Dysregulation of Plasmalogen Homeostasis Impairs Cholesterol Biosynthesis

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Plasmalogen biosynthesis is regulated by modulating fatty acyl-CoA reductase 1 stability in a manner dependent on cellular plasmalogen level. However, physiological significance of the regulation of plasmalogen biosynthesis remains unknown. Here we show that elevation of the cellular plasmalogen level reduces cholesterol biosynthesis without affecting the isoprenylation of proteins such as Rab and Pex19p. Analysis of intermediate metabolites in cholesterol biosynthesis suggests that the first oxidative step in cholesterol biosynthesis catalyzed by squalene monooxygenase (SQLE), an important regulator downstream of the rate-limiting step catalyzed by the enzyme squalene monooxygenase (SQLE), is reduced by degradation of SQLE upon elevation of cellular plasmalogen level. By contrast, the defect of plasmalogen synthesis causes elevation of SQLE expression, resulting in the reduction of 2,3-epoxysqualene required for cholesterol synthesis, hence implying a novel physiological consequence of the regulation of plasmalogen biosynthesis.

Physiological significance of plasmalogen homeostasis remains unknown. Physiological consequences of the homeostasis of plasmalogen in cells are broad, including myelination, paranode organization, and movement of cell innervation is shown in a knock-out mouse defective in plasmalogen synthesis (6). We earlier demonstrated that Far1 activity is regulated by modulating its stability in response to the cellular level of plasmalogens (6, 7). Very recently, a plasmalogen-deficient disorder with intellectual disability, epilepsy, and cataract was shown in a FARI-defective patient, implying the essential function of Far1 in plasmalogen synthesis (8). However, the physiological consequence of the homeostasis of plasmalogen in cells remains poorly understood.

In the present study, we assessed the synthesis of lipids by modulating the cellular level of plasmalogens and found that homeostasis of plasmalogen is linked to cholesterol synthesis. Cholesterol synthesis is regulated by posttranslational and transcriptional mechanisms. The third step of cholesterol synthesis catalyzed by HMG-CoA reductase (HMGCR) is generally accepted as a rate-limiting step. HMGCR activity is mainly regulated via a sterol-mediated feedback mechanism at the level of transcription and endoplasmic reticulum-associated degradation of HMGCR (9). Recently, epoxidation of squalene catalyzed by the enzyme squalene monooxygenase (SQLE) is proposed as the second potential rate-limiting step in chole-

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†This article contains supplemental Table S1.

1 To whom correspondence should be addressed. Tel.: 81-92-642-4232; Fax: 81-92-642-4233; E-mail: yfujiki@kyudai.jp.

2 The abbreviations used are: ADAPS, alkyl-dihydroxyacetonephosphate synthase; DOS, 2,3;22,23-diepoxysqualene; 24,25-EC, 24,25-epoxycholesterol; Etn, ethanolamine; Far1, fatty acyl-CoA reductase 1; GPE, glycerylphosphoethanolamine; HMGCR, HMG-CoA reductase; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; LSS, lanosterol synthase; MARC6, membrane-associated RING finger 6; MOS, 2,3-monoepoxysqualene; PlsEttn, purified plasmalogens; SM, sphingomyelin; SQLE, squalene monooxygenase; SREBP, sterol regulatory element-binding protein.
terol biosynthesis (10–12). In the present study, we investigated if the cellular plasmalogen level plays a role in cholesterol homeostasis by modulating the cellular level of plasmalogens. We report here a novel consequence of cellular plasmalogen level in cholesterol homeostasis.

**Experimental Procedures**

**Materials**—Restriction enzymes and DNA-modifying enzymes were purchased from Nippon Gene and Takara. Ham’s F-12 and DMEM were from Invitrogen. [14C]Acetate and [14C]palmitate were purchased from Moravek Biocemi-cals. Ethanolamine (Etnt), lovatstatin, Ro48-8071, 2,3-monoepoxy-squalene (MOS), cholesterol, lanosterol, and squalene were purchased from Sigma. 24,25-Epoxycholesterol (24,25-EC) was purchased from Pep-pydrin Institute. Epoxomicin was purchased from Sigma. 24,25-Epoxycholesterol (24,25-EC) was purchased from Enzo Life Sciences. Epoxomicin was purchased from Pep-tide Institute.

**Cell Culture**—HeLa, HEK293, and human fibroblasts (6) were cultured in DMEM supplemented with 10% fetal bovine serum in 5% CO2 and 95% air (6). CHO-K1, were cultured in DMEM supplemented with 10% fetal bovine serum in 5% CO2 and 95% air (13, 14). These culture mediums were used for the metabolic labeling with [14C]acetate and [14C]palmitate. Plasmalogen level in HeLa and HEK293 cells was increased by adding 5 or 2 μM Etn, respectively (Fig. 2C). Elevation of plasmalogens in CHO-K1 cells were performed as described (6, 7). To increase cellular cholesterol, cells were cultured in the presence of 20 μg/ml of cholesterol complexed with methyl-β-cyclodextrin (15) for 16 h (12) or the indicated time.

