Cardiolipin synthesis is required to support human cholesterol biosynthesis from palmitate upon serum removal in Hela cells[1]

Kristin D. Hauff, Seok-Yong Choi, Michael A. Frohman, and Grant M. Hatch

Abstract: We examined whether cardiolipin (CL) synthesis was required to support cholesterol (CH) production from palmitate in Hela cells. Knockdown of human cardiolipin synthase-1 (*hCLS1*) in Hela cells has been shown to reduce CL synthesis. Therefore Hela cells stably expressing shRNA for *hCLS1* and mock control cells were incubated for 16 h with [^{14}C(U)]palmitate bound to albumin (1:1 molar ratio) in the absence or presence of serum. Knockdown of *hCLS1* in Hela cells resulted in a reduction in [^{14}C(U)]palmitate incorporation into CL and CH. This reduction in [^{14}C(U)]palmitate incorporation into CH was most pronounced during incubation under serum-free conditions. The reduction in [^{14}C(U)]palmitate incorporation into CH was not due to alterations in total uptake of [^{14}C(U)]palmitate into cells or altered palmitate metabolism, since [^{14}C(U)]palmitate incorporation into phosphatidylcholine, the major [^{14}C(U)]palmitate-containing lipid, and its immediate precursor, 1,2-diacyl-*sn*-glycerol, were unaffected by *hCLS1* knockdown. In addition, knockdown of *hCLS1* did not affect CH pool size, indicating that CH catabolism was unaltered. Hydroxymethylglutaryl coenzyme A reductase enzyme activity and its mRNA expression were reduced by knockdown of *hCLS1* and this was most pronounced in Hela cells cultured under serum-free conditions. These data indicate that CL synthesis is required to support human de novo CH biosynthesis under conditions of increased demand for CH.

Key words: cardiolipin synthesis, cholesterol synthesis, human, hydroxymethylglutaryl coenzyme A reductase, palmitate, fatty acid, Hela cells, serum, metabolism, cardiolipin synthase, short hairpin RNA, phosphatidylcholine, phospholipids.

Résumé : Nous voulons vérifier si la synthèse de cardiolipine (CL) est essentielle à la production de cholestérol (CH) à partir du palmitate dans les cellules Hela. La suppression de l'activité de la cardiolipine synthase-1 humaine (*hCLS1*) dans les cellules Hela a pour effet de diminuer la synthèse de CL. Des cellules Hela exprimant bien l'ARN court en épingle à cheveux (shRNA) pour l'*hCLS1* et des simili-cellules de contrôle sont incubées durant 16 h avec du [^{14}C(U)]palmitate lié à de l'albumine (rapport molaire 1 : 1) en l'absence ou en présence de sérum. La suppression de la *hCLS1* dans les cellules Hela suscite une diminution de l'incorporation du [^{14}C(U)]palmitate dans la CL et le CH. La diminution de l'incorporation du [^{14}C(U)]palmitate dans le CH est plus importante quand l'incubation se fait en l'absence de sérum. Cette diminution n'est pas due à des modifications de l'apport total de [^{14}C(U)]palmitate dans les cellules ou à une modification du métabolisme du palmitate, car l'incorporation du [^{14}C(U)]palmitate dans la phosphatidylcholine, le lipide contenant le plus de [^{14}C(U)]palmitate, et dans le 1,2-diacyl-*sn*-glycérol, son précurseur, n'est pas affectée par la suppression de l'activité de la *hCLS1*. De plus, la suppression de l'activité de la *hCLS1* ne modifie pas la dimension du pool du CH, ce qui signifie que le catabolisme du CH n'est pas altéré. La suppression de l'activité de la *hCLS1* diminue l'activité enzymatique de l'hydroxyméthylglutaryl coenzyme A réductase et de l'expression de l'ARNm et cette diminution est plus importante dans les cellules Hela incubées en l'absence de sérum. D'après ces observations, la synthèse de la CL est essentielle à la biosynthèse de novo du CH lorsque la demande de CH augmente.

Mots-clés : synthèse de cardiolipine, synthèse du cholestérol, humain, hydroxyméthylglutaryl coenzyme A réductase, palmitate, acide gras, cellules Hela, sérum, métabolisme, cardiolipine synthase, ARN court en épingle à cheveux, phosphatidylcholine, phospholipide.

[Traduit par la Rédaction]

Received 7 January 2009. Accepted 9 June 2009. Published on the NRC Research Press Web site at cjpp.nrc.ca on 29 September 2009.

K.D. Hauff. Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, Winnipeg, Canada.
S.Y. Choi. Department of Biomedical Sciences, Chonnam National University, Medical School, Gwangju, Korea.
M.A. Frohman. Department of Pharmacology, Center for Developmental Genetics, Stony Brook University, Stony Brook, New York, USA.
G.M. Hatch.[2] Departments of Pharmacology and Therapeutics, Biochemistry and Medical Genetics, and Internal Medicine, Faculty of Medicine, and Centre for Research and Treatment of Atherosclerosis, University of Manitoba, Winnipeg, MB R3E OT6, Canada.

[1]This article is one of a selection of papers published in a special issue celebrating the 125th anniversary of the Faculty of Medicine at the University of Manitoba.
[2]Corresponding author (e-mail: hatchgm@ms.umanitoba.ca).

