostasis is regulated not only by the cholesterol level in cells, but also by cholesterol metabolites, especially oxysterols. 24S-Hydroxycholesterol (24S-HC), 25-hydroxycholesterol (25-HC) and 27-hydroxycholesterol (27-HC) are well-studied bioactive side-chain oxysterols. In the transcriptional regulation of cholesterol homeostasis, 25-HC binds to liver X receptors (LXRs) to activate LXR-mediated transcription, increasing cholesterol efflux and elimination [5]. 25-HC also inhibits the sterol regulation element binding protein (SREBP) maturation process and the subsequent transcription of genes involved in cholesterol biosynthesis and uptake [6]. In contrast to cholesterol, which directly interacts with SREBP cleavage-activating protein (SCAP), 25-HC enhances the interaction between SCAP and insulin-induced-genes (Insig) proteins, resulting in retention of the SREBP-2-SCAP complex in the ER [7,8].

Recently, we reported that mRNA levels of *Pcyt2* and *Hmgcr* increased after serum starvation, and the increases were clearly suppressed by supplementation with FBS or oxysterols such as 24S-HC, 25-HC and 27-HC [9-11]. Therefore, we were interested in the regulation of endogenous oxysterol production in cells. These bioactive side-chain oxysterols are generated enzymatically in cells, with cytochrome P450 46A1 (Cyp46A1) for 24S-HC production [12], cytochrome P450 27A1 (Cyp27A1) for 27-HC and 25-HC production [13], cytochrome P450 3A11 (Cyp3A11) for 25-HC production [14] and cholesterol 25-hydroxylase (CH25H) (hydroxylation of cholesterol 25-carbon) [15]. Expressions of these oxysterol producing enzymes

are dependent on cell types. Thus, in this study, the mRNA levels of these oxysterol producing enzymes were examined at first.

Insulin-like growth factor (IGF)-I and IGF-II play crucial roles in growth and development. IGFs are synthesized by a variety of cell types and are involved in linear growth, cell proliferation and differentiation, and in apoptosis [16]. In circulation, most IGFs are present as 150 kDa ternary complexes comprising one molecule each of IGF, IGF-binding protein (IGFBP)-3 or IGFBP-5, and an 85 kDa glycoprotein, the acid-labile subunit (ALS) [17].

In this study, only Cyp46A1 was expressed at high levels in NIH3T3 cells, and other oxysterol producing enzyme mRNA were hardly detected. Therefore, we were interested in the machinery that regulates Cyp46A1 enzymatic activity and its endogenous production of oxysterols. We examined the effect of fetal bovine serum (FBS) on the transcription of *Cyp46A1* in NIH3T3 cells and clarified that the transcription of *Cyp46A1* is negatively regulated by IGFs and insulin, suggesting that intracellular oxysterol levels may be altered by this classic hormonal stimulus.

2. Materials and methods

2.1. Materials

High-glucose Dulbecco's modified Eagle's medium (DMEM) (FUJIFILM Wako Pure Chemicals, Osaka, Japan) and fetal bovine serum (FBS) (Invitrogen, Waltham, MA) were used for cell culture. 4β-Hydroxycholesterol (HC), 7α-HC, 7β-HC, 20(*S*)-HC, 22(*R*)-HC, 24(*S*)-HC, 25-HC, 27-HC, cholestan-3β,5α,6β-triol, 7-ketocholesterol, cholesterol-d7 and lanosterol-d_6 were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Methanol for liquid chromatography/tandem mass spectrometry (LC-MS/MS), formic acid, ethyl acetate, sodium hydrogen carbonate, trimethylamine, and actinomycin D were purchased from FUJIFILM Wako Pure Chemicals. Acetonitrile for LC-MS/MS was purchased from Kanto Kagaku (Tokyo, Japan). Tetrohydrofuran for LC-MS/MS was purchased from Nacalai Tesque (Kyoto, Japan). Picolinic acid, 2-methyl-6-nitrobenzoic anhydride, 4-dimethylaminopyridine and tetrahydrofuran (dehydration) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Ultrapure water for LC-MS/MS was prepared with a PURELAB ultra apparatus (Organo Co., Ltd., Tokyo). Insulin from bovine pancreas was obtained from Sigma-Aldrich (St Louis, MO), and human IGF-I and IGF-II were obtained from Cell Signaling Technology (Danvers, MA). Nanosep centrifugal filter devices (membrane NMWL 100-kDa and 300-kDa filters) were purchased from Pall (Port Washington, NY). [^{14}C]Cholesterol was obtained from Perkin Elmer (Waltham, MA). Anti-V5 antibody was purchased from Thermo Fisher Scientific (Waltham, MA). Polyclonal antibodies against Cyp46A1 and PDI were obtained from Proteintech Japan (Tokyo, Japan) and Enzo Life Sciences, Inc. (Farmingdale, NY), respectively.

2.2. Tissue culture

NIH3T3 cells were supplied by the RIKEN Cell Bank (Tsukuba, Japan) and maintained in DMEM with 10% FBS, then treated with trypsin-EDTA (Invitrogen) for cell dispersion.

