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Review Article

Regulation of Glycerophospholipid and Cholesterol Synthesis in Cells by Hydroxylated Cholesterols (Oxysterols)

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Summary

Phosphatidylethanolamine (PE) and cholesterol are major lipid components of mammalian cell membranes. The rate limiting enzymes for the synthesis of PE and cholesterol are CTP: phosphoethanolamine cytidylyltransferase (Pcyt2) and 3-hydroxy-3-methylglutaryl-CoA reductase (Hmgcr), respectively. The transcription and activity of these enzymes in NIH3T3 cells are suppressed by fetal bovine serum (FBS) and hydroxylated cholesterol (oxysterols) in similar dose-dependent manners. These results suggested that the intrinsic oxysterol concentration in cells is important for maintaining membrane lipid components, leading us to investigate the regulation of oxysterol-producing enzyme activity in cells. Using NIH3T3 cells, we found that of all oxysterol-producing enzymes, only the 24S-hydroxycholesterol producing enzyme cytochrome P450 46A1 (Cyp46A1) is expressed. Transcription of Cyp46A1 is suppressed by FBS, and we identified insulin-like growth factor II (IGF-II) in FBS as the factor suppressing Cyp46A1 transcription. The oxysterol level in NIH3T3 cells is also suppressed by IGF-II. These results suggested that the classical hormone IGF-II regulates the oxysterol content in cells and transduces cellular signals.

Key Words: Oxysterols, Phosphatidylethanolamine, Cholesterol, Pcyt2, Hmgcr, 25-Hydroxycholesterol, Cytochrome P450 46A1

Abbreviations: phosphatidylethanolamine, PE; phosphatidylcholine, PC; CTP: phosphoethanolamine cytidylyltransferase, Pcyt2; CTP: phosphocholine cytidylyltransferase, Pcyt1; phosphatidylserine decarboxylation, Psd; site 1 protease, S1P; site 2 protease, S2P; C/EBP CCAAT/enhancer-binding protein, C/EBP; specificity protein 1, Sp1; 3-hydroxy-3-methylglutaryl-CoA reductase, Hmgcr; 3-hydroxy-3-methylglutaryl-CoA, HMG-CoA; sterol regulatory element, SRE; hydroxylated cholesterol, HC; Liver X receptor, LXR; insulin-induced gene, Insig; sterol regulatory element-binding protein, SREBP; sterol regulatory element-binding protein cleavage activation protein, SCAP; fetal bovine serum, FBS; low-density lipoprotein, LDL; 25-hydroxycholesterol, 25-HC; cytochrome P450 46A1, Cyp46A1,

Glycerophospholipids such as phosphatidylethanolamine (PE), phosphatidylcholine (PC) and cholesterol are major components of mammalian cell membranes (Table 1). The distribution of these lipids in cell membranes is strictly regulated, with PE found mainly in the inner leaflet and PC in the outer leaflet[1]. Cholesterol is found in both leaflets.

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Table 1 Lipid contents of cell membranes. From Voet Biochemistry (%: lipid weight/total lipid weight).

	Human erythroid plasma membrane	Bovine heart mitochondria
Glycerophospholipids	48	73.5
Phosphatidylcholine (PC)	19	39
Phosphatidylethanolamine (PE)	18	27
Phosphatidylserine (PS)	8.5	0.5
Phosphatidylinositol (PI)	1	7
Phosphatidic acid (PA)	1.5	0
Sphingolipids	27.5	0
Sphingomyelin	17.5	0
Glycosphingolipid	10	0
Cholesterol	25	3
Cardiolipin	0	22.5

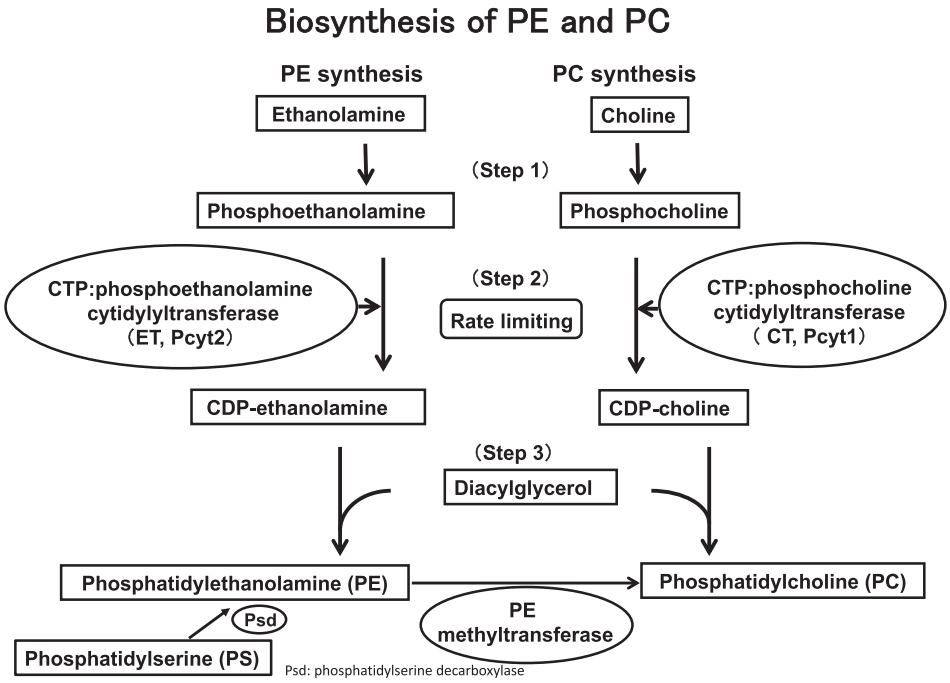

Figure 1 Biosynthesis of phosphatidylethanolamine (PE) and phosphatidylcholine (PC).