Introduction

Cardiolipin (CL) was the first polyglycerolphospholipid discovered and was first isolated from beef heart by Mary Pangborn (reviewed in Hostetler 1982). CL is both synthesized and localized exclusively within mammalian mitochondria (Daum et al. 1986; Hostetler 1982). CL is localized to both inner and outer mitochondrial membranes and within contact sites in mammalian cells (Nicolay et al. 1990). CL is required for the reconstituted activity of a number of key mitochondrial enzymes involved in cellular oxidative metabolism (reviewed in Chicco and Sparagna 2007; Hatch 1998; and Hoch 1992). CL anchors cytochrome *c* to the inner mitochondrial membrane (Tuominen et al. 2002) and may play a key role in cytochrome *c* release and apoptosis (McMillin and Dowhan 2002; Ostrander et al. 2001; Ott et al. 2002). CL also plays an essential role in mitochondrial biogenesis (Schlame et al. 2000) and the assembly of respiratory enzyme supercomplexes (Pfeiffer et al. 2003). Hence, CL may be the 'glue' that holds the respiratory chain together (Zhang et al. 2002).

CL is synthesized in mammalian cells by the CDP-DG pathway (reviewed in Hatch 2004). Phosphatidic acid is converted to cytidine diphosphate 1,2-diacyl-*sn*-glycerol (CDP-DG) catalyzed by cytidine diphosphate 1,2-diacyl-*sn*-glycerol synthetase (CDS). There are 2 isoforms of this enzyme in mammalian tissues, CDS-1 and CDS-2 (Halford et al. 1998). CDP-DG condenses with glycerol-3-phosphate to form phosphatidylglycerol phosphate catalyzed by phosphatidylglycerol phosphate synthase (PGS). Phosphatidylglycerol phosphate does not accumulate in mammalian cells and is rapidly converted to phosphatidylglycerol by a phosphatidylglycerol phosphate phosphatase. The final step in the CL biosynthetic pathway involves the condensation of phosphatidylglycerol with another molecule of CDP-DG to form CL catalyzed by cardiolipin synthase (CLS) (Hostetler et al. 1972). The human CLS-1 gene (*hCLS1*) was recently cloned by 4 independent laboratories (Chen et al. 2006; Houtkooper et al. 2006; Lu et al. 2006; Choi et al. 2007).

De novo cholesterol (CH) biosynthesis occurs in all nucleated cells and is upregulated in response to low intracellular sterol concentrations (Vance and Vance 2002). 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is the major rate-limiting step in de novo CH synthesis (Brown and Goldstein 1999). HMGR activity in cells is regulated by sterols, primarily at the level of expression of *HMGR* mRNA (Goldstein and Brown 1990). It has been estimated that approximately two-thirds of all cellular CH is from de novo biosynthesis (Endo and Hasumi 1989). Previous studies have shown that palmitate may be utilized for CH biosynthesis in mammalian cells and in the de novo biosynthesis of phospholipids (Vance and Vance 2002).

Evidence suggests there may be a link between CL and CH. The mitochondrial enzyme cytochrome P-450$_{scc}$ (CYP11A1) is responsible for the oxidative side-chain cleavage of CH. It was found that this enzyme has a CL binding site that can enhance the enzyme–substrate interaction (Lambeth 1981; Pember et al. 1983). In a rat model of diabetes, CL has been identified as one of the first lipids to be altered (Han et al. 2007), often a consequence of metabolic X syndrome, which has been linked to altered CH metabolism (Holvoet et al. 2008). Human diseases such as Barth syndrome (BTHS) (Kelley et al. 1991) and Tangier's disease (Fobker et al. 2001) reportedly have altered metabolism of both CL and CH. Spencer et al. (2006) found that 56% of a small cohort of BTHS patients exhibited a reduced low-density lipoprotein (LDL) profile of 1.55 mmol/L (<60 mg/dL). It was unknown whether CL synthesis was required to support CH biosynthesis. Here we show that knockdown of *hCLS1* results in a reduced ability to synthesize CH de novo from palmitate upon serum removal in Hela cells. The results suggest that CL synthesis is required to support CH biosynthesis under conditions of increased demand for CH.

Materials and methods

Materials

[^{14}C(U)]Palmitate was obtained from Perkin-Elmer, Woodbridge, Canada, and DuPont, Mississauga, Canada. DL-3-[glutaryl-3-^{14}C]hydroxy-3-methylglutaryl coenzyme A was obtained from American Radiolabeled Chemicals, St. Louis, USA. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and antibiotics were products of Canadian Life Technologies (Gibco), Burlington, Canada. Lipid standards were obtained from Serdary Research Laboratories, Englewood Cliffs, USA. Thin-layer chromatography plates (silica gel G, 0.25 mm thickness) were obtained from Fisher Scientific, Winnipeg, Canada. Ecolite scintillant was obtained from ICN Biochemicals, Montreal, Canada. Qiagen OneStep RT-PCR kit was used for PCR studies. All other chemicals were certified ACS grade or better and obtained from Sigma Chemical, St. Louis, USA, or Fisher Scientific, Winnipeg, Canada.

Cell culture

Hela cells were obtained from American Type Culture Collection. Hela cells were transfected with plasmids containing short hairpin RNA (shRNA) to human *CLS* as previously described (Choi et al. 2007). Cells were maintained in DMEM containing 10% FBS, 100 U penicillin, and 100 µg/mL streptomycin. In addition, transfected cells had 10 µg/mL blasticidin in their media as a selective reagent for propagation, but shared the same media as the untransfected cells (lacking in blasticidin) during all experimental treatments. Hela cells were incubated overnight with media either containing or deficient in FBS and [^{14}C(U)]palmitic acid (bound to albumin in a 1:1 molar ratio), and cells were incubated for 16 h. Cellular lipids were then isolated as described below.