**Lipid Analysis**—Cells were cultured for 5 h in the presence of 1 μCi/ml of [14C]acetate or [14C]palmitate (5, 6). Lipids were extracted from aliquots (100 μg protein) of cell lysates by the Bligh and Dyer method (16). For analysis of squalene, the extracted lipids were subjected to alkaline methanolysis (6) to separate cholesteryl ester from squalene on TLC. Lipids were analyzed on TLC plates (Silica Gel 60, Merck) with hexane/diethyl ether/acetic acid solution (v/v/v: 80/20/1.5). 14C-Labeled lipids were detected by autoradiography using a FLA-5000 imaging analyzer and quantified using an image analyzer software (Multi Gauge, Fuji Film). Plasmalogens and phosphatidylethanolamine were analyzed by TLC (6, 7, 13) or LC-ESI-MS/MS (17). Cellular free cholesterol was determined by the enzymatic method using an Amplex Red cholesterol assay kit according to the manufacturer’s instruction (Molecular Probes).

**Immunoblot Analysis**—Cells were harvested in homogenizing buffer (13). Aliquots of equal amounts of proteins were separated by SDS-PAGE, and subjected to immunoblotting with rabbit polyclonal antibodies to SQLE (Proteintech group), Pex19p (18), and Pex3p (19), and goat anti-lactate dehydroge-nase antibody (20). Mouse monoclonal antibodies to P450 reductase (Santa Cruz), actin (Millipore), Rab5 (BD Biosciences), Rab4 (BD Biosciences), α-tubulin (Thermo Scientific), and myc peptide (Santa Cruz) were used. After probing with HRP-conjugated secondary antibodies, immunoblots were developed with ECL reagents (GE Healthcare), and visualized by an LAS-4000 Mini luminescent image analyzer (Fuji Film). The intensity of bands was quantified by Multi Gauge version 3.0 software (Fuji Film).

**Construction of Expression Vector for MARCH6 and SQLE**—First strand cDNA was prepared from total RNA of HeLa cells. Human MARCH6 was amplified using a RT product as a template with a set of primers, HsMARCH6Fw.NotI (5′-ccgcgcgcc-cacctgcaccccggaggaagacatatgag-3′) and HsMARCH6 Rv.SalI (5′-tctccacagtctacagcgctcc-3′) and was digested with NotI and SalI. The resultant fragment was cloned into the NotI-Xhol site of pcDNA3.1/Zeo/PMP22-Myc6 and termed pcDNA3.1/Zeo/MARCH6-Myc6. pcDNA3.1/Zeo/MARCH6C9A-Myc6 was generated by inverse PCR using a set of primers HsMARCH6.C9A.Fw (5′-gggagagatcatacagctaggtgtg-3′) and HsMARCH6.C9A.Rv (5′-gggagagatcatacagctaggtgtg-3′). Human SQLE was likewise amplified using a set of primers, HsSQLE.Fw.NotI (5′-gggcggcgcctgagtttggttggtgctcagc-3′) and HsSQLE.Rv.Spel (5′-actagatagctagctagctagctagc-3′), and was digested with NotI and Spel. The resultant fragment was cloned into the NotI-Nhel site of pUCD2HygSRoPEX16-HA2 (21).

**Immunoprecipitation—SOLEHA2**—MARCH6-Myc6 and MARCH6C9A-Myc6 were transfected into CHO cells as described (22) and cultured for 2 days in the presence or absence of Etn. The cells were lysed for 5 min on ice with ice-cold phosphate-buffered saline containing 0.2% Triton X-100 and a mixture of protease inhibitors, and further solubilized at 4°C for 20 min. After centrifugation, cell lysates were subjected to immunoprecipitation using rabbit anti-Myc antibody. Polyclonal antibody to myc peptide was raised in rabbits by injection of the c-Myc peptide, CYILSVQAEEQKLISEEDL.

**siRNA-mediated Knockdown of MARCH6**—MARCH6 knockdown in HeLa cells was performed using siRNA purchased from MISSION RNA (Sigma). EGFP siRNA designed by Stealth™siRNA was used for a control (Invitrogen). Target sequences of siRNA are as follows: human MARCH6, 5′-CTT-AGTCATCGCGGATT-3′ and EGFP, 5′-CACATGAGACGCACGACTTCTCA-3′.

**Real-time PCR**—Extraction of total RNA from cells, synthesis of first-strand cDNA, and quantitative real-time PCR were performed using the housekeeping gene porphobilinogen deami-nase (PBGD) (23) or ribosomal protein 3 (RPL3) (24) as internal control (17). Primers for Chinese hamster SQLE (12) and LSS (25), and human LSS and HMGCR (24) were listed in supplemental Table S1.

**Data Presentation**—Quantitative data were shown as mean ± S.D. from three independent experiments.

**Results**

**Elevation of Plasmalogen Level Reduces Cholesterol Synthesis**—To explore physiological consequences of the homeostasis of plasmalogens, cellular plasmalogens were elevated by supplementing CHO-K1 cells with purified plasmalogens (PlsEtn) or Etn and assessed the biosynthesis of lipids by labeling with either [14C]acetate or [14C]palmitate. Supplementation of Etn increased cellular plasmalogens and phosphatidylethanolamine about 1.5 times more than those in CHO-K1 cells. When cells were cultured in the presence of