2.3. RT-PCR

For RT-PCR analysis, NIH3T3 cells (1×10^5) were dispensed onto 35-mm plates and cultured overnight at 37^{O}C. To analyze the effect of FBS on the transcription of *Cyp46A1*, *Ch25h*, *Cyp27A1* and *Cyp3A11*, cells were cultured in 0.5-10% FBS for 0-48 h with or without actinomycin D depending on the experiment. We identified the component in FBS that inhibits the transcription of *Cyp46A1* by culturing cells in serum-starved medium (0.5% FBS) for 48 h before adding one of the following materials: FBS; the lipid or aqueous phase extracted from FBS using the Bligh & Dyer method; FBS dialyzed against PBS (pH 7.4); the flow-through fractions of FBS separated using a Nanosep centrifugal filter device (membrane NMWL 100-kDa and 300-kDa filters) at 14,000×g for 1.5 h, and fractions of the aqueous phase of FBS separated using the Bligh & Dyer method, followed by HPLC-C18 column separation for 24 h. To analyze the effects of hormones, 0.5 μg/ml of IGF-II, 0.5 μg/ml of IGF-I, or 100 nM insulin was added to the medium after 48 h starvation. The cells were harvested after 6 or 4 h and total RNA was collected using RNeasy mini kits (Qiagen, Hilden, Germany). We quantified the mRNA levels of *Cyp46A1*, *Ch25h*, *Cyp27A1* and *Cyp3A11*, *Pcyt2*, *Hmgcr*, and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) in NIH3T3 cells by reverse transcribing 0.8-1 μg of total RNA at 37°C for 15 min using a ReverTra Ace qPCR RT Master mix kit (TOYOBO, Osaka, Japan). In case for *Ch25h* mRNA level analysis, reverse transcribing was performed or not performed. Reverse-transcribed samples or samples without reverse-transcription were then subjected to 40 cycles of amplification using FastStart Universal SYBR Green Master (ROX) (Roche, Basel, Switzerland) or ABI TaqMan Universal

PCR Master Mix (Thermo Fisher Scientific) for RT-PCR using a 7300 Real-Time PCR System (Applied Biosystems, Waltham, MA). The oligonucleotide primers for mouse *Gapdh* were MA050371-(F)(R) (Gapdh) (TAKARA, Shiga, Japan), for mouse *Cyp46A1* the primers were mouse *Cyp46A1*-F (5'- ACACCTACTTTGAAGACCCAT-3') and mouse *Cyp46A1*-R (5'-TGACAACTTTCACCTCCAT-3'), for mouse *Ch25h* the primers were mouse *Ch25h*-F (5'-CCAGCTCCTAAGTCACGTC-3') and mouse *Ch25h*-R (5'- CACGTCGAAGAAGGTCAG-3'), for mouse Cyp27A1 the primers were mouse *Cyp27A1*-F (5'-CTTCCTGCTGACCAATGAAT-3') and mouse *Cyp27A1*-R (5'- AGCTTTTAGCAGAGGCATGT-3'), for mouse Cyp3A11 the primers were mouse *Cyp3A11*-F (5'-GGCAGCATTGATCCTTATG-3') and mouse *Cyp3A11*-R (5'-AAGAACTCCTTGAGGGAGAC-3'), and for mouse *Hmgcr* the primers were mouse *Hmgcr*-F (5'- CCTCTCTACAGTACCTGCCTTACA-3') and mouse *Hmgcr*-R (5'-CCGATCACATTCTCACAGCA-3'). The TaqMan® primers for mouse *Pcyt2* were Mm00470327_m1 (Applied Biosystems).

We prepared a mouse liver cDNA library, then constructed *Cyp46A1*, *Ch25h*, *Cyp27A1* and *Cyp3A11* expression vectors. Mouse *Cyp46A1* cDNA was obtained using the primers *Cyp46A1*-F (5'-GAGCCATGAGCCCCGGGC-3') and *Cyp46A1*-R (5'-GCAGGGTGGGGGTGGGGG-3'), mouse *Ch25h* cDNA was obtained using the primers *Ch25h*-F1 (5'-CAGCATGGGCTGCTACAACGGTTC-3') and *Ch25h*-R1 (5'-AGTCTGTTTCTTCTTCTGGATCAAGTGTAC-3'), mouse *Cyp27A1* cDNA was obtained using the primers *Cyp27A1*-F (5'-ATCCATGGCTGCGTGGAGCC-3') and *Cyp27A1*-R (5'-CTGTCTCTGCAGAAAATGCAGCC-3'), and mouse *Cyp3A11* cDNA was obtained using the primers *Cyp3A11*-F (5'-AGGGATGGACCTGGTTTCAGCTC-3') and *Cyp3A11*-R (5'-TGCTCCAGTTATGACTGCATCCC-3'). The cDNA fragments were inserted into the pcDNA vector in-frame with a V5-tag (Invitrogen). The construct containing GAPDH cDNA was also prepared.

2.4. [^{14}C]Cholesterol radiolabeling, and determining the whole activity of the oxysterol-producing enzymes

The whole enzyme activity of oxysterol-producing enzymes (*Cyp46A1* et cetera) was measured as previously described [12], with some modifications. NIH3T3 cells (3×10^5) were dispensed onto 35-mm plates and cultured overnight. Next, the cells were cultured in medium containing 10% FBS or serum-starved medium (0.5% FBS) for 1 h, then labeled with [^{14}C]cholesterol (0.16 μCi/dish, 1.57 μM cholesterol). Cells from both cultures were collected after 24 h incubation. For the serum starvation experiments, NIH3T3 cells were cultured in serum-starved medium (0.5% FBS) for 24 h, then medium was changed to 10% FBS or 0.5% FBS. After 1 h incubation, the cells were labeled with [^{14}C]cholesterol (0.16 μCi/dish) for 24 h and the cells were collected. Total lipids in the cells and medium were extracted using the method of Bligh & Dyer. Briefly, the collected cells were suspended in 1 ml of lysis buffer [20 mM Tris-Cl pH 8.0, 0.1% Triton-X100], and then chloroform and methanol were added to the cell lysate or the medium to a final ratio of chloroform:methanol:water of 1.78:0.89:1.0 (v/v/v). The total lipid-containing chloroform phase was spotted onto silica gel plates (Merck Millipore) and oxysterols were separated by two-dimensional TLC using a solvent system comprising chloroform:methanol:ammonium (65:35:5, v/v/v) for the first dimension and ethyl acetate/toluene (4:6, v/v) for the second dimension. The spots were visualized and quantified using a bioimaging analyzer (Typhoon FLA 7000; GE Healthcare, Piscataway, NJ), and the quantified radio activity/0.16 μCi in μg protein were calculated as %/μg protein.