Lipid isolation and cholesterol determination

CH was isolated as described with modification (Folch et al. 1957). Briefly, after the initial treatment of cells described above, the medium was removed and cells washed with 2 mL PBS. Cells were scraped from the plates using a plastic scraper into 2 mL of a methanol:H_2O (1:1, *v*/*v*) solution. Cells were transferred with a Pasteur pipette to silanated glass tubes, and aliquots were taken for total radioactivity and protein (25 and 50 µL, respectively) determination. Protein assays were performed with a BioRad protein assay kit. Bovine serum albumin (BSA) was used as a

standard. An additional 0.5 mL of H_2O and 2 mL of chloroform were added to each sample, followed by brief mixing using a vortex mixer. Samples were then centrifuged at 600*g* in a swinging bucket rotor for 10 min. The upper, aqueous layer was removed by suction, along with the protein interphase. Addition of 2 mL theoretical upper phase (methanol : 0.9% NaCl : chloroform 48:47:3 by volume) was followed by a second brief mixing as above and then centrifugation at 750*g* for 5 min. Removal of the aqueous phase was followed by drying of the organic phase under nitrogen gas. Samples were capped and stored at –20 °C. A 50 µL portion of the total sample was spotted onto silica thin-layer chromatography plates for one-dimensional separation of CH. Plates were developed for approximately 1 h in hexanes : diethyl ether : glacial acetic acid (70:30:2 by volume). A portion of the total sample was spotted onto silica thin-layer chromatography plates for separation of phosphatidylcholine (PC) and 1,2-diacyl-*sn*-glycerol (DG) as previously described (Hatch and McClarty 1996). Spots corresponding to lipids were visualized with iodine vapor and removed into plastic scintillation vials; radioactivity in the sample was determined by liquid scintillation counting in a Beckman model LS6500 scintillation counter. In some experiments, CH content of the cells was determined by colorimetric reaction of the Amplex Red CH assay kit from Invitrogen's Molecular Probes. After isolation of cellular lipids as described above, the lipid residue was reconstituted with 1% Triton X-100 in isopropanol, and CH assays were performed as per protocol. All isolates were measured immediately after drying with nitrogen, as fresh samples appeared to yield the best results.

Real-time PCR

Cells were incubated as above and then RNA was isolated using the Trizol method of phenol extraction and stored at –80 °C. The isolated RNA was combined with Qiagen RT-PCR master mix and the appropriate primers to a total reaction volume of 25 µL. Reverse transcription was performed immediately before PCR as part of the same cycler protocol. The cycler protocol for real-time PCR on an Eppendorf Mastercycler ep realplex 2 consisted of reverse transcription at 50 °C for 30 min, *Taq* activation at 95 °C for 15 min, separation at 95 °C for 1 min, followed by a melting curve that increased in temperature incrementally from 60 °C to 95 °C over the course of 20 min. Fluorescence readings were taken throughout to determine product quality. The primers used for *CDS-1*, *CDS-2*, and *PGS* in Hela cells have been previously described (Hauff et al. 2009). The following primers were used for *HMGR* and housekeeping gene *18s* RNA. *HMGR* (GenBank NM 000859): forward cggttGGAAGAGACAGGGA-TAAAC[FAM]G, reverse GGGTATCTGTTTCAGCCAC-TAAGG; and *18s* (X03205): forward CTC GGGCCTGCTTTGAACAC, reverse cggg TGCTCTTAG CTGAGTGTCC[FAM]G. The PCR program for *HMGR* (400 nmol/L) and *18s* rRNA (100 nmol/L) primers ran 50 cycles of separation at 94 °C for 15 s and annealing/elongation at 60 °C for 45 s. Fluorescence readings were taken at the end of every elongation step. The changes in gene expression were analyzed on Eppendorf Mastercycler ep realplex software (version 1.5.474), and the data presented as mean fold change ($2^{-\Delta\Delta Ct}$) in mRNA expression (Livak and Schmittgen 2001) relative to *18s* rRNA, a gene not affected by short-term incubation of cells under altered serum conditions (Schmittgen and Zakrajsek 2000).

Assay of in vitro HMGR

Cells were incubated as above, and then removed from the plates and suspended on ice in 2 mL homogenizing buffer (10 mmol/L Tris–HCl, pH 7.4, 0.25 mol/L sucrose), followed by homogenization with 2 × 20 strokes of a Dounce A homogenizer. The homogenate was centrifuged at 4 °C, 1 000*g* for 5 min and the supernatant centrifuged at 4 °C, 10 000*g* for 15 min. The resulting supernatants were then further centrifuged at 4 °C, 100 000*g* for 1.5 h, and the resulting microsomal pellets were resuspended in homogenizing buffer and kept on ice while protein content was determined. Protein assays were performed with BioRad protein assay. BSA was used as a standard. In vitro HMGR assays were performed as described (Ohashi et al. 2003). Reaction buffer containing, at final concentration, 5 mmol/L β-NADPH tetrasodium salt (freshly made), 10 mmol/L EDTA, 10 mmol/L dithiothreitol (DTT), 100 mmol/L Tris-HCl (pH 7.4), was added to 50 µg microsomal protein per sample. The reactions were initiated by the addition of DL-3-[glutaryl-3-^{14}C]hydroxy-3-methylglutaryl coenzyme A (4.5 µCi/µmol) and cold 3-hydroxy-3-methylglutaryl coenzyme A to 110 µmol/L, and then incubated at 37 °C for 30 min. Addition of HCl to 1 mol/L and incubation for a further 30 min at 37 °C were required to lactonize the mevalonate formed. Samples were stored in the freezer overnight and then spotted onto dried silica thin-layer chromatography plates. Plates were developed for 2 h in thin-layer chromatography tanks containing acetone:benzene (1:1, *v*/*v*). Subsequently, the plates were exposed to iodine vapor and spots corresponding to a retention factor of R_f = 0.6–0.9 were removed into scintillation vials and radioactivity ([^{14}C]mevalonate formation) was determined.