3 Y. Yagita and Y. Fujiki, unpublished data.
Plasmalogens Regulate Cholesterol Biosynthesis

PleS, plasmalogens were increased nearly by 2-fold, whereas
phosphatidylethanolamine levels were not altered (Fig. 1, A and
C). By liquid chromatography-electrospray ionization-tandem
mass spectrometry (LC-ESI-MS/MS) analysis, plasmalogens
containing 18:0 and 18:1, but not 16:0 fatty alcohols at the
respective sn-1 position were shown to be increased in the pre-
sence of PleS, which was most likely due to the lesser amount
of bovine brain-derived plasmalogens containing 16:0 fatty
alcohol (data not shown). In contrast, all species of plasmalo-
gens were elevated in the presence of Etn (Fig. 1, D–I). When
CHO-K1 cells were metabolically labeled with [14C]acetate in
the presence of Etn or PleS, cholesterol synthesis was signif-
icantly reduced, whereas synthesis of glycerophospholipids
including phosphatidylcholine and serine- and inositol-con-
taining phospholipids (phosphatidylerine and phosphatidyl-
inositol, respectively) were not altered (Fig. 1, A and B). In these
conditions, cellular free cholesterol was not altered (Table 1).
Similarly, reduction of cholesterol synthesis was also observed
in HeLa and HEK293 cells upon culturing with Etn (Fig. 2, A
and B). The cellular level of plasmalogens in HeLa and HEK293
cells was increased about 1.5 times as compared with that in the
untreated respective cells (Fig. 2C). Taken together, these
results strongly suggest that cholesterol biosynthesis is specifi-
cally reduced by elevation of the cellular level of plasmalogens.

Elevation of Plasmalogen Level Down-regulates Cholesterol
Synthesis Steps at Post-isoprenoid Biosynthetic Pathway—We
further investigated which step of the cholesterol synthesis is
affected upon elevation of plasmalogens. Cholesterol is synthe-

**FIGURE 1. Elevation of cellular plasmalogens level lowers cholesterol synthesis.** A, CHO-K1 cells were cultured for 43 h in the absence (−) or presence of purified plasmalogens (PleS) from bovine brain or Etn, and further incubated for 5 h with [14C]acetate. The biosynthesis of lipids including cholesterol (upper panel) and phospholipids (upper middle panel) were detected with a FLA-5000 imaging analyzer. Cellular levels of cholesterol (lower middle panel), plasmalogens (2-acyl-GPE), phosphatidylcholine (PC), and sphingomyelin (SM) were detected (lower panel) with iodine vapor. Note that biosynthesis of cholesterol is reduced in cells cultured in the presence of PleS or Etn (lanes 2 and 3), as compared with that under the normal culture condition (lane 1). PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol. B, relative levels of newly synthesized cholesterol show the level of cholesterol synthesis in CHO-K1 (−) was designated as 100. *p < 0.05; Student’s t test compared with CHO-K1 (−). C, lipids were extracted from CHO-K1 cells cultured as described in A and analyzed by LC-ESI-MS/MS. Total amounts of plasmalogens (left panel) and PE (right panel) from cells cultured in the absence (−, solid bar) or presence (−) of PleS (gray bar) or Etn (open bar) were shown. *, p < 0.01; t test versus CHO-K1 (−). D, lipids were extracted as described in A. Total amounts of plasmalogens with alkanyl 16:0 (left panel), 18:0 (middle panel), and 18:1 (right panel) at the sn-1 position are shown. *, p < 0.05; t test versus CHO-K1.

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<th>Table 1 Quantitation of free cholesterol</th>
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<td>CHO-K1</td>
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*a Cell lines were cultured with plasmalogens purified from bovine brain, Etn, ethanolamine, and cholesterol as described under “Experimental Procedures.”
*b Free cholesterol in cell lines was determined by the enzymatic method using an Amplex Red cholesterol assay kit as described under “Experimental Procedures.”
*c Values are mean ± S.D.
sized via the isoprenoid biosynthetic pathway. If the increased level of plasmalogens suppresses the rate of any steps of isoprenoid synthesis including HMGCR, a rate-limiting enzyme of the cholesterol synthesis, farnesylation, and/or geranylgeranylation of proteins such as Pex19p (18, 26) and Rab5 (27) would be reduced as reported (28). Therefore, we verified the isoprenylation of Pex19p, Rab5, and Rab4 (Fig. 3A). When cells were treated with lovastatin, an HMGCR inhibitor, the non-farnesylated form of Pex19p and non-geranylgeranylated form of Rab5 and Rab4 were recovered in the cytosol fraction, each with a slower mobility than the modified form in SDS-PAGE, consistent with a previous report (28). However, these unmodified Pex19p, Rab5, and Rab4 were not observed when plasmalogen levels were elevated in CHO-K1, hence indicating that isoprenoid synthesis was not altered by the elevation of plasmalogens.