2.5. LC-MS/MS analysis of the extracted cholesterol, oxysterols and cholesterol derivertives

NIH3T3 cells (1×10^5) was dispensed onto 35-mm plates and cultured overnight, the cells were cultured in medium with 10% FBS or serum-starved medium (0.5% FBS) for 48 h. To determine the intrinsic oxysterol derivatives, cells were washed with PBS twice, then collected. To determine cholesterol derivatives, medium was changed with 10% FBS or 0.5% FBS again for 1h, and cholesterol derivatives were labeled with deuterium by adding cholesterol-7d (1.25 μM) to the cell medium for 23 h. Then, the cells were washed with PBS twice, and collected. For hormonal stimulating experiments, cells were stimulated with insulin, IGF-I and IGF-II for 6 h after 48 h serum starvation, then washed PBS and cells were collected. To determine cholesterol derivatives produced by *Cyp46A1*, cells were transfected with the plasmids encoding this cDNAs for overnight, and cholesterol derivatives were labeled with deuterium by adding cholesterol-7d (1.25 μM) to the cell medium for 6 h. Then, the cells were washed with PBS twice, and collected. Total lipids in the cells and in the medium were extracted using the Bligh & Dyer method. Briefly, the cells were suspended in 1 ml of lysis buffer [20 mM Tris-Cl pH 8.0, 0.1% Triton-X100] and protein concentrations in the cells were analyzed using the Bradford method. Chloroform and methanol were added to the cell lysate (1 ml) or the medium (2 ml) to a final ratio of chloroform:methanol:water of 1.78:0.89:1 (v/v/v) and 50 ng of lanosterol-d_6 was added to all samples as an internal standard.

We used slightly modified literature methods for derivatization and LC-MS/MS analysis of the extracted cell samples [18-20]. In brief, sample extracts were transferred to 1.5 ml polypropylene tubes and dried under a nitrogen gas stream at 40°C. Picolinic acid (80 mg), 100 mg of 2-methyl-6-nitrobenzoic anhydride and 30 mg of 4-dimethylaminopyridine were dissolved in 1.5 ml of tetrahydrofuran and 0.2 ml of trimethylamine, then 170 μl of the reagent mixture was added each dried cellular sample and incubated at 80°C for 30 min, then dried. Sodium hydrogen carbonate (5%, 200 μl) and 1 ml of ethyl acetate were added to the mixture and mixed. After centrifugation and separation of the two phases, the upper organic layer was dried and dissolved in 100 μl of

methanol, then a 20 μl aliquot was analyzed by LC-MS/MS using a Nexera ultra high performance liquid chromatograph system (Shimadzu Co., Kyoto, Japan) and a Qtrap6500 quadrupole-linear ion trap hybrid tandem mass spectrometer (SCIEX, Framingham, MA) in positive ion mode and equipped with an electrospray ion source probe. The curtain gas, ionspray voltage, turbo gas temperature, turbo gas, nebulizer gas and collision activation gas were set at 10 psi, 5000 V, 300°C, 50 psi, 70 psi, and 10 units, respectively. Selected reaction monitoring transitions for MS/MS are shown in Supplementary Table S1. A Sunshell C30 core-shell column (2.1 mm i.d. × 150 mm, 2.6 μm, ChromaNik Technologies Inc., Osaka, Japan) was used as the LC separation column. A mixture of formic acid/water (0.1:100, v/v) and a mixture of methanol/acetonitrile/tetrahydrofuran (45:45:10, v/v/v) was used as mobile phase A and B, respectively. The flow rate and column oven temperature were set at 0.4 ml/min and 40°C, respectively. The gradient program was as follows: B (%), 50>50>80>90>100>100; time (min), 0>3>3.01>33>43>45. The equilibration time was set at 5 min. All data were obtained and analyzed with Analyst 1.6.2 software (SCIEX) and SCIEX OS-Q software (SCIEX), respectively. The peaks detected at the retention times shown in Supplementary Table S1 were integrated and quantified by individual absolute calibration curves (ranging from 0.1 to 100 ng/ml, containing lanosterol-d6 set as an internal standard).

2.6. Western blotting analysis

NIH3T3 cells ($3x10^5$) were cultured for 24h in 6cm culture dishes with 4 ml of 10% FBS in DMEM after cell dispersion, then medium was changed to 10% FBS or 0.5% FBS with DMEM. After 16 h incubation, cells were collected, suspended and sonicated in 100μl of homogenizing buffer (50mM Tris/HCl, pH7.5, 1mM EDTA, 1mM PMSF, 0.25M sucrose). After 100,000 x g for 30 min centrifugation, pelleted proteins were resuspended in 30 μl of homogenizing buffer, and 25

μg of proteins separated with 12% acrylamide gel SDS-PAGE were transferred to PVDF membranes (FluoroTrans, Pall Corp., Port Washington, NY) using a Trans-Blot SD Semi-Dry Transfer blotter (Bio-Rad Laboratories, Hercules, CA), then incubating the membranes with 5% (w/v) skim milk in TBS for 3 h and washing three times with T-TBS (TBS containing 0.05% Tween 20). The membranes were then incubated with primary antibodies against Cyp46a1 overnight at 4 °C, washed three times with T-TBS, then incubated with horseradish peroxidase-conjugated IgGs for 1 hour at room temperature. The membranes were washed three times with T-TBS and stained with Clarity Western ECL Substrate (Bio-Rad) according to the manufacturer's instructions and visualized using a ChemiDoc Touch (Bio-Rad). For PDI protein detection, PDI polyclonal antibody (Enzo Life Sciences, Inc.) was used as primary antibody. Band densities were calculated using Image Lab (Bio-Rad). The relative band densities of Cyp46A1/PDI were calculated, and fold-increases of cells cultured in 0.5% FBS compared with cells in 10% FBS were determined. Values are shown as the mean±SD from three independent culture dishes.