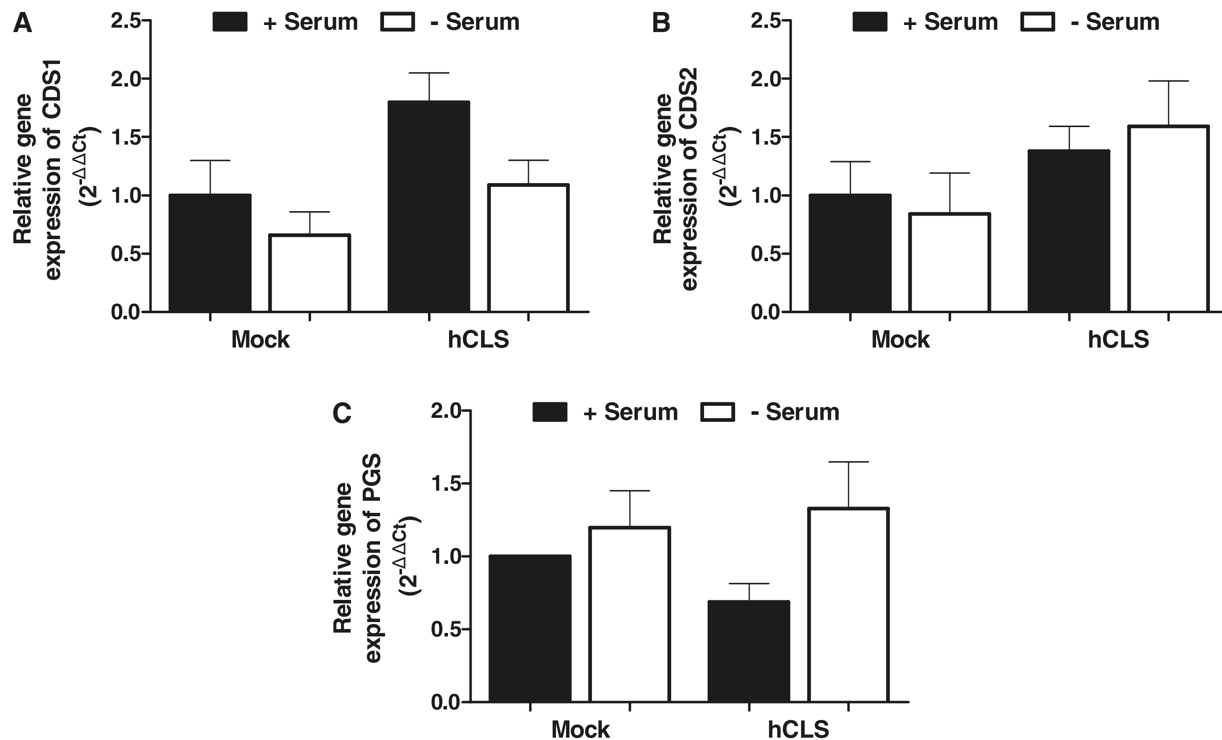
Statistics

One-way or two-way ANOVA, with Dunnett or Bonferroni post tests, respectively, was performed using GraphPad Prism software version 5.00 for Windows (www.graphpad.com). Data were reported as means ± SE, and the level of significance was defined as $p < 0.05$.

Results and discussion

We previously demonstrated that knockdown of *hCLS1* reduced *hCLS1* mRNA expression and CL synthesis in Hela cells (Choi et al. 2007). In this study, we began by examining the expression of genes of the CDP-DG pathway of CL biosynthesis in serum-supplemented and serum-depleted Hela cells in which *hCLS1* was knocked down. Hela cells stably expressing shRNA to *hCLS1* (hCLS cells) or mock control cells were incubated for 16 h with medium in the absence or presence of serum. Total RNA was prepared and mRNA expression of *CDS-1*, *CDS-2*, *PGS*, and *hCLS1* determined. Knockdown of *hCLS1* did not affect expression of *CDS-1*, *CDS-2*, or *PGS* in Hela cells incubated in serum-containing or serum-free medium (Figs. 1A–1C). As previously demonstrated, (Choi et al. 2007) knockdown of

Fig. 1. Expression of genes encoding enzymes of the CDP-DG pathway of cardiolipin biosynthesis in Hela cells. Hela cells stably expressing shRNA to *hCLS1* (hCLS) or mock controls were incubated for 16 h with medium in the absence or presence of serum. Total RNA was prepared and mRNA expression of *CDS-1* (A), *CDS-2* (B), and *PGS* (C) was determined. Data are means ± SE (n = 3). shRNA, short hairpin RNA; *hCLS1*, human cardiolipin synthase 1 gene; *CDS*, cytidine diphosphate 1,2-diacyl-*sn*-glycerol synthetase gene; *PGS*, phosphatidylglycerol phosphate synthase gene.