We next determined the level of sterol intermediates in the cholesterol biosynthetic pathway. Lanosterol, the first sterol intermediate in cholesterol biosynthesis, is synthesized via condensation of isoprene to squalene and subsequent oxidation of squalene to MOS, followed by cyclization of MOS. 24,25-EC is generated de novo by a shunt of the cholesterol biosynthetic pathway through synthesis of 2,3,22,23-diepoxyoxsqualene (DOS) from MOS (29). When CHO-K1 cells were metabolically labeled with $[^{14}C]$acetate, synthesis of MOS, cholesterol, and 24,25-EC was apparently reduced upon elevation of plasmalogens (Fig. 3B). In HeLa cells, MOS, lanosterol, cholesterol, and 24,25-EC were detected at a reduced level upon the elevation of plasmalogens. When lanosterol synthase (LSS) activity was partially inhibited by Ro48-8071, an inhibitor of LSS (30, 31) in HeLa cells, synthesis of lanosterol and cholesterol was lowered, whereas marked accumulation of MOS and slight elevation of 24,25-EC were observed (Fig. 3C), distinct from the results obtained from the elevation of plasmalogens, suggesting that LSS activity is not suppressed upon the elevation of plasmalogens. Moreover, the squalene level was slightly elevated by the increase of plasmalogens in CHO-K1 and HeLa cells (Fig. 3, B and C). Taken together, these results suggest that the oxidation step catalyzed by SQLE is most likely down-regulated by the elevation of plasmalogens.
Elevation of Plasmalogens Causes Degradation of SQLE in a MARCH6-dependent Manner—We next assessed the expression level of SQLE in HeLa cells. Upon elevation of the plasmalogen level, expression of SQLE was reduced to about 50% as compared with the untreated cells, whereas the expression level of P450 reductase (P450R), an endoplasmic reticulum enzyme, was not altered by the elevation of plasmalogens. The reduced SQLE level was fully recovered to that of untreated cells by treatment of the cells with epoxomicin, a proteasomal inhibitor (Fig. 4, A, lane 4). The transcription level of SQLE, LSS, and HMGCR was not altered upon the elevation of plasmalogens (Fig. 4B), where the transcription level of these enzymes was lowered with rapamycin (24). Furthermore, cycloheximide chase experiments using HeLa cells in the presence of Etn revealed that elevation of plasmalogens stimulated degradation of SQLE as compared with the turnover of SQLE in the absence of Etn (Fig. 4C). Taken together, these results suggest that SQLE is specifically degraded upon elevation of plasmalogens in a proteasome-dependent manner.

SQLE is reported to be degraded by membrane-associated RING finger 6 (MARCH6) in a manner dependent on cholesterol (32, 33). Therefore, we investigated whether plasmalogen-dependent degradation of SQLE is also mediated by MARCH6 by expressing MARCH6 or MARCH6C9A, an inactive form of
Plasmalogens Regulate Cholesterol Biosynthesis

MARCH6 (Fig. 4D). Expression of MARCH6C9A-MycC6 elevated the level of SQLE, whereas the expression of MARCH6-MycC6 slightly reduced the SQLE level, consistent with the earlier studies (32). The expression level of MARCH6 was lower than MARCH6C9A, most likely due to the degradation mediated by autoubiquitination of wild-type MARCH6 (32, 34). Degradation of SQLE was stimulated by expression of MARCH6-MycC6 in the presence of Etn, which was partially inhibited by the expression of MARCH6C9A-MycC6. Moreover, plasmalogen-dependent degradation of SQLE was interfered by the treatment of dsRNA against MARCH6 (Fig. 4E), where the transcriptional level of MARCH6 was reduced about 50% (data not shown). In addition, turnover of a cholesterol-dependent degradation of SQLE in plasmalogen-elevated cells had a similar tendency to that in the cells cultured in the absence of Etn, where the cellular free cholesterol level was slightly lowered in plasmalogen-elevated cells (Fig. 4F). Taken together, these results strongly suggested that SQLE is degraded in a MARCH6-dependent manner upon elevation of plasmalogens.

Cholesterol Synthesis in Plasmalogen-deficient Cells—We next investigated cholesterol synthesis in plasmalogen-deficient cells such as fibroblasts from an ADAPS-deficient patient and an ADAPS-defective CHO mutant, adaps ZPEG251 (6, 13). Cholesterol was more efficiently synthesized than 24,25-EC in human fibroblasts from a normal control (Fig. 5A). In contrast, 24,25-EC was more effectively synthesized than cholesterol in fibroblasts from an ADAPS-deficient patient. In addition, there was the same tendency in the sterol synthesis in plasmalogen-deficient CHO cell mutant, adaps ZPEG251 (Fig. 5B), as indicated in the ratio the 24,25-EC/cholesterol (Fig. 5B, lower panel). These results suggest that the abnormally low level of plasmalogens causes suppression of cholesterol synthesis.

In the cholesterol synthetic pathway, MOS is converted to cholesterol by the action of two enzymes: LSS and ADAPS. Therefore, synthesis of cholesterol by lowering the cellular amount of MOS available for the synthesis of cholesterol is most likely due to the limited amount of MOS available for the synthesis of cholesterol.

Expression of SQLE Is Elevated in Plasmalogen-deficient Cells—Synthesis of 24,25-EC is modulated by the relative activities of SQLE and LSS (36). Partial inhibition of LSS activity or overexpression of SQLE stimulates 24,25-EC synthesis (31, 38). However, accumulation of DOS was not observed in the absence of Ro48-8071 in ZPEG251 (data not shown), suggesting that the reduced activity of LSS is unlikely for the preferential DOS production in plasmalogen-deficient cells. Therefore, we investigated the expression level of SQLE in plasmalogen-deficient cells including ADAPS-defective fibroblasts and ZPEG251 and found that SQLE was expressed at about a 2-fold higher level in plasmalogen-deficient cells, suggesting that the expression level of SQLE is elevated in plasmalogen-deficient cells.