2.7. HPLC

FBS aqueous phase (1 ml) obtained using the Bligh & Dyer method was injected onto an HPLC-C18 column (C18M 4D, Showdex, Showa Denko, Tokyo, Japan) using a Hitachi HPLC system (L-7100 and L-7300, Hitachi High-Tech Corpoaration, Tokyo, Japan), and detected using a UV/VIS detector (SPD-20A, Shimadzu Corporation, Kyoto, Japan) at 280 nm. The gradient went from 100% buffer A (100% distilled water) to 100% buffer B (100% acetonitrile) over 15 min at 0.2 ml/min. Fractions were collected every one minute, and each HPLC separation was performed in duplicate. Total samples (0.4 ml) of each fraction were evaporated using a refrigerated vapor trap (Speed Vac Plus, Savant Instruments Inc., Farmingdale, NY), dissolved in 200 μl of PBS, and added to cell culture medium.

2.8. Identification of the protein in FBS that represses Cyp46A1 transcription using a nano liquid chromatography Triple TOF 6600 mass spectrometer

Each fraction separated on the HPLC-C18 column (60 μl) was evaporated and dissolved in PBS (30 μl). Proteins in each fraction were precipitated with acetone (120 μl), dissolved in 50 μl of ammonium carbohydrate, reduced with DTT (10 mM), alkylated with iodoacetoamide (50 mM), then digested with 10 ng/μl trypsin overnight at 37°C. The samples were applied onto a GL-Tip SDB (GL Sciences, Tokyo, Japan), then washed with 0.1% formic acid, and desalted by eluting with 80% acetonitrile (v/v) containing 0.1% formic acid. The eluted samples were evaporated, then dissolved in 4 μl of 0.1% formic acid. The samples were analyzed using nano-liquid chromatography (nanoLC)-Triple TOF mass spectrometry (Triple TOF MS/MS) using a TripleTOF 6600 mass spectrometer (SCIEX) coupled to a nanoLC Eksigent 400 system comprising a reverse-phase LC with a nano column (75 μm×15 cm ChromXP C18-CL, 3 μm, 120 Å). The MS and MS/MS spectral data were collected using Analyst software (SCIEX), and peptides were identified using protein plot software (SCIEX).

2.9. Immunocytochemistry

NIH3T3 cells (2.5×10^4) were dispensed onto 24-well plates and cultured overnight in 10% FBS, then transfected with the Cyp46A1 tagged with V5 at C terminus expression vectors using Lipofectamine 2000 (Thermo Fisher) according to the manufacturer's instructions. Six hours after transfection, the medium was changed to 10% FBS. After 24 h incubation, the cells were fixed with 4% paraformaldehyde in PBS for 15 min, washed with PBS, permeabilized with 0.1% Triton X-100 (w/v) for 10 min, then blocked with 5% skim milk for 30 min. The cells were then

incubated with primary antibody overnight at 4°C, followed by washing and immunostaining with fluorescently labeled secondary antibodies conjugated with Alexa Fluor 488 (green) (Thermo Fisher) for 1 h at room temperature. Nuclei were stained with DAPI (blue) (Dojindo, Ltd., Kumamoto, Japan). Samples were observed with a confocal microscope (FV10i; Olympus, Tokyo, Japan or LSM780; Zeiss, Oberkochen, Germany). The ER was visualized by co-transfecting a plasmid vector pDsRed-ER (TAKARA).

2.10. Statistical analysis

All values are expressed as means ± S.D. Group means were compared using the Student's *t*-test after analysis of variance to determine the significance of the differences between individual mean values. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Enhanced Cyp46A1 transcription and oxysterol-producing enzyme activity by serum starvation in NIH3T3 cells

Previously, we reported that mRNA levels of *Pcyt2* and *Hmgcr* increased after serum starvation, and the increases were clearly suppressed by supplementation with FBS or oxysterols such as 24-HC, 25-HC and 27-HC [9-11]. We were therefore interested in the regulation of endogenous oxysterol production in cells and thus here analyzed the mRNA levels of oxysterol producing enzymes. Ch25h produces 25-HC from cholesterol [15], Cyp46A1 produces 24S-hydroxycholesterol [12], Cyp27A1 produces 27-HC and 25-HC [13], and Cyp3A produces 25-HC [14]. To certify the expression of these enzymes in NIH3T3 cells, we measured the mRNA levels

of *Ch25h*, *Cyp46A1*, *Cyp27A1* and *Cyp3A11*. As shown in Fig. 1 and Supplementary Fig. S1, only *Cyp46A1* was expressed in NIH3T3 cells, and the mRNA levels of *Ch25h*, *Cyp27A1* and *Cyp3A11* could not be detected. *Ch25h* is a single-exon gene, and equal levels of PCR products of *Ch25h* could be detected with or without reverse-transcription (Supplementary Fig. S1A). As shown in Fig. 1A, *Cyp46A1* mRNA levels in NIH3T3 cells over 24 h serum starvation were significantly increased compared to cells cultured in 10% FBS, and the increased levels of *Cyp46A1* mRNA were suppressed by actinomycin D treatment. Thus, we concentrated on investigating the transcriptional regulation of *Cyp46A1*. As shown in Fig. 1B, the *Cyp46A1* mRNA levels in cells cultured in serum-starved medium were about 1.5 times higher than in cells cultured in 10% FBS for 24 hours. The *Cyp46A1* mRNA levels in serum-starved NIH3T3 cells were clearly suppressed by the addition of FBS in a dose-dependent manner (Fig. 1C). These results clearly showed that a factor in FBS suppresses the transcription of *Cyp46A1*.