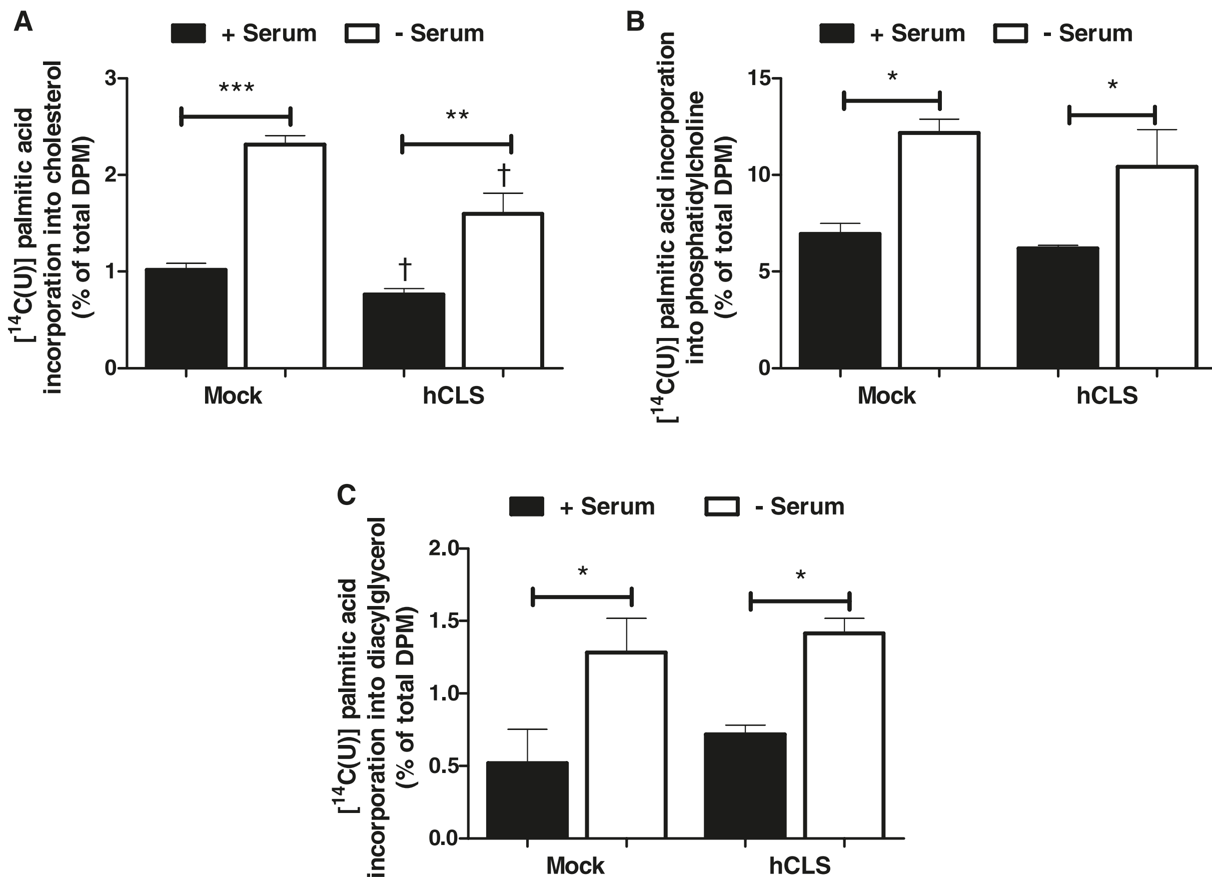


hCLS1 reduced *hCLS1* mRNA expression approximately 20%–40% (data not shown). Thus, knockdown of *hCLS1* using shRNA reduces only *hCLS1* mRNA expression but not other enzymes of the CDP-DG pathway of CL synthesis.

We next examined CL synthesis from [^{14}C(U)]palmitate. Hela cells stably expressing shRNA to *hCLS1* (hCLS cells) or mock control cells were incubated for 16 h in medium containing [^{14}C(U)]palmitate in the absence or presence of serum. The cells were then harvested and the radioactivity incorporated into CL was determined. We used 0.1 mmol/L palmitate bound to albumin (in a 1:1 molar ratio) to achieve the labeling because this is representative of circulating plasma palmitate levels and higher palmitate:albumin ratios result in apoptosis (Ostrander et al. 2001). Total uptake of [^{14}C(U)]palmitate into growing cells was unaltered between mock (0.68 ± 0.02 × 10^7 dpm/mg) and hCLS (0.70 ± 0.04 × 10^7 dpm/mg) cells (60 dpm = 1 Bq). In addition, total uptake of [^{14}C(U)]palmitate into growth-arrested (serum-free) cells was unaltered between mock (0.69 ± 0.05 × 10^7 dpm/mg) and hCLS (0.67 ± 0.03 × 10^7 dpm/mg) cells. Incorporation of [^{14}C(U)]palmitate into CL was reduced 42% ($p < 0.05$) by knockdown of *hCLS1* in serum-supplemented Hela cells (from 0.07% ± 0.01% to 0.04% ± 0.01% of the total incorporated radioactivity) and was reduced 50% ($p < 0.05$) by knockdown of *hCLS1* in Hela cells incubated in serum-free conditions (from 0.08% ± 0.01% to 0.04% ± 0.01% of the total incorporated radioactivity). Thus, knockdown of *hCLS1* reduced CL biosynthesis from [^{14}C(U)]palmitate in Hela cells grown in serum-containing or serum-free medium.

To examine whether CL synthesis is required to support CH synthesis from palmitate, Hela cells stably expressing shRNA to *hCLS1* (hCLS cells) or mock control cells were incubated for 16 h in medium containing [^{14}C(U)]palmitate in the absence or presence of serum, and the radioactivity incorporated into CH was determined. Serum removal would be expected to upregulate CH biosynthesis (Fogelman et al. 1977). Serum removal resulted in elevated CH synthesis from [^{14}C(U)]palmitate in mock transfected Hela cells (Fig. 2A). Thus, palmitate can be used as a carbon source for CH synthesis in Hela cells. However, knockdown of *hCLS1* resulted in a 20% decrease ($p < 0.05$) and 40% decrease (p <0.05) in [^{14}C(U)]palmitate incorporation into CH in Hela cells grown in serum-containing or serum-free medium, respectively. Hela cell growth is attenuated under serum-free conditions (Hauff et al. 2009). Since the total uptake of [^{14}C(U)]palmitate into cells was unaltered and a reduction in [^{14}C(U)]palmitate incorporation into CH was observed for cells incubated in either the absence or presence of serum, the decrease in [^{14}C(U)]palmitate incorporation into CH was not simply due to a potential growth defect caused by reduced CL synthesis. Thus, knockdown of *hCLS1* reduces [^{14}C(U)]palmitate utilization for CH biosynthesis in Hela cells grown in serum-containing or serum-free medium. However, the effect was most significant when the

Fig. 2. Synthesis of lipids from [^{14}C(U)]palmitate in Hela cells. Hela cells stably expressing shRNA to *hCLS1* (hCLS) or mock controls were incubated for 16 h in medium containing [^{14}C(U)]palmitate in the absence or presence of serum. Cells were harvested and the radioactivity incorporated into cholesterol (A), phosphatidylcholine (B), and 1,2-diacyl-*sn*-glycerol (C) was determined. Data are means ± SE ($n = 3$). *, significant at $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. †, $p < 0.05$ vs. corresponding control.