The Kennedy pathway and the rate limiting enzymes, Pcyt2 for PE synthesis and Pcyt1 for PC synthesis

In the de novo biosynthesis of phospholipids, PE and PC are synthesized via the CDP-ethanolamine and CDP-choline pathways (Fig. 1), which together are referred to as the Kennedy pathway. Synthesis occurs on the endoplasmic reticulum (ER), and each of these pathways involves a similar series of three sequential enzymatic reactions[2]. In step 1, ethanolamine/choline is phosphorylated by ethanolamine/choline kinase, producing phosphoethanolamine/phosphocholine. In step 2, CTP: phosphoethanolamine cytidylyltransferase (Pcyt2) or CTP: phosphocholine cytidylyltransferase (Pcyt1) catalyzes the transfer of CTP to phosphoethanolamine or phosphocholine, generating CDP-ethanolamine or CDP-choline. This second step is considered as the rate limiting step in these pathways. Finally in step 3, ethanolamine/choline phosphotransferase transfers CDP-ethanolamine/CDP-choline to diacylglycerol, producing PE or PC.

There are three other PE synthesis pathways: (1)

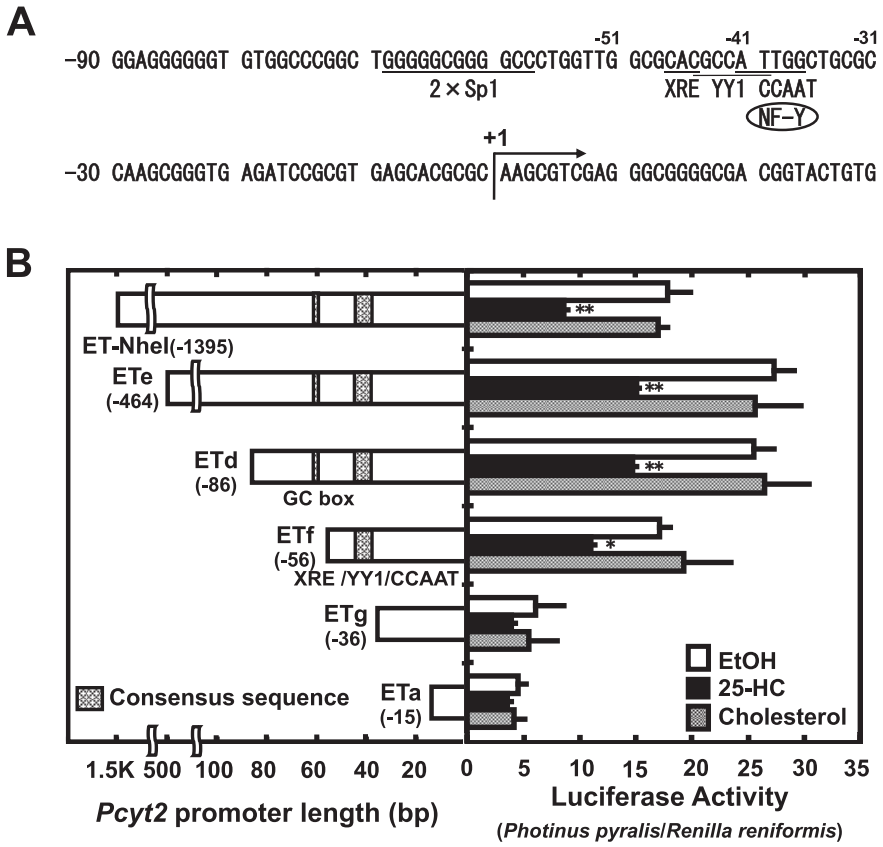

Figure 2 Mouse *Pcyt2* proximal promoter sequence and putative binding sites for transcriptional factors, and luciferase activities of *Pcyt2* promoter-reporter constructs.