Western blotting analysis also showed that relative Cyp46A1 protein levels were clearly increased after serum starvation (Fig. 2) as mRNA levels. Then, to determine oxysterol species produced by *Cyp46A1*, cDNA encoding *Cyp46A1* expression plasmid were transfected with cholesterol-d7 and intrinsic oxysterols and deuterium labeled cholesterol derivatives were analyzed. As shown in Fig. 3A, intrinsic oxysterols such as not only 24-HC but also 25-HC and 27-HC were increased by *Cyp46a1* over-expression. After cells were labelled with cholesterol-d7, 24S-HC-d7 was increased.

Next, we examined the effect of FBS concentration on the synthesis of oxysterols in NIH3T3 cells by incubating the cells with [^{14}C]cholesterol. As shown in Fig. 4, the synthesis of oxysterols from cholesterol in cells subjected to serum starvation were clearly higher than in cells grown in medium with 10% FBS. These results show that enzyme activities for oxysterol production increased under FBS-depleted conditions.

We confirmed the effect of FBS on the synthesis of oxysterols by adding 1.25 μM cholesterol-d7 to the cells, then identifying and precisely quantifying the amounts of deuterium-labeled oxysterols in the cells and medium with or without serum starvation using LC-MS/MS after both cells were treated with 48 h serum starvation at first. The amount of 25-, 24S- and 27-HC-d7 in the cells and medium increased when the cells were cultured under serum-starved conditions compared with the cells in 10% FBS (Fig. 5A, B and C), suggesting that the amounts of newly synthesized oxysterols increased in cells cultured in serum-starved medium. 25-HC-d7 was most produced in this condition, this amount is much higher than 24S-HC-d7 and 27-HC-d7. Overcame cholesterol-d7 was metabolized about 10 times more to 25-HC than 24S-HC or 27-HC (Fig. 5).

Next, we analyzed the amounts of intrinsic oxysterols and cholesterol in cells cultured with or without serum starvation after both cells were treated with 48 h serum starvation at first. The intrinsic amounts of 25-HC, 24S-HC and cholesterol increased in cells cultured in 0.5% FBS medium for 24 h compared with cells cultured in 10% FBS (Fig. 6). These results showed that the production of endogenous 25-HC, 24S-HC and cholesterol increased in cells cultured in serum-starved medium. In contrast, the amounts of 27-HC increased in 10% FBS medium (Fig. 6C) and could be derived from oxysterols contained in FBS. Intrinsic oxysterol amounts (25-HC, 27-HC and 24S-HC) are almost equal order around 1~4 ng/mg protein in cells in serum starved medium (Fig. 6), however, overcame cholesterol-d7 was metabolized about 10 times more to 25-HC-d7 than 24S-HC-d7 or 27-HC-d7 (Fig. 4).

3.2. Intracellular localization of Cyp46A1

A V5-tagged *Cyp46A1* expression vector was prepared and transfected into NIH3T3 cells. The localization of *Cyp46A1*-V5 protein in the cells was evaluated by immunocytochemistry using

anti-V5 antibody. As shown in Supplementary Fig. S2, *Cyp46A1*-V5 was mainly located in the ER.

3.3. Determination of the factor inhibiting Cyp46A1 transcription in FBS

To identify the factor in FBS responsible for inhibiting *Cyp46A1* transcription, cells cultured in serum-starved medium were incubated with the aqueous phase or lipid phase obtained from FBS using the Bligh & Dyer method. As shown in Fig. 7A, the *Cyp46A1* mRNA levels in serum-starved cells were significantly suppressed by the aqueous phase but not by the lipid phase. In contrast, as reported previously, *Pcyt2* and *Hmgcr* mRNA levels in serum-starved cells were clearly suppressed by the lipid phase [9] but not by the aqueous phase (Supplementary Fig. S3). Oxysterols in the lipid phase respond to the suppressive effect of FBS against *Pcyt2* and *Hmgcr* transcription [9] . Taken together, these results showed that the FBS suppressive machinery for *Cyp46A1* is completely different from that for *Pcyt2* and *Hmgcr* mRNA transcription.

We thus embarked on identifying the inhibition factor in the liquid phase obtained from FBS effective against *Cyp46A1* transcription using the Bligh & Dyer method. The liquid phase was filtered through molecular size separating filters and cells cultured in serum-starved medium were incubated with the filtered samples. As shown in Fig. 7A, the *Cyp46A1* mRNA levels were significantly suppressed using the dialyzed aqueous phase and samples filtered through a 300 kDa molecular filter. Therefore, we expected that the factor responsible for the inhibition of *Cyp46A1* mRNA transcription might be a water soluble protein with a mass between 100 kDa and 300 kDa.

To identify this factor, the aqueous phase of FBS separated using the Bligh & Dyer method was fractionated using C18-reverse phase HPLC, then twice concentrated fractions (100 μl concentrated from 200 μl of each fraction) were added to serum-starved NIH3T3 cells and *Cyp46A1* mRNA was quantified. As shown in Fig. 7B, fraction No. 23 and 27 most potently

suppressed *Cyp46A1* mRNA levels in serum-starved NIH3T3 cells. We identified the molecular species in fraction No. 27 by analyzing this using nanoLC-Triple TOF MS/MS (Sciex 6600) and identified IGF-II in the fraction, as shown in Supplementary Fig. S4.