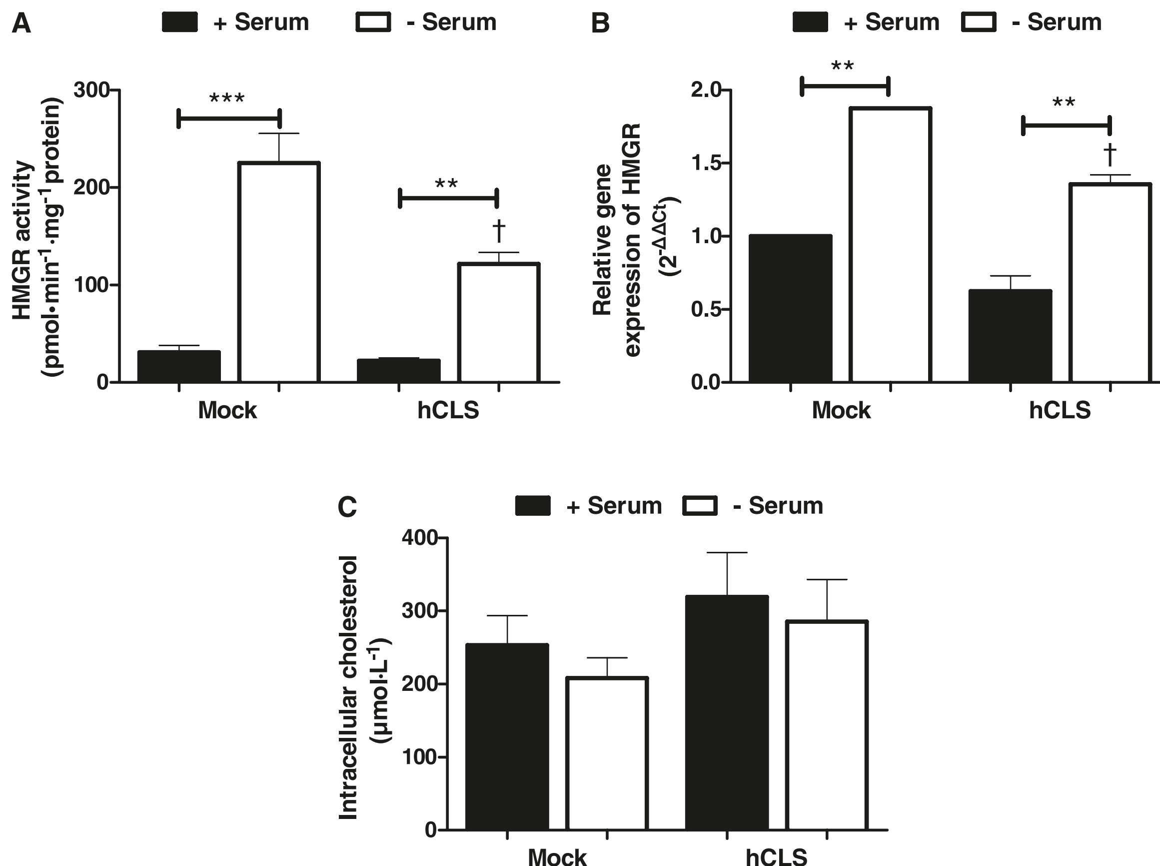
cells were grown in the absence of serum, a condition where CH demand is highly upregulated (Fogelman et al. 1977).

Palmitate is a saturated fatty acid that enters into most phospholipids via de novo biosynthesis (Vance and Vance 2002). It could be argued that general [^{14}C(U)]palmitate metabolism was disrupted by knockdown of *hCLS1*. To address this, Hela cells stably expressing shRNA to *hCLS1* (hCLS cells) or mock controls were incubated for 16 h with medium containing [^{14}C(U)]palmitate in the absence or presence of serum, and the radioactivity incorporated into the major palmitate-containing lipid PC and its immediate precursor DG was determined. Serum removal resulted in an elevation in [^{14}C(U)]palmitate incorporation into PC and DG compared with serum-supplemented cells (Figs. 2B, 2C). Knockdown of *hCLS1* did not affect incorporation of [^{14}C(U)]palmitate into PC or DG in Hela cells grown in serum-containing or serum-free medium. Thus, the reduction in [^{14}C(U)]palmitate incorporation into CH with knockdown of *hCLS1* was not due to changes in metabolism of the major palmitate-containing lipid or its immediate precursor in Hela cells.

The reason for the reduction in [^{14}C(U)]palmitate incorporation into CH with knockdown of *hCLS1* was examined. Hela cells stably expressing shRNA to *hCLS1* (hCLS cells) or mock controls were incubated for 16 h with medium in the absence or presence of serum, microsomal fractions were prepared, and the HMGR enzyme activity was determined. Serum removal resulted in an increase in HMGR activity in both mock and hCLS cells (Fig. 3A). Knockdown of *hCLS1* in Hela cells reduced in vitro HMGR activity 46% ($p < 0.05$) in the absence of serum compared with control. The effect was only significant when cells were grown in the absence of serum. In addition, we examined expression of *HMGR* mRNA in these cells using real-time PCR. Hela cells stably expressing shRNA to *hCLS1* (hCLS cells) or mock controls were incubated in the absence or presence of serum for 16 h, total RNA was prepared, and *HMGR* mRNA expression was determined. Knockdown of *hCLS1* in Hela cells reduced *HMGR* mRNA expression 28% ($p < 0.05$) in the absence of serum compared with control (Fig. 3B). The effect was significant when cells were grown in the absence of serum and paralleled the reduced HMGR enzyme activity. These data indicate that the reduction in [^{14}C(U)]palmitate incorporation into CH might be due in part to reduced activity and mRNA expression of the key rate-limiting enzyme of CH biosynthesis in Hela cells grown under serum-free conditions.