Truncated *Pcyt2* promoter fragments ET-Nhe (-1395/+ 56), ETe (-464/+ 56), ETd (-86/+ 56), ETf (-56/+ 56), ETg (-36/+ 56) and ETa (-15/+ 56) were cloned into the luciferase reporter vector pGL4.24 and the constructs (375 ng) and pRL-CMV (5 ng) were transfected into NIH3T3 cells. 25-Hydroxycholesterol (25-HC) (black bars) or cholesterol (grey bars) (1.25 μM), or control vehicle (white bars), was added to the culture medium 12 h after transfection, and reporter activities were measured 24 h later. Luciferase activities (*Photinus pyralis*) were normalized for transfection efficiency relative to pRL-CMV (*Renilla reniformis*). $^{**}p < 0.01$ and $^{*}p < 0.03$ compared with cells treated with control vehicle (EtOH) or cholesterol. 25-HC, 25-hydroxycholesterol. All values are expressed as means ± S.D. Group means were compared by using Student's t test after ANOVA or one-way ANOVA with Tukey test to determine the significance of the differences between individual mean values. $P < 0.05$ was considered statistically significant. (Ando et al. BJ 2015 [12])

the phosphatidylserine decarboxylation (Psd) pathway in mitochondrial inner membranes (Fig. 1), (2) the acylation of Lyso-PE to PE, and (3) the base exchange pathway. The predominant PE biosynthesis pathways are the CDP-ethanolamine and Psd pathways[3]. Both Pcyt2-/- and Psd-/- are embryonic lethal phenotypes in mice[4,5], indicating that both pathways are essential for mouse development. In mitochondria[6], and especially in the absence of ethanolamine in vitro, the Psd pathway predominantly contributes to PE biosynthesis[7].

In humans, rats and mice, Pcyt2 (rate-limiting enzyme for PE biosynthesis) is a cytosolic enzyme[8] and is encoded by a single gene, *Pcyt2*. Two alternatively spliced forms of *Pcyt2* have been identified[9,10]. The human *PCYT2* promoter is TATA-less and driven by a functional CAAT box, and is regulated by early growth response factor-1 and nuclear factor-κB[11]. In C2 C12 cells, C/EBP CCAAT/enhancer-binding protein (C/EBP), specificity protein 1 (Sp1), Sp3 and MyoD are bound to the *Pcyt2* promoter and enhance its transcription during muscle cell differentiation[11]. Mouse *Pcyt2* and mouse *Hmgcr* promoters are shown in Fig. 2A and Fig. 3A, respectively, and consensus elements for several transcription factor binding sites important for these enzyme transcriptions are indicated[12].

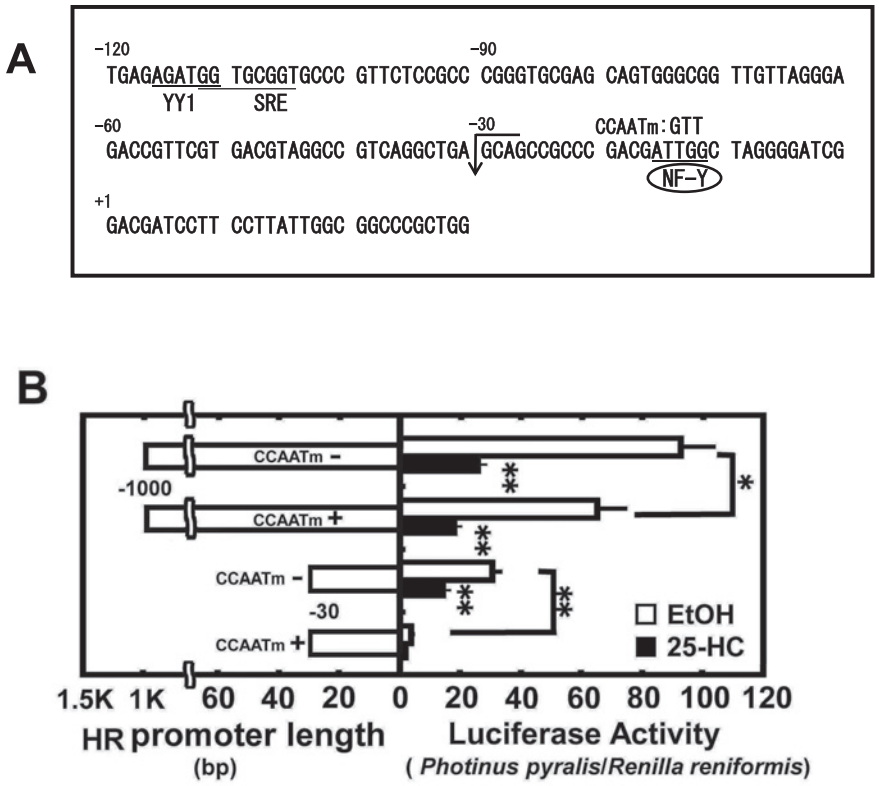

Figure 3 Mouse *Hmgcr* proximal promoter sequence and putative binding sites for transcriptional factors, and luciferase activities of *Hmgcr* promoter-reporter constructs.