3.4. Effect of IGF-II, IGF-1, and insulin on Cyp46A1 mRNA levels and intrinsic oxysterol concentrations

To confirm the effects of IGF-II on *Cyp46A1* mRNA levels, the commercial obtained IGF-II, IGF-I or insulin, were added to serum-starved NIH3T3 cells for 48 h. As shown in Fig. 8A, the *Cyp46A1* mRNA levels in serum-starved cells were significantly suppressed by the addition of IGF-II for 6 h. We were also interested in the effects of IGF-I and insulin because IGFs bind to insulin receptor [21]. As expected, IGF-I and insulin also suppressed *Cyp46A1* mRNA levels (Fig. 8A). These results suggest that the mRNA level of *Cyp46A1* is regulated negatively by these classic hormones. We were interested in the effects of these hormones on *Hmgcr* mRNA levels. *Hmgcr* mRNA levels were increased by these hormonal stimulations for 4 h (Fig. 8B). We were also interested in intrinsic oxysterol amounts after these hormonal stimulations. As shown in Fig. 8C, intrinsic oxysterol amounts, such as 24S-HC, 25-HC and 27-HC, were decreased 6 h after insulin, IGF-I and IGF-II stimulations. These results suggested that insulin, IGF-I and IGF-II stimulation decreased transcription of *Cyp46A1* and oxysterol amounts in cells, and increased *Hmgcr* mRNA levels.

4. Discussion

Previously, we reported that *Pcyt2* and *Hmgcr* mRNA levels in NIH3T3 cells significantly increased after the cells were cultured in serum-starved medium, and the increases were suppressed by supplementation with low-density lipoprotein or oxysterols [9]. Therefore, we were interested in the synthesis of endogenous oxysterols in cells. As shown in this study, we found that FBS contains bioactive compounds that reduce the level of *Cyp46A1* transcription, *Cyp46A1* activity, and the amounts of oxysterols in NIH3T3 cells (Figs. 1-6). These bioactive compounds were contained in the aqueous phase of FBS separated using the Bligh & Dyer method (Fig. 7). Partial purification using HPLC, followed by nanoLC-Triple TOF MS/MS analysis, identified IGF-II as the likely transcriptional suppressor of *Cyp46A1* (Figs. 7 and 8 and Supplementary Fig.S4). It is noteworthy that the plasma IGF-II level is high before birth in the fetus [22], therefore we detected the effects of FBS and IGF-II on *Cyp46A1* mRNA levels. We performed these experiments in FBS starved conditions in this study because study for oxysterol producing enzymes using cells in 10% FBS containing IGF-II may be complicated and confused.

We also investigated the effects of commercial obtained IGF-II on *Cyp46A1* levels in NIH3T3 cells. As shown in Fig. 8A, *Cyp46A1* mRNA levels were significantly suppressed by the addition of the IGF-II, as expected. Both IGF-II and IGF-I can bind to insulin receptor [21], and insulin and IGF-I belong to be in the same family of hormones [23], and thus we investigated the effects of IGF-I and insulin on *Cyp46A1* mRNA levels in NIH3T3 cells. As expected, IGF-I and insulin suppressed levels similar to IGF-II (Fig. 8A). In contrast, *Hmgcr* mRNA levels were increased after IGF-II, IGF-I or insulin treatment. Therefore, mRNA levels of *Cyp46A1* and *Hmgcr* were likely affected by these hormones in NIH3T3 cells. At the same time, as shown in Fig. 8C, intrinsic 24S-HC, 25-HC and 27-HC concentrations in cells were decreased after these hormonal stimulations. These results suggested that mRNA levels of *Cyp46A1* are negatively

regulated by IGFs and insulin, and that the intercellular levels of oxysterols were decreased, and then mRNA levels of *Hmgcr* may be increased by these hormones. It was reported that *in vivo* inhibition of Cyp46A1 improved insulin signaling [24], and this result may be coincided with our results.

It is commonly accepted that IGF-I is clinically more noteworthy than IGF-II. IGF-II-producing tumors are linked to IGF-II-associated disease, but the disease itself is relatively rare and much less frequent than endocrine disorders such as acromegaly and growth hormone deficiency [25]. On the other hand, IGF-I is a major mediator for growth hormone signaling [26], and IGF-I is often used as an indicator of growth hormone secretion because the quantification of growth hormone is highly dependent on the assay method used [27], and levels change according to the stress levels of the patient [28]. However, the efficacy of IGF-I is closely related to IGF-II concentration because IGF-II can bind to the IGF-I receptor [29], although it preferentially binds to the IGF-II receptor [25]. Since both IGF-I and IGF-II can bind to the insulin receptor [29], a similar process in cell signaling likely occurs.

Since insulin, IGF-I, and IGF-II can bind to each other's receptor [29], each has a significant effect on the insulin receptor family. IGF-II mainly binds to the IGF-II receptor and the IGF-II/mannose-6-phosphate receptor [30]. IGF-II binding to IGF-II receptor promotes sphingosine kinase recruitment and activation, releasing sphingosine 1-phosphate (S1P) into the extracellular fluid and activating intracellular signaling events by S1P [31,32]. IGF-II binding to Insulin receptor A (IR-A) enhances the She/ERK/MAPK pathway (growth effect pathway) potentially while insulin binding to IR-A enhances the IRS-1/PI3K/AKT pathway (metabolic effect pathway) effectively, leading to the activation of transcription factors [33]. It is reported that the affinity between IGF-II and insulin receptor B (IR-B) may be stronger than that between IGF-I and IR-B [29]. We therefore need to take into accounts that *Cyp46A1* expression is reduced by a

unique IGF-II route. Unfortunately, the details of this interaction are currently poorly understood, but future studies will reveal details of the cross-reaction between IGF-I, IGF-II and insulin.