FIGURE 4. Increase in cellular plasmalogens reduces the levels of SQLE. A, left panel. HeLa cells were cultured with Etn (lanes 3 and 4) for 43 h and further cultured for 5 h in the presence (lanes 1 and 4) or absence (lanes 2 and 3) of 10 μM epoxomicin (Epoxy), an inhibitor of proteasome. Expression level of SQLE, P450 reductase (P450R), and α-tubulin (α-Tub) was assessed by immunoblotting with antibodies as indicated. Left: Cellular plasmalogens were detected with iodine vapor (lower panel) and LC-ESI-MS/MS (lower graph), respectively. The relative plasmalogens levels was represented by taking as 1 that in mock-treated HeLa cells. *, p < 0.01; † test versus a control; ‡, p < 0.01; † test versus Etn, B, cells were cultured in the absence (solid bar) or presence of Etn (gray bar) for 48 h or 50 nm rapamycin, an inhibitor for mammalian target of rapamycin (mTOR) (open bar) for 18 h. Expression levels of SQLE, LSS, and HMGCGR are represented by taking those as 100 in mock-treated cells (n = 3). Analysis of the expression level of the respective mRNAs in the presence of rapamycin was performed (n = 1). C, left panel. HeLa cells were cultured in the absence (upper panel) or presence (lower panel) of Etn for 40 h and further incubated for 8 h in the presence of cycloheximide (CHX, 100 μg/ml) and assessed for the expression of SQLE at each time point. Middle panel. SQLE bands at each time point were quantified. Relative amounts of SQLE in the absence (square) or presence (triangle) of Etn at each time point were represented by taking as 100 that at the time point of cycloheximide addition. *, p < 0.05; † test versus SQLE level at the same time in the absence of Etn. Right panel, cellular plasmalogens were detected by LC-ESI-MS/MS and relative plasmalogens levels at the time point of cycloheximide addition were shown. *, p < 0.05; † test versus a control. Dot indicates a truncated form of SQLE. D, left panel. HeLa cells transfected with mock vector (−), an E3 ligase, MARCH9-MycC(Wt), or a MARCH mutant, MARCH6C9A-MycC (C9A) were divided into two dishes and cultured for 2 days in the absence (−) or presence (+) of Etn. Expression level of SQLE, MARCH6, and tubulin and plasmalogens levels were analyzed as in A. Dot indicates a truncated form of SQLE. Right panel, the relative expression level of SQLE was represented by taking as 100 that in mock-treated HeLa cells. *, p < 0.05; † test versus a control, ‡, p < 0.05; † test versus Etn without MARCH6 expression, PC, phosphatidyicholine. E, HeLa cells treated with either control or dsRNA against MARCH6 were cultured for 48 h in the presence (+) or absence (−) of Etn. The expression levels of SQLE and actin were assessed by immunoblotting with specific antibodies as indicated on the left (upper panel). Dot indicates a truncated form of SQLE. Cellular plasmalogens were detected as in A and relative plasmalogens levels were represented by taking as 1 that in control dsRNA-treated HeLa cells (middle panel). Relative expression level of SQLE (lower panel) was represented by taking as 1 that in control dsRNA-treated HeLa cells (lane 1). *, p < 0.05; † test versus a control dsRNA-treated HeLa cells, n.s., not significant. F, left panel. HeLa cells were cultured as described in C, except that the cells were further incubated for 4 h in the presence of cycloheximide (CHX, 100 μg/ml) plus cholesterol (Chol) (20 μg/ml). The cells were assessed for the level of SQLE at each time point. Dot indicates a truncated form of SQLE. Middle panel, relative amounts of SQLE in the absence (square) or presence (triangle) of Etn at each time point were represented by taking as 100 that at the time point of cycloheximide addition and cholesterol addition (+Chol). The relative expression level of SQLE in the absence (diamond) or presence (blue triangle) of Etn without adding cholesterol (−Chol) was obtained from C. *, p < 0.05; † test versus SQLE level at the same time point indicated by a square. Right panel, cellular cholesterol (upper panel) and plasmalogens (lower panel) were determined by the enzymatic method and LC-ESI-MS/MS, respectively. Relative cholesterol levels at the time point of cycloheximide addition and after culturing for 4 h in the presence of cholesterol were represented by taking 1 that in HeLa cells at the time point of cycloheximide addition. Plasmalogens level was likewise represented. *, p < 0.05; † test versus a control, ‡, p < 0.05; † test versus cholesterol loading-HeLa cells.
**Plasmalogens Regulate Cholesterol Biosynthesis**

**FIGURE 5. Synthesis of sterols in plasmalogen-deficient cells.** A, fibroblasts derived from a healthy control (Cont.) and a patient defective in ADAPS (adaps) were metabolically labeled for 5 h with [14C]acetate and assessed for sterol synthesis. Origin indicates the spots where the extracted lipids were placed on TLC (upper panel). Synthesis levels of 24,25-EC and cholesterol in control (solid bar) and adaps-deficient (open bar) fibroblasts were shown (lower panel). *, p < 0.01; t test versus control fibroblasts. B, synthesis of MOS (upper panel) and sterols (middle panel) in CHO-K1, adaps ZPEG251, and ZPEG251/ADAPS-HA2 were analyzed. Note that synthesis of cholesterol and MOS was specifically abrogated in ZPEG251. The ratio of 24,25-EC to cholesterol is presented (lower panel). *, p < 0.01; t test versus CHO-K1. **, p < 0.01; t test versus ZPEG251. C, ZPEG251/ADAPS-HA2, ZPEG251, and ZPEG251 that had been treated with double-strand RNA against FAR1 were metabolically labeled with [14C]acetate and analyzed by autoradiography for synthesis of MOS (upper panel) and accumulation of fatty alcohol (middle panel). Relative amount of MOS was determined as taking as 100 that in ZPEG251/ADAPS-HA2 lower panel. *, p < 0.01; t test versus ZPEG251/ADAPS-HA2. CHO-K1, ZPEG251, and ZPEG251/ADAPS HA were metabolically labeled with [14C]acetate in the presence of the inhibitor. Synthesis of DOS (upper panel) and sterols (middle panel) was analyzed. Relative amount of DOS was determined as taking as 100 that in CHO-K1 lower panel. *, p < 0.01; t test versus CHO-K1. **, p < 0.01; t test versus ZPEG251.