The effect of 25-hydroxycholesterol (25-HC) was analyzed by the luciferase reporter activity of the prepared constructs. The HR (-1000/+ 61) or HR (-30/+ 61) promoter–luciferase plasmid, with or without mutation in the NF-Y-binding consensus sequence (T $^{-14}$GG $^{-12}$ changed to G $^{-14}$TT $^{-12}$), HR (-1000/+ 61) CCAATm or HR (-30/+ 61) CCAATm (500 ng) and pRL-CMV (25 ng) were transfected into NIH3T3 cells for 12 h. Then, 25-HC (1.25 μM) (black bars) or control vehicle (white bars) was added to the culture medium, and reporter activities were measured after 24 h incubation. Luciferase activities (*Photinus pyralis*) were normalized for transfection efficiency relative to pRL-CMV (*Renilla reniformis*). $^{**}p < 0.01$ and $^{*}p < 0.02$ compared with cells treated with control vehicle (EtOH). 25-HC, 25-hydroxycholesterol. (Ando et al. BJ 2015 [12])

The rate limiting enzyme, Hmgcr, for cholesterol synthesis

Cholesterol, the other major lipid in the cell membrane, is produced from acetyl-CoA by sequential enzymatic reactions, and its synthesis is regulated by a feedback system. The rate-limiting enzyme for cholesterol biosynthesis is HMGCR (3-hydroxy-3-methylglutaryl-CoA reductase), which synthesizes mevalonate from HMG-CoA and is regulated at both the transcriptional and post-translational levels[13]. This enzyme is the target of statins for treating hypercholesterolemia. Transcription of the *Hmgcr* gene is enhanced when cellular cholesterol levels are low. Specifically, SCAP [SREBP (sterol regulatory element-binding protein) cleavage activation protein] binds to SREBPs and escorts them from the ER to the Golgi, then active domains of SREBPs are released by two sequential cleavages by site 1 protease (S1P) and S2P. The released active domain binds to sterol regulatory element (SRE) and stimulates Hmgcr transcription. By contrast, cholesterol inhibits the release of SREBPs from ER[14]. Cholesterol derivatives such as hydroxylated cholesterol (oxysterols) also inhibit the release of SREBPs mediated by the product of the insulin-induced gene (Insig)[15] (Fig. 3A and B).

Transcriptional regulation of *Pcyt2* and *Hmgcr* by oxysterols

Previously, we reported that the enhanced transcription of *Pcyt2* in cells cultured in serum-starved medium can be suppressed by the addition of fetal bovine serum (FBS) or the lipid phase of FBS obtained by chlo-

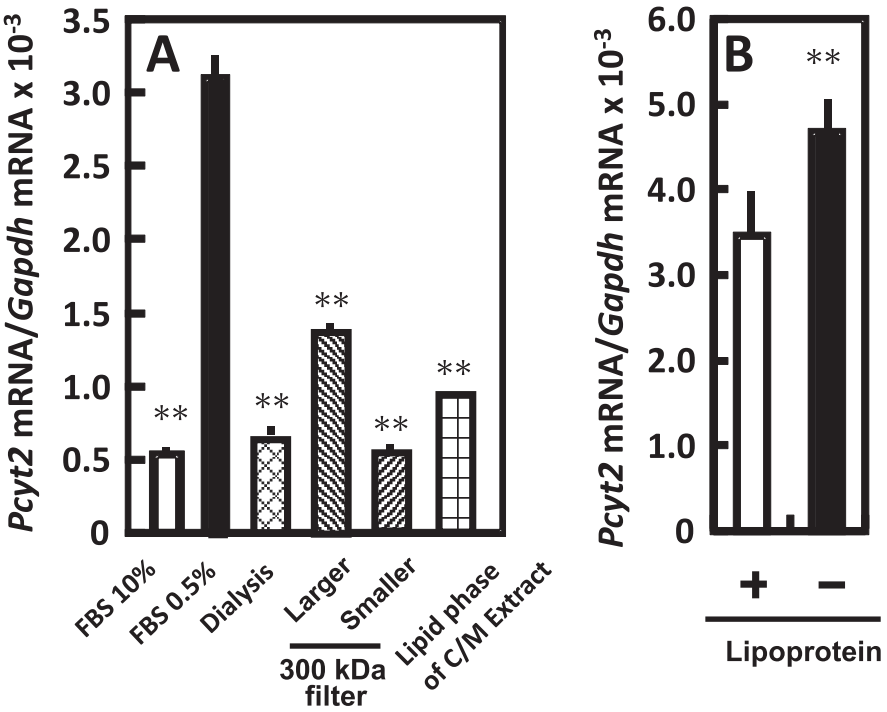

Figure 4 Inhibition of *Pcyt2* transcription by FBS and fractionated FBS samples, and effect of lipoprotein on *Pcyt2* transcription in NIH3T3 cells.