Cyp46A1 protein is localized in the ER as previously reported using HepG2 cells [34]. This localization may be linked to the physiological relevance of the product oxysterols for regulating cholesterol biosynthesis. Oxysterols in the ER enhances the binding of SREBP cleavage-activating protein (SCAP) to insulin-induced-genes (Insigs), thereby abrogating the movement of the SCAP-SREBP-2 complex to the Golgi apparatus, inactivating SREBP-2, and inhibiting HMGCR expression [35]. Therefore, the regulation of *Cyp46A1* enzymatic activity is important for the production of oxysterols in the ER, and for directly controlling the expression of HMGCR and other proteins regulated by oxysterols.

Cyp46A1 is expressed in brain, and well investigated concerning cholesterol metabolism in brain. Currently, there are no disease-modifying treatments for Alzheimer's disease (AD), and all treatments are symptomatic. Therefore, a variety of targets and pathways are being evaluated for the development of disease-modifying therapies for these progressive and debilitating diseases. Among them, CYP46A1, a cholesterol 24-hydroxylase and a key brain enzyme, is being studied [36]. Recently, it was reported that 24-HC is a positive allosteric modulator of N-methyl-D-aspartate receptors, which are responsible for excitatory neurotransmission throughout the central nervous system and play an important role in synaptic plasticity and learning [37]. Therefore, understanding the machinery for regulation of Cyp46A1 activity will be important.

This study showed that intracellular oxysterol levels can be controlled by reducing the mRNA levels of *Cyp46A1* by IGFs and insulin in NIH3T3 cells. Cellular concentrations of oxysterols controlled by these hormones may be responsible for intracellular signaling by these hormones. In the future, we will study further how IGF-II leads to changes in this intracellular signaling process for controlling intracellular oxysterol concentrations.

Data Availability Statement

All data are contained in the manuscript and supplementary information.

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Author Contributions

Y.S., H.A., M.M., M.A. and Y.H. performed the experiments, analyzed the results, and wrote the manuscript. M.S., T.J., I.U. and Y.A. helped with the experiments. H.S. designed and edited the manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

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Figure legends

Fig. 1. The effects of FBS concentrations in culture medium on *Cyp46A1* transcription in NIH3T3 cells.

(A) NIH3T3 cells were cultured in 10% FBS (white bar) or serum-starved medium (0.5% FBS) (black bar) for 24 h, then treated with or without actinomycin D (1 μM) for 16 h. The mRNA levels of *Cyp46A1* were quantified relative to *Gapdh* mRNA levels. (B) NIH3T3 cells were cultured in medium with 10% FBS (white bar) or serum-starved medium (0.5% FBS) (black bar). At time points at 0, 10 and 24 h incubation, the mRNA levels of *Cyp46A1* were quantified relative to *Gapdh* mRNA levels. (C) NIH3T3 cells were cultured in serum-starved medium (0.5% FBS) for 48 h, then FBS was added at concentrations ranging from 0.5% to 10%. After 12 h incubation, the mRNA level of *Cyp46A1* was quantified relative to *Gapdh* mRNA levels. Values shown are means ± S.D. from three independent culture dishes. Each experiment was repeated at least three times with similar results. * and ** indicate significant differences as compared to cells cultured in 0.5% FBS medium ($p<0.05$ and $p<0.01$, respectively).

Fig. 2. Western blot analysis for Cyp46A1 after serum starvation.

(A) NIH3T3 cells were cultured for 24h with 10% FBS in DMEM, then medium was changed to 0.5% FBS (lane 1-3) or 10% FBS (lane 4-6) with DMEM. After 16 h incubation, cells were collected, homogenized and centrifuged at 100,000g for 30 min. Pellets were resuspended and western blotting (25 μg protein) was performed with anti-Cyp46A1-antibody or anti-PDI-antibody. Cells were transfected and overexpressed with Cyp46A1 cDNA, and proteins were collected (lane 7). (B) The band densities in A were calculated using Image Lab. The relative band densities of Cyp46a1/PDI were calculated, and fold-increases of cells cultured in 0.5% FBS

compared with cells in 10% FBS were determined. Values are shown as the mean±SD from three independent culture dishes. *p < 0.05 as compared with control cells cultured with 10% FBS.

Fig. 3. Effects of *Cyp46A1* overexpression on intrinsic oxysterols and production of oxysterol derivatives from cholesterol-d7.

(A and B) *Cyp46A1* expressing vector was transfected and overexpressed for 24 h, and then cholesterol-d7 (1.25 μM) was added (B). After 6 h incubation, the lipid phases from the cells were extracted using the Bligh & Dyer method and analyzed by LC/MS/MS. Oxysterols were quantified relative to protein levels. Values shown are means ± S.D. from three independent culture dishes. Each experiment was repeated at least three times with similar results. ** indicates significant differences as compared to cells transfected with vector control (p<0.01).

Fig. 4. The effects of serum starvation on the synthesis of oxysterols from radio-labeled cholesterol.