SQLE Interacts with MARCH6 in a Plasmalogen-dependent Manner—Knockdown of MARCH6 stabilizes SQLE under normal culture conditions and abolishes cholesterol-dependent degradation of SQLE (32, 33). Therefore, we investigated if the absence of plasmalogens results in degradation or inactivation of MARCH6, giving rise to the elevation of the expressed levels of SQLE. However, addition of cholesterol in wild-type CHO-K1 and ZPEG251 similarly promoted degradation of SQLE (Fig. 7A), suggesting that expression and activity of MARCH6 was not altered in the absence of plasmalogens.

We further assessed whether plasmalogens regulate interaction of SQLE with MARCH6 by a coimmunoprecipitation assay. HA-tagged SQLE (SQLE-HA2) plus MARCH6-Myc6 or MARCH6C9A-Myc6 were coexpressed in ZPEG251 and CHO-K1 cells, and subjected to immunoprecipitation using anti-Myc antibody. SQLE-HA2 was coimmunoprecipitated with MARCH6C9A-Myc6 in ZPEG251 and CHO-K1 (Fig. 7C), consistent with the earlier study (32). Moreover, a larger amount of SQLE-HA2 was coimmunoprecipitated with MARCH6C9A-Myc6 in ZPEG251 and CHO-K1 (Fig. 7C), consistent with the earlier study (32). Moreover, a larger amount of SQLE-HA2 was coimmunoprecipitated with MARCH6C9A-Myc6 in ZPEG251 and CHO-K1 (Fig. 7C), consistent with the earlier study (32). Moreover, a larger amount of SQLE-HA2 was coimmunoprecipitated with MARCH6C9A-Myc6 in ZPEG251 and CHO-K1 (Fig. 7C), consistent with the earlier study (32). Moreover, a larger amount of SQLE-HA2 was coimmunoprecipitated with MARCH6C9A-Myc6 in ZPEG251 and CHO-K1 (Fig. 7C), consistent with the earlier study (32). Moreover, a larger amount of SQLE-HA2 was coimmunoprecipitated with MARCH6C9A-Myc6 in ZPEG251 and CHO-K1 (Fig. 7C), consistent with the earlier study (32). Moreover, a larger amount of SQLE-HA2 was coimmunoprecipitated with MARCH6C9A-Myc6 in ZPEG251 and CHO-K1 (Fig. 7C), consistent with the earlier study (32).

**Discussion**

In the present study, we show that the cellular plasmalogen level regulates cholesterol synthesis by modulating SQLE stability. Cholesterol synthesis is shown to be controlled at multiple steps, including sterol regulatory element-binding protein (SREBP)-mediated transcriptional regulation and post-translational regulation of HMGCRC, a rate-limiting enzyme of cholesterol synthesis (39, 40). In addition, recent studies revealed that the activity of SQLE is controlled at a post-translational level through the cholesterol-dependent ubiquitination and proteasomal degradation (12, 32, 33). In the present study, our finding that elevation of cellular plasmalogens also causes suppression of cholesterol synthesis in several cell lines (Figs. 1 and 2), implies that plasmalogen-dependent degradation of SQLE is a conserved mechanism for the regulation of cholesterol synthesis.

MARCH6 is an E3 ligase responsible for the degradation of SQLE in response to an exogenous cholesterol influx (32, 33). SQLE expression is elevated by reduction of MARCH6 (32, 33) (Fig. 4E), expression of an inactive form of MARCH6 (33) (Fig. 4D), or treatment of cells with epoxymycin (Fig. 4A), hence suggesting that SQLE is constitutively degraded in a MARCH6-dependent manner. Indeed, SQLE is constitutively degraded in SRD-1 cells (32), HeLa (Fig. 4C), and normal control fibroblasts (Fig. 6C). Moreover, SQLE interacts with MARCH6 without addition of external cholesterol (Fig. 7B) (32), where the interaction is increased upon elevation of cellular plasmalogens. Taken together, we suspect that the constitutive degradation of SQLE is more likely stimulated by the elevation of plasmalogens, but not cholesterol. Exogenous supplementation of cholesterol to the culture cells induces degradation of SQLE (12, 32, 33).

higher level in plasmalogen-deficient cells than that in control fibroblasts and CHO-K1 cells (Fig. 6, A and B). Quantitative RT–PCR analysis showed that the mRNA expression level of SQLE in plasmalogen-deficient cells was not altered as compared with that in control cells (Fig. 6, A and B, lower panels). Furthermore, cycloheximide chase experiments revealed that SQLE in plasmalogen-deficient fibroblasts was more stable than that in control cells (Fig. 6C). Collectively, these results suggest that SQLE is stabilized in plasmalogen-deficient cells more likely by a post-translational mechanism.
by enhancing MARCH6-mediated ubiquitination (32). However, cholesterol-dependent degradation of SQLE in plasmalog-en-deficient cells was likewise detected as that in wild-type cells (Fig. 7A). Moreover, elevation of plasmalogens did not synergistically augment the cholesterol-dependent degradation of SQLE in HeLa cells (Fig. 4F). Accordingly, we suggest that plasmalogens augment the interaction of SQLE with MARCH6 by a mechanism distinct from that involving cholesterol, although the mechanism underlying cholesterol-dependent interaction between SQLE and MARCH6 is not defined.

In humans, MARCH6 is expressed in several different tissues including heart, brain, kidney, and liver (41). However, only three proteins, including SQLE, HMGCR, and type 2 iodothyronine deiodinase, have been so far identified as a substrate for MARCH6.