(A) After serum starvation (0.5% FBS) for 48 h, NIH3T3 cells were treated with 10% FBS (white bar), 0.5% FBS (black bar), dialyzed FBS (oblique cross striped bar), the larger (right oblique striped bar) or smaller (left oblique striped bar) phase of FBS after 300 kDa filter separation or the lipid phase (crossed striped bar) of FBS extracted using the Bligh & Dyer method for 12 h. Then, total RNA was extracted and mRNA levels of *Pcyt2* were quantified relative to *Gapdh*. $^{**}p < 0.01$ as compared to cells cultured in 0.5% FBS (black bar) **(B)** NIH3T3 cells were cultured in medium with lipoprotein containing serum (10% FBS) (white bar) or lipoprotein-deficient serum (black bar), then the *Pcyt2* mRNA levels were quantified relative to *Gapdh* after 24 h incubation. $^{**}p < 0.01$ as compared to cells cultured in 10% FBS. (Ando et al. BBA 2010 [16])

roform/ethanol separation (Bligh & Dyer Method) to the culture medium (Fig. 4). We identified low-density lipoprotein (LDL) and oxysterols such as 25-hydroxycholesterol (25-HC) as the compounds responsible for this suppression, in contrast to the regulation of *Pcyt1*α (most active and ubiquitous form of *Pcyt1*) transcription (Fig. 5). LDL and 25-HC can also suppress *Hmgcr* transcription in a similar dose-dependent manner[16] (Fig. 4 and 5). Oxysterols such as 25-HC, 24-HC and 27-HC suppress the transcription of *Pcyt2* and *Hmgcr* (Fig. 6). These oxysterols are known as LXR agonist, although *Pcyt2* and *Hmgcr* mRNA levels were not affected by treatment with the synthetic LXR agonists[12,17]. These results suggest that oxysterols are important negative regulators for maintaining the PE and cholesterol content in cell membranes by controlling the levels of *Pcyt2* and *Hmgcr* mRNAs, which in turn affect the levels of the Pcyt2 and Hmgcr enzymes.

In mammalian tissues, four cDNA isoforms of *Pcyt1* (rate-limiting enzyme for PC biosynthesis) have been reported[18,19]. Transcription factors that are involved in cell growth and division are important for the transcription of most active Pcyt1α in the cell[20,21]. We showed that the mRNA levels of *Pcyt1*α are not affected by treatment with LDL or 25-HC[16] (Fig. 5).

Importance of nuclear factor-Y for the regulation of *Pcyt2* and *Hmgcr* transcription by oxysterols

In the mouse *Pcyt2* promoter, SRE was not detected at the promoter region, as shown in Fig. 2A, in contrast to the *Hmgcr* promoter as shown in Fig. 3A. Therefore, we were interested in the transcriptional regulation of *Pcyt2*. We identified and characterized an element in the *Pcyt2* promoter at position -56 to -36 that is regulated by 25-HC by preparing and analyzing mouse deleted or mutated *Pcyt2* promoter-luciferase

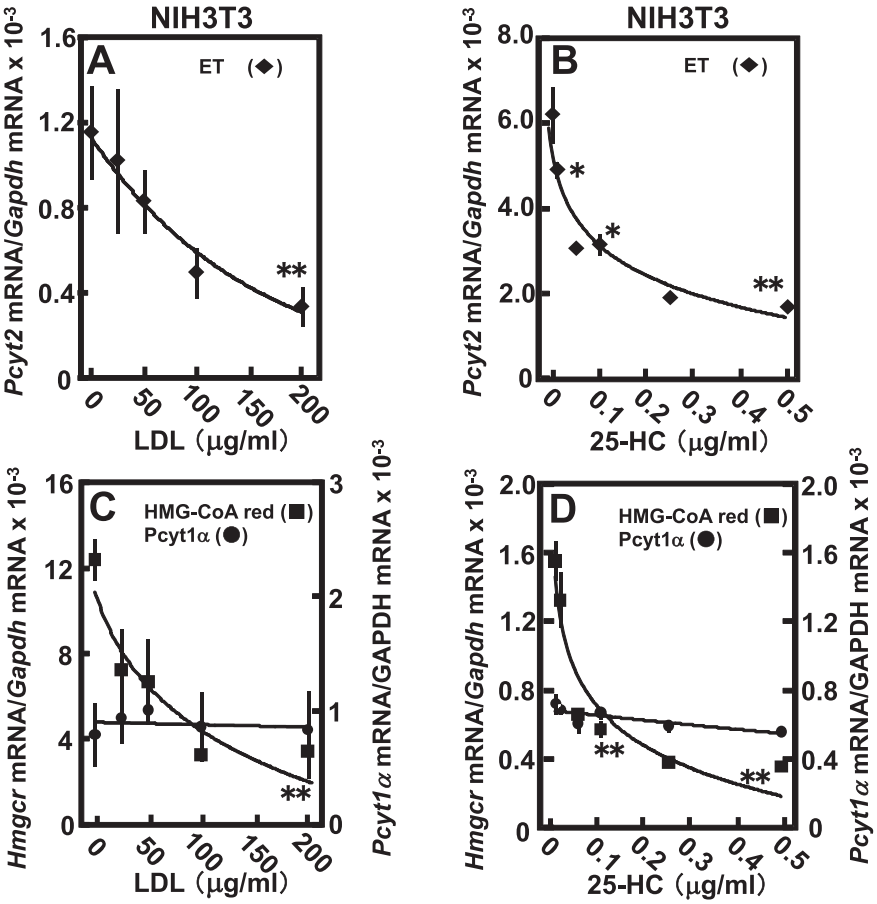

Figure 5 Inhibition of *Pcyt2* and *Hmgcr* transcriptions by LDL and 25-hydroxycholesterol.
After 48 h of serum starvation (0.5% FBS), NIH3T3 cells were incubated with several concentrations of **(A)** and **(C)** LDL (0-200 μg/ml) or **(B)** and **(D)** 25-hydroxycholesterol (25-HC) (0-0.5 μg/ml) for 12 h, then **(A)** and **(B)** mRNA levels of *Pcyt2* (closed diamonds) and **(C)** and **(D)** the mRNA levels of *Hmgcr* (closed squares) and *Pcyt1*α (closed circles) were quantified relative to *Gapdh*. $^{**}p < 0.01$ and $^{*}p < 0.05$ as compared to control cells incubated with vehicle. (Ando et al. BBA 2010 [16])