Finally, to examine whether knockdown of *hCLS1* altered

Fig. 3. HMGR enzyme activity (A), mRNA expression (B), and cholesterol pool size (C) in Hela cells stably expressing shRNA to *hCLS1* (hCLS) or mock controls incubated for 16 h with medium in the absence or presence of serum. Data are means ± SE (n = 3). **, significant at $p < 0.01$ and ***, $p < 0.001$. HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase. †, $p < 0.05$ vs. corresponding control.



CH degradation, Hela cells stably expressing shRNA to *hCLS1* (hCLS cells) or mock controls were incubated for 16 h in the absence or presence of serum and the pool size of CH was determined. Knockdown of *hCLS1* did not affect the CH pool size when cells were incubated in either in the absence or presence of serum (Fig. 3C). Thus, knockdown of *hCLS1* did not alter CH degradation.

Palmitate is oxidized to acetyl coenzyme A in the mitochondria of mammalian cells, and the acetyl residues may be transported back into the cytosol in the form of citrate where they may be converted back to acetyl coenzyme A and thus serve as a carbon source for synthesis of CH (Lehninger 1976). The question must be asked: why does knockdown of *hCLS1* affect CH biosynthesis from palmitate? A previous study has shown that bovine heart CL is an effective activator of CH side-chain cleavage activity of CYP11A1 by maintenance of the membrane curvature at a value optimal for activity (Schwarz et al. 1996). CYP11A1 is localized to mitochondria (Pelletier et al. 2001). In addition to reduced enzyme activity and mRNA expression of *HMGR*, it is possible that newly synthesized CL may be required to support CYP11A1 activation and the subsequent metabolism of CH to steroid hormones. Thus, a decrease in this reaction might result in reduced utilization of CH for steroid synthesis and accumulation of CH in the endoplasmic reticulum, which could result in a reduction in CH synthesis via repression of *HMGR* expression. However, since Hela cells are not steroidogenic, it is unlikely that a lowered CYP11A1 activity would contribute to a reduced synthesis of CH in these cells.

Conclusion

Exogenous palmitate may serve as a carbon source for de novo CH biosynthesis in Hela cells grown under conditions of serum-supplemented or serum-free medium. Knockdown of *hCLS1* and hence decreased CL synthesis reduces CH synthesis from palmitate, and the effect is most pronounced under a condition in which CH synthesis needs to be upregulated, that is, growth in serum-free medium. Thus, CL synthesis is required to support human CH biosynthesis from palmitate under conditions of CH demand in Hela cells.

Acknowledgements

This work was funded by grants from the Barth Syndrome Foundation of Canada, the Canadian Institutes of Health Research, and the Heart and Stroke Foundation of Manitoba (to G.M.H.) and the United Mitochondrial Disease Foundation (to M.A.F.). K.D.H was funded by studentships from the Manitoba Institute of Child Health, the Manitoba Health Research Council, and the Health Sciences Centre Foundation.

The authors also thank Marilyne Vandel, William A. Taylor, and Fred Y. Xu for technical assistance. G.M.H is a Canada Research Chair in Molecular Cardiolipin Metabolism.

References

Brown, M.S., and Goldstein, J.L. 1999. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc. Natl. Acad. Sci. U.S.A. **96**(20): 11041–11048. doi:10.1073/pnas.96.20.11041. PMID:10500120.

Chen, D., Zhang, X.Y., and Shi, Y. 2006. Identification and functional characterization of hCLS1, a human cardiolipin synthase localized in mitochondria. Biochem. J. **398**(2): 169–176. doi:10.1042/BJ20060303. PMID:16716149.

Chicco, A.J., and Sparagna, G.C. 2007. Role of cardiolipin alterations in mitochondrial dysfunction and disease. Am. J. Physiol. Cell Physiol. **292**(1): C33–C44. doi:10.1152/ajpcell.00243.2006. PMID:16899548.

Choi, S.Y., Gonzalvez, F., Jenkins, G.M., Slomianny, C., Chretien, D., Arnoult, D., et al. 2007. Cardiolipin deficiency releases cytochrome *c* from the inner mitochondrial membrane and accelerates stimuli-elicited apoptosis. Cell Death Differ. **14**(3): 597–606. doi:10.1038/sj.cdd.4402020. PMID:16888643.

Daum, G., Heidorn, E., and Paltauf, F. 1986. Intracellular transfer of phospholipids in the yeast, *Saccharomyces cerevisiae*. Biochim. Biophys. Acta, **878**(1): 93–101. PMID:3524689.

Endo, A., and Hasumi, K. 1989. Biochemical aspect of HMG CoA reductase inhibitors. Adv. Enzyme Regul. **28**: 53–64. doi:10.1016/0065-2571(89)90063-0. PMID:2696346.

Fobker, M., Voss, R., Reinecke, H., Crone, C., Assmann, G., and Walter, M. 2001. Accumulation of cardiolipin and lysocardiolipin in fibroblasts from Tangier disease subjects. FEBS Lett. **500**(3): 157–162. doi:10.1016/S0014-5793(01)02578-9. PMID:11445077.

Fogelman, A.M., Seager, J., Edwards, P.A., and Popják, G. 1977. Mechanism of induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase in human leukocytes. J. Biol. Chem. **252**(2): 644–651. PMID:833148.