**FIGURE 6. Expression level of SQLE is elevated in the absence of plasmalogens.** A, expression level of SQLE was assessed by immunoblotting in fibroblasts derived from a control (Cont.) and a patient defective in ADAPS (adaps) (upper panel). Actin was used as a loading control. Relative expression levels of SQLE were represented by taking as 100 that in control fibroblasts (middle panel). *, p < 0.05; t test versus control. Transcription levels of SQLE and LSS in control (Cont., solid bar) and ADAPS-defective fibroblasts (adaps, open bar) were assessed by real-time PCR (lower panel). Relative expression levels of SQLE and LSS are represented by taking that as 100 in control fibroblasts. B, expression level of SQLE was assessed as in A in CHO-K1, ZPEG251, and ZPEG251/ADAPS-HA2 (upper panel). Tubulin (α-Tub) was used as a loading control. The relative expression level of SQLE was represented by taking as 100 that in CHO-K1 (middle panel). *, p < 0.01; t test versus CHO-K1. **, p < 0.01; t test versus ZPEG251. Relative expression levels of SQLE and LSS to the housekeeping gene PBGD (porphobilinogen deaminase) (12, 23) was analyzed by real-time PCR using total RNA prepared from CHO-K1 (solid bar), adaps ZPEG251 (gray bar), and ZPEG251/ADAPS-HA2 (open bar) (lower panel). Relative expression levels of SQLE and LSS are represented by taking that as 100 in CHO-K1 cells (n = 3). C, SQLE is more stable in adaps fibroblasts. Control (Cont.) and ADAPS-defective (adaps) fibroblasts were cultured for 9 h in the presence of cycloheximide (100 μg/ml) and assessed for the expression level of SQLE at each time point (left panel). Relative amounts of SQLE in control (triangle) and ADAPS-defective (square) fibroblasts at each time point were represented by taking as 100 that at the time point of cycloheximide addition (right panel).
MARCH6-mediated degradation in mammals (32, 33, 42). However, MARCH6-mediated degradation of HMGCR seems to be independent of plasmalogens because isoprenylation of Pex19p, Rab5, and Rab4 (Fig. 3A), and squalene synthesis (Fig. 3, B and C) were not reduced by the increment of plasmalogens. Further studies including identification of more potential substrates of MARCH6 and the effects of plasmalogens on the degradation of such substrates are clearly required.

Sterol-dependent degradation of SQLE seems to be a conserved mechanism in higher eukaryotes (43). Sterol-dependent degradation of ERG1, the SQLE homologue, is likewise mediated by MARCH6 homologue Doa10 in Saccharomyces cerevisiae (33). A similar mechanism was postulated (32, 44) from the findings that accumulation of squalene in the SQLE-defective plant was restored when a mutant allele of the plant homologue of MARCH6, SUD1, was crossed into this background (32, 44). However, plasmalogens are not synthesized in plant (2, 45), whereas only trace amounts of plasmalogens are detected in S. cerevisiae (46). Given these findings, we suggest that plasmalogen-mediated degradation of SQLE is specific for mammals. SQLE apparently spans the endoplasmic reticulum membrane via its hydrophobic segment located in the N-terminal region (12, 47), although the membrane topology of SQLE is not yet defined. Interestingly, the region encompassing the 100-amino acid sequence including a potential transmembrane domain of human SQLE is sufficient for cholesterol-dependent degradation (12). However, this region is absent from yeast ERG1, and only a few amino acids are conserved between plant and human

**FIGURE 7. Plasmalogens modulate the interaction of SQLE with MARCH6.** A, CHO-K1 and adaps ZPEG251 were cultured for 16 h in the presence of cholesterol (20 μg/ml). CHO-K1 and ZPEG251 were cultured for 16 h in the presence of cholesterol (20 μg/ml). Tubulin (α-Tub) was used as a loading control. Dot indicates a truncated form of SQLE. Cell-free cholesterol was detected with iodine vapor (left lower panel) and by the enzymatic method (Table 1). Right panel, relative amount of full-length SQLE upon elevation of cholesterol (open bar) is represented by taking those at 100 in mock-treated respective cells (solid bar). *p < 0.05; t test versus respective cells cultured in the absence of cholesterol. B, upper panel, SQLE-HA2 was coexpressed with mock vector (lanes 1–6) or MARCH6-Myc6 (lanes 7 and 8) in CHO-K1 treated with Etn, and further cultured for 5 h in the presence of 10 μM epoxomycin. Cell lysates were subjected to coimmunoprecipitation (IP) using rabbit anti-Myc antibody as indicated at the top. MARCH6-Myc6 and SQLE-HA2 were detected with monoclonal antibodies to Myc and HA tags, respectively. Input (In.), 1% of cell lysates used for immunoprecipitation. Lower panel, coimmunoprecipitation of MARCH6-Myc6 with SQLE-HA2 was assessed and represented as a ratio of SQLE-HA2 versus MARCH6-Myc6 by taking as 1 that in CHO-K1 treated with Etn. **p < 0.01; t test versus Etn. C, upper panel, SQLE-HA2 was coexpressed with mock vector (lanes 1 and 2) or MARCH6C9A-Myc6 (lanes 3–8) in CHO-K1 treated with Etn. **p < 0.01; t test versus Etn. C, upper panel, SQLE-HA2 was coexpressed with mock vector (lanes 1 and 2) or MARCH6C9A-Myc6 (lanes 3–8) in CHO-K1 treated with Etn. **p < 0.01; t test versus Etn.
SQLE. Accordingly, we propose that plasmalogens affect the interaction of SQLE with MARCH6 via the transmembrane domain in the N-terminal region of SQLE. 