reporter constructs using NIH3T3 cells (Fig. 2B). Nuclear factor Y (NF-Y) and Yin Yang 1 (YY1) transcription factors were identified as proteins that can bind to this element (-41/-37) using a yeast one-hybrid system[12] and a chromatin immunoprecipitation (ChIP) assay (Fig. 7) and regulate transcription. The data suggest that 25-HC suppresses the co-operative activities of NF-Y and YY1, suppresses the interaction with RNA polymerase II at the *Pcyt2* promoter, and thus suppresses *Pcyt2* transcription.

NF-Y is a ubiquitous transcription factor that binds to the CCAAT box, which is present in 30% of eukaryotic promoters. NF-Y is composed of three subunits (NF-YA and NF-YB/NF-YC), all of which are necessary for binding to the CCAAT box. These three subunits are well conserved evolutionarily[22], and are important for RNA polymerase II recruitment for transcription initiation[23]. NF-Y is known to recruit histone acetyltransferase, such as p300, and enhance histone acetylation and transcription. We found that p300 binds to *Pcyt2* promoter, and that p300 binding and histone acetylation (H3K27) in *Pcyt2* promoter is suppressed by 25-HC[17].

In the mouse *Hmgcr* promoter, the NF-Y binding site was also detected at -16/-12 as analyzed by deleted and mutated promoter-luciferase reporter constructs (Fig. 3A). The -16/-12 element is important for cellular response to 25-HC[12]. NF-Y binds to this element, and binding of RNA polymerase II to this element was suppressed by 25-HC. The *Pcyt2* promoter region was also verified by ChIP analysis[12]. These results supported the new transcriptional regulatory machinery by oxysterols illustrated in Fig. 8A. This regulatory machinery is proposed for mouse cells and tissues because

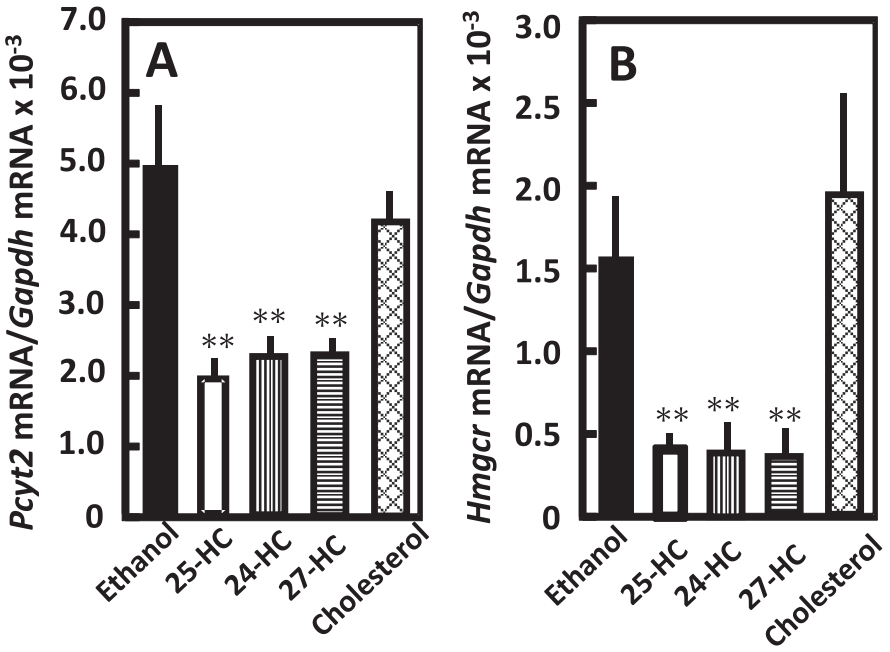

Figure 6 Inhibition of *Pcyt2* and *Hmgcr* transcription by several oxysterol species.
After serum starvation (0.5% FBS) for 48 h, NIH3T3 cells were treated with 0.5 μg/ml of the indicated oxysterols (25-hydroxycholesterol (25-HC) (white bar), 24-hydroxycholesterol (24-HC) (vertical striped bar) and 27-hydroxycholesterol (27-HC) (horizontal striped bar)) and cholesterol (oblique crossed striped bar) for 12 h. Then, total RNA was extracted and mRNA levels of *Pcyt2* **(A)** and *Hmgcr* **(B)** were quantified relative to *Gapdh*. $^{**}p < 0.01$ as compared to control cells incubated with control vehicle. (Ando et al. BBA 2010 [16])