(A, B and C) NIH3T3 cells were cultured in medium with 10% FBS or serum-starved medium (0.5% FBS) for 1 h, then labeled with [^{14}C]cholesterol (0.16 μCi/dish, 1.57 μM cholesterol). After 24 h incubation, the lipid phases from the cells and medium were extracted using the Bligh & Dyer method and the radio-labeled oxysterols were separated by TLC. The densities of the spots on the TLC were quantified using ImageQuant software, and the quantified radio activity/0.16 μCi in μg protein was calculated as %/μg protein (B, C). (D, E) NIH3T3 cells were cultured in serum-starved medium (0.5% FBS) for 24 h, then the medium was changed to 10% FBS or serum-starved medium (0.5% FBS) (black bar). After 1 h incubation, the cells were labeled with [^{14}C]cholesterol (0.16 μCi/dish), then after 24 h incubation, the lipid phases from the cells and medium were extracted using the Bligh & Dyer method and radio-labeled oxysterols were separated by TLC as

in (A). The densities of the spots on the TLC were quantified using ImageQuant software, and the quantified radio activity/0.16 μCi in μg protein was calculated as %/μg protein. Values are means ± S.D. from three independent culture dishes. Each experiment was repeated at least three times with similar results. * and ** indicate significant differences as compared to cells cultured in 10% FBS medium (p<0.05 and p<0.01, respectively).

Fig. 5. The effects of serum starvation on the synthesis of oxysterols from cholesterol-d7.

(A-C) NIH3T3 cells were cultured in serum-starved medium (0.5% FBS) for 48 h, and then medium was changed to 10% FBS (white bar) or serum-starved medium (0.5% FBS) (black bar). After 1 h incubation, 1.25 μM cholesterol-d7 was added, and after 23 h, the lipid phases from the cells and medium were extracted using the Bligh & Dyer method, and the amounts of oxysterol species, 24S-HC-d7 (A), 25-HC-d7 (B), 27-HC-d7 (C), and cholesterol-d7 (D) were quantified by LC-MS/MS per mg protein. Values are means ± S.D. from three independent culture dishes. Each experiment was repeated at least three times with similar results. * and ** indicate significant differences as compared to cells treated with cells cultured in 10% FBS medium (p<0.05 and p<0.01, respectively).

Fig. 6. The effects of serum starvation on intrinsic oxysterol concentrations in cells and medium.

(A-D) NIH3T3 cells were cultured in serum-starved medium (0.5% FBS) for 48 h, then cultured in medium with 10% FBS (white bar) or serum-starved medium (0.5% FBS) (black bar) for 24 h, then the lipid phases from the cells and medium were extracted using the Bligh & Dyer methods. The amounts of cholesterol derivatives, 24S-HC (A), 25-HC (B), 27-HC (C), and cholesterol (D) were quantified by LC-MS/MS per mg protein. Values are means ± S.D. from three independent

culture dishes. Each experiment was repeated at least three times with similar results. * and ** indicate significant differences as compared to cells cultured in 10% FBS medium ($p<0.04$ and $p<0.01$, respectively).

Fig. 7. The effects of FBS fractions separated by using the Bligh & Dyer method or molecular size, or fractions of the aqueous phase separated using a HPLC-C18 column on *Cyp46A1* transcription.

(A) NIH3T3 cells were cultured in serum-starved medium (0.5% FBS) for 48 h, then treated with 10% FBS (white bar), dialyzed FBS (gray bar), the aqueous phase (vertical striped bar) or the lipid phase (horizontal striped bar) of FBS extracted using the Bligh & Dyer method, and the smaller molecular fractions of the aqueous phase of FBS passed through a 300 KDa filter (right oblique striped bar) or 100 KDa filter (left oblique striped bar). After 24 h incubation, the mRNA level of *Cyp46A1* was quantified relative to *Gapdh* mRNA levels. Each fraction was added at a concentration equivalent to 10% FBS. Values are means ± S.D. from three independent culture dishes. Each experiment was repeated at least three times with similar results. ** indicate significant differences as compared to cells cultured in 0.5% FBS ($p<0.02$). (B) FBS (9 ml) was fractionated using the Bligh-Dyer method (1:1:0.9 (v/v/v) = chlorohorm:methanol:water) and 9 ml of aqueous phase was obtained. The aqueous phase (1 ml) was applied onto a C18-reverse phase HPLC column and fractions were collected every 1 min (flow rate: 0.2 ml/min) using a linear water-acetonitrile gradient (line). Each fraction (200 μl) was evaporated and dissolved with 100 μl of PBS. Twice concentrated fractions (30 μl concentrated from 60 μl of each fraction) was added to 48 h starved NIH3T3 cells. After 24 h incubation, the mRNA level of *Cyp46A1* (Bar) was quantified relative to *Gapdh* mRNA levels.

Fig. 8. The effects of IGF-II, IGF-I and insulin on *Cyp46A1* and *Hmgcr* transcription, and intrinsic oxysterol amounts in NIH3T3 cells.

(A and B) NIH3T3 cells were cultured in serum-starved medium (0.5% FBS) for 48 h, then treated with insulin (100 nM), IGF-I (0.5 μg/ml) or IGF-II (0.5 μg/ml). After 6 and 4 h incubation, the mRNA levels of *Cyp46A1* (A) and *Hmgcr* (B) were quantified relative to *Gapdh* mRNA levels, respectively. Values are means ± S.D. from three independent culture dishes. Each experiment was repeated at least three times with similar results. * and ** indicate significant differences as compared to cells treated with control vehicle ($p<0.05$ and $p<0.01$, respectively). (C) NIH3T3 cells were cultured with serum-starved medium (0.5% FBS) for 48 h, and then incubated with insulin, IGF-I or IGF-II. After 6 h, the lipid phases from the cells were extracted using the Bligh & Dyer methods. The amounts of oxysterols were quantified by LC-MS/MS per mg protein. Values are means ± S.D. from three independent culture dishes. Each experiment was repeated at least three times with similar results. * and ** indicate significant differences as compared to cells treated with control vehicle ($p<0.05$ and $p<0.01$, respectively).