Folch, J., Lees, M., and Sloane Stanley, G.H. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. **226**(1): 497–509. PMID:13428781.

Goldstein, J.L., and Brown, M.S. 1990. Regulation of the mevalonate pathway. Nature, **343**(6257): 425–430. doi:10.1038/343425a0. PMID:1967820.

Halford, S., Dulai, K.S., Daw, S.C., Fitzgibbon, J., and Hunt, D.M. 1998. Isolation and chromosomal localization of two human CDP-diacylglycerol synthase (*CDS*) genes. Genomics, **54**(1): 140–144. doi:10.1006/geno.1998.5547. PMID:9806839.

Han, X., Yang, J., Yang, K., Zhao, Z., Abendschein, D.R., and Gross, R.W. 2007. Alterations in myocardial cardiolipin content and composition occur at the very earliest stages of diabetes: a shotgun lipidomics study. Biochemistry, **46**(21): 6417–6428. doi:10.1021/bi7004015. PMID:17487985.

Hatch, G.M. 1998. Cardiolipin: biosynthesis, remodeling and trafficking in the heart and mammalian cells. Int. J. Mol. Med. **1**(1): 33–41. [Review.] PMID:9852196.

Hatch, G.M. 2004. Cell biology of cardiac mitochondrial phospholipids. Biochem. Cell Biol. **82**(1): 99–112. doi:10.1139/o03-074. PMID:15052331.

Hatch, G.M., and McClarty, G. 1996. Regulation of cardiolipin biosynthesis in H9c2 cardiac myoblasts by cytidine 5′-triphosphate. J. Biol. Chem. **271**(42): 25810–25816. doi:10.1074/jbc.271.42.25810. PMID:8824210.

Hauff, K., Linda, D., and Hatch, G.M. 2009. Mechanism of the elevation in cardiolipin during HeLa cell entry into the S-phase of the human cell cycle. Biochem. J. **417**(2): 573–582. doi:10.1042/BJ20080650. PMID:18808366.

Hoch, F.L. 1992. Cardiolipins and biomembrane function. Biochim. Biophys. Acta, **1113**(1): 71–133. PMID:1550861.

Holvoet, P., Lee, D.-H., Steffes, M., Gross, M., and Jacobs, D.R., Jr. 2008. Association between circulating oxidized low-density lipoprotein and incidence of the metabolic syndrome. JAMA, **299**(19): 2287–2293. doi:10.1001/jama.299.19.2287. PMID:18492970.

Hostetler, K.Y. 1982. Polyglycerolphospholipids. *In* Phospholipids. *Edited by* J. Hawthorne and G. Ansell. Elsevier, Amsterdam. pp. 215–242.

Hostetler, K.Y., van den Bosch, H., and van Deenen, L.L. 1972. The mechanism of cardiolipin biosynthesis in liver mitochondria. Biochim. Biophys. Acta, **260**(3): 507–513. PMID:4556770.

Houtkooper, R.H., Akbari, H., van Lenthe, H., Kulik, W., Wanders, R.J., Frentzen, M., and Vaz, F.M. 2006. Identification and characterization of human cardiolipin synthase. FEBS Lett. **580**(13): 3059–3064. doi:10.1016/j.febslet.2006.04.054. PMID:16678169.

Kelley, R.I., Cheatham, J.P., Clark, B.J., Nigro, M.A., Powell, B.R., Sherwood, G.W., et al. 1991. X-linked dilated cardiomyopathy with neutropenia, growth retardation, and 3-methylglutaconic aciduria. J. Pediatr. **119**(5): 738–747. doi:10.1016/S0022-3476(05)80289-6. PMID:1719174.

Lambeth, J.D. 1981. Cytochrome P-450_{scc}: cardiolipin as an effector of activity of a mitochondrial cytochrome P-450. J. Biol. Chem. **256**(10): 4757–4762. PMID:6894445.

Lehninger, A.L. 1976. Catabolism and the generation of phosphate-bond energy. *In* Biochemistry. 2nd ed. *Edited by* A.L. Lehninger. Worth Publishers, New York. pp. 361–587.

Livak, K.J., and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods, **25**(4): 402–408. doi:10.1006/meth.2001.1262. PMID:11846609.

Lu, B., Xu, F.Y., Jiang, Y.J., Choy, P.C., Hatch, G.M., Grunfeld, C., and Feingold, K.R. 2006. Cloning and characterization of a cDNA encoding human cardiolipin synthase (hCLS1). J. Lipid Res. **47**(6): 1140–1145. doi:10.1194/jlr.C600004-JLR200. PMID:16547353.

McMillin, J.B., and Dowhan, W. 2002. Cardiolipin and apoptosis. Biochim. Biophys. Acta, **1585**(2-3): 97–107. PMID:12531542.

Nicolay, K., Rojo, M., Wallimann, T., Demel, R., and Hovius, R. 1990. The role of contact sites between inner and outer mitochondrial membrane in energy transfer. Biochim. Biophys. Acta, **1018**(2-3): 229–233. doi:10.1016/0005-2728(90)90255-3. PMID:2203472.

Ohashi, K., Osuga, J.-i., Tozawa, R., Kitamine, T., Yagyu, H., Sekiya, M., et al. 2003. Early embryonic lethality caused by targeted disruption of the 3-hydroxy-3-methylglutaryl-CoA reductase gene. J. Biol. Chem. **278**(44): 42936–42941. doi:10.1074/jbc.M307228200. PMID:12920113.

Ostrander, D.B., Sparagna, G.C., Amoscato, A.A., McMillin, J.B., and Dowhan, W. 2001. Decreased cardiolipin synthesis corresponds with cytochrome *c* release in palmitate-induced cardiomyocyte apoptosis. J. Biol. Chem. **276