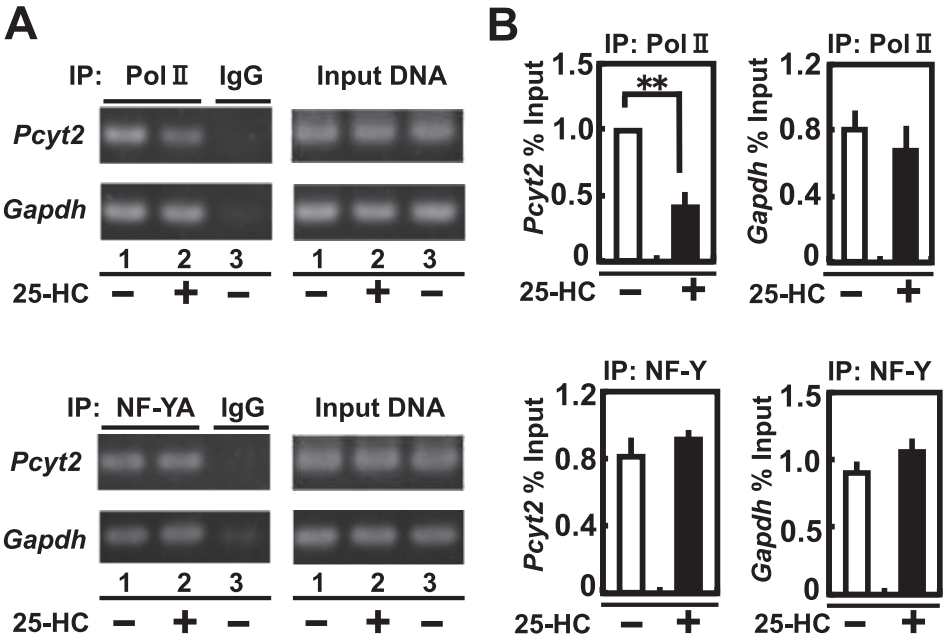

Figure 7 Binding of NF-Y and RNA polymerase II to the *Pcyt2* promoter region and the effects of 25-hydroxycholesterol determined by ChIP analysis.
(A) NIH3T3 cells were cultured in serum-starved medium (0.5% FBS) for 48 h, then were treated with or without 1.25 μM 25-hydroxycholesterol (25-HC). After 8 h of incubation, ChIP analysis was performed using anti-RNA polymerase II (Pol II) or anti-NF-YA antibody, or control IgG. The promoter region of *Pcyt2* (-96/+ 44) and *Gapdh* (-21/+ 144) were amplified using specific primer sets. **(B)** The band densities in **(A)** were analyzed. The data are densitometric analyses of the percentage input (n = 3). $^{*}p < 0.02$ and $^{**}p < 0.01$ compared with cells treated with control vehicle. (Ando et al. SBMB 2019 [17])

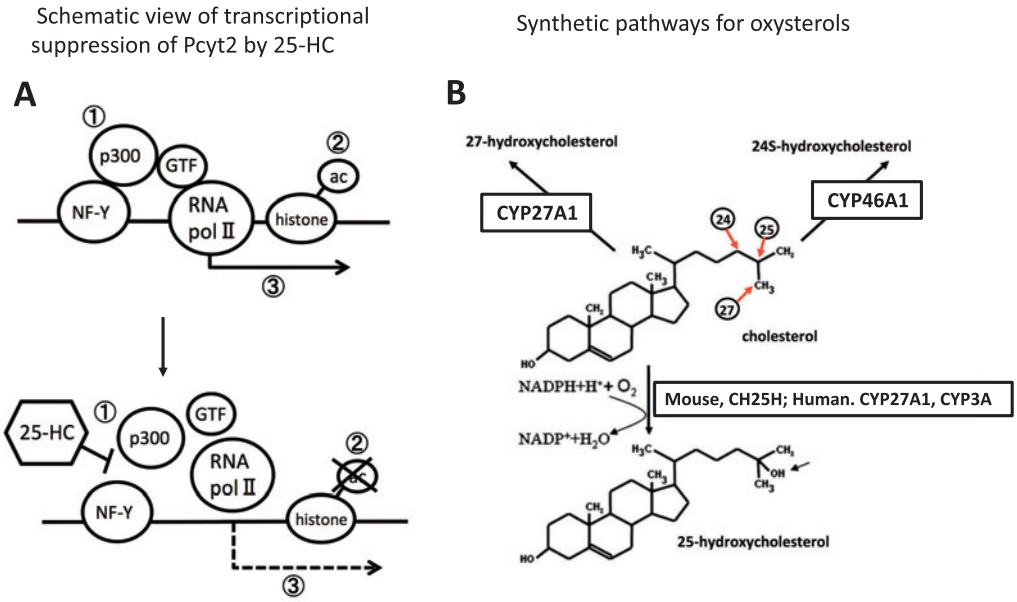

Figure 8 Schemes showing the inhibition of promoter activity by 25-hydroxycholesterol, and synthetic pathways for oxysterols.