SQLE is proposed as the second rate-limiting enzyme in cholesterol synthesis (10–12). Inhibition of SQLE efficiently reduces cholesterol synthesis (48, 49), where accumulation of squalene does not cause any major adverse effects (50). Furthermore, the reduced level of cholesterol synthesis in plasmalogen-deficient cells is consistent with the lowered level of HMGCR activity (51) and cholesterol synthesis (12) in the cholesterol-elevated cells. Therefore, suppression of SQLE expression by elevation of plasmalogens might be an alternative potential way to reduce cholesterol synthesis without affecting the synthesis of physiologically important metabolites in the mevalonate pathway, including dolichol, ubiquinone, heme A, and preynylated proteins.

In the present study, we found that synthesis of cholesterol was specifically reduced in plasmalogen-deficient cells such as adaps CHO mutant ZPEG251, whereas 24,25-EC synthesis was elevated in plasmalogen-deficient ZPEG251 and fibroblasts derived from an ADAPS-deficient patient when cultured in the presence of FCS (Fig. 5). Our result is not compatible with that of the earlier study addressing newly synthesized cholesterol in the presence of lipoprotein-deficient serum (52). The HMGCR activity (51) and synthesis of cholesterol (52) are dramatically increased in the presence of lipoprotein-deficient serum, suggesting that the ablation of cholesterol synthesis in plasmalogen-deficient cells is hindered by stimulating mevalonate synthesis. Interestingly, endoplasmic reticulum stress in the PEX2−/− mouse liver causes an elevation of cholesterol synthesis via activation of the SREBP-2 pathway (53–55), whereas cholesterol synthesis in the PEX5−/− mouse is not affected (56). Transcription of SQLE and LSS, targets in the SREBP-2 pathway (36, 57), were not elevated in adaps ZPEG251 and fibroblasts derived from a patient defective in ADAPS (Fig. 6, A and B), suggesting that elevation of cholesterol synthesis in the PEX2−/− mouse liver is caused by multiple peroxisomal dysfunctions as well as the defect in plasmalogen synthesis.

Expression of SQLE is at a very low level in most non-cholesterogenic tissues, whereas SQLE is highly abundant in liver, followed by gut, skin, and neural tissue (58). Interestingly, the cellular plasmalogen level is very low in several tissues such as liver and small intestine (59). Therefore, the limited amount of plasmalogens in liver may contribute to the high level of SQLE expression by suppressing the MARCH6-mediated degradation of SQLE, thereby resulting in the efficient synthesis of 24,25-EC as observed in plasmalogen-deficient cells (Fig. 5).

Synthesis of 24,25-EC is regulated by the relative activity of SQLE and LSS (36). LSS activity is high in cholesterogenic and non-cholesterogenic tissues (58). Therefore, it is likely that synthesis of 24,25-EC is dependent on the activity of SQLE. However, the regulation mechanism of SQLE activity remains unknown. Synthesis of DOS and 24,25-EC is elevated in plasmalogen-deficient cells (Fig. 5) and 24,25-EC synthesis is increased by overexpression of SQLE (60). Taken together, the synthesis rate of 24,25-EC more likely depends on the expression level of SQLE, although we cannot exclude the possibility that unidentified post-translational modification of SQLE contributes to the regulation of SQLE activity.

24,25-EC is a physiological ligand for liver X receptor (LXR) in liver (61), playing a role in the reverse transport of cholesterol by stimulating transcription of ABCA1 encoding ATP-binding cassette transporter A1 (62–65) and IDOL coding for the inducible degrader of the LDL receptor (66). Because 24,25-EC synthesis is elevated in plasmalogen-deficient cells, we suspect that a limited amount of plasmalogens in liver manipulates the synthesis of 24,25-EC toward the effective reverse cholesterol transport. Plasmagen deficiency causes the impaired high-density lipoprotein (HDL)-mediated cholesterol efflux from murine macrophage-like cells (67), the altered transport of internalized cholesterol to the endoplasmic reticulum (3), and abnormal cellular distribution of cholesterol (68). Moreover, addition of cis-(-)-2-O-docosahexaenoyl-1-O-hexadecylglycerol increases cholesterol esterification by raising the sterol-O-acetyltransferase 1 expression level in HEK293 cells (69) and elevation of plasmalogens reduces the cholesterol level in HeLa cells cultured in the presence of exogenously added cholesterol (Fig. 4F). Taken these together with our findings, we suggest that the cellular plasmalogen level plays an important role at multiple steps in cholesterol homeostasis.

Plasmalogens are the major glycerophospholipids in brain and nerve tissue (70–72). Cholesterol, which is required for the proper function of neuronal cells, is mainly provided by glial cells in the brain, because the blood-brain barrier separates the brain from the circulating cholesterol (73). Therefore, the regulation of plasmalogen synthesis most likely plays a pivotal role in the homeostasis of cholesterol especially in the central nervous system. Neurons do not efficiently synthesize cholesterol and mainly take up the cholesterol produced by astrocytes (74), hence suggesting that regulation of plasmalogen homeostasis unequivocally contributes to regulation of cholesterol synthesis in astrocytes. Our findings reported here open a way to address cholesterol homeostasis involving plasmalogen physiology.

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Plasmalogens Regulate Cholesterol Biosynthesis