(A) Upper panel: (1) NF-Y recruits p300 to the *Pcyt2* promoter, then (2) p300 acetylates H3K27 in the *Pcyt2* promoter, and (3) enhances *Pcyt2* transcription. Lower Panel: (1) 25-HC suppresses the recruitment of p300 to the *Pcyt2* promoter, (2) suppresses H3K27 acetylation, and (3) suppresses RNA polymerase II (pol II) binding to the *Pcyt2* promoter. GTF, general transcription factors. **(B)** Oxysterol production from cholesterol by cytochrome p450 46A1 (CYP46A1) (mainly for 24S-HC production), cytochrome p450 27A1 (CYP27A1) (for 27-HC production), cytochrome p450 3A (CYP3A) (for 25-HC production) and cholesterol 25-hydroxylase (CH25H) (for 25-HC production) in mouse.

NIH3T3 mouse cells were used in our experiments. The *Pcyt2* mRNA levels in mouse Hepa1 cells were also suppressed by FBS[16]. SRE systems may also be critical in human cells and tissues, since human HeLa cells respond to serum starvation similar to NIH3T3 cells[16].

IGF-II suppresses the 24-HC-producing enzyme cytochrome P450 46A1 (Cyp46A1)

In these experiments, oxysterols were applied to cells from the outside (Fig. 6) whereas intrinsic oxysterol-producing enzymes are expressed in cells (Fig. 8B)[24]. Therefore, we were interested in the regulation of oxysterol-producing enzyme activity inside cells. We recently showed that the 24-HC-producing enzyme cytochrome P450 46A1 (Cyp46A1) is the only enzyme expressed in NIH3T3 cells that synthesizes oxysterols, and its transcription and enzyme activity in NIH3T3 cells cultured under FBS starvation conditions were suppressed by FBS, as found for *Pcyt2* and *Hmgcr* (Fig. 9A). However, the lipid phase of FBS obtained by chloroform/methanol separation (Bligh & Dyer Method) showed no effect on *Cyp46A1* transcription whereas the aqueous phase clearly suppressed its transcription. We attempted to identify the suppressing factor in FBS, and identified insulin-like growth factor II (IGF-II) in the aqueous phase as the factor suppressing *Cyp46A1* transcription (Fig. 9B). The bovine IGF-II concentration in FBS was 324 ng/ml and in the aqueous phase obtained by the Bligh & Dyer method it was 135 nm/ml. Furthermore, IGF-II, IGF-I and insulin suppressed the oxysterol content in cells[24]. Insulin, IGF-I and IGF-II can affect to each other's receptor[25]. These results show that classical hormones such as IGFs and insulin suppress the transcription of oxysterol-producing enzymes and thus the oxysterol content in cells, and transduce cellular signaling. *CYP46A1*mRNA levels in the T98G human glioblastoma cell line were also increased by serum starvation but not by FBS supplementation, and IGF-II did not suppress the increase. Further studies on the regulation of oxysterol production by IGFs and insulin in cells are warranted.

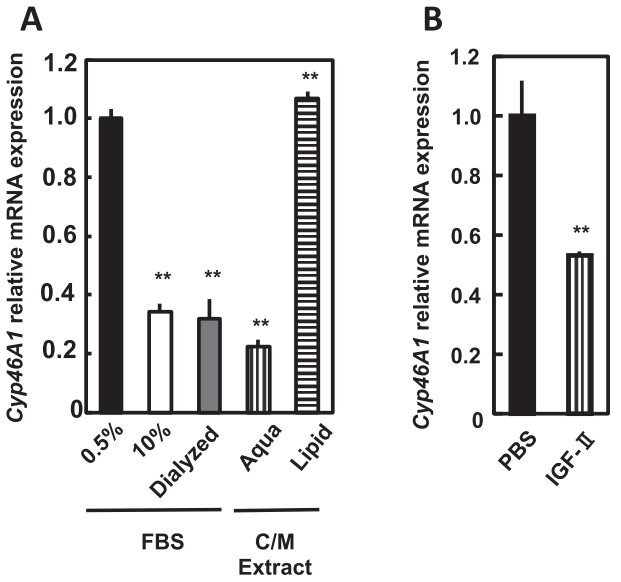

Figure 9 Inhibition of 24S-hydroxycholesterol-producing enzyme (Cyp46A1) transcription in NIH3T3 cells by FBS and fractionated FBS samples, and IGF-II.

(A) NIH3T3 cells were cultured in serum-starved medium (0.5% FBS) for 48 h, then treated with 0.5% FBS (black bar), 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. 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. $^{**}p < 0.02$ indicates significant differences as compared to cells cultured in 0.5% FBS. **(B)** NIH3T3 cells were cultured in serum-starved medium (0.5% FBS) for 48 h, then treated with IGF-II (0.5 μg/ml). After 6 h incubation, the mRNA levels of *CYP46A1* were quantified relative to *Gapdh* mRNA levels. $^{**}p < 0.01$ indicates significant differences as compared to cells treated with control vehicle. (Shinohara et al. BBA 2022 [24])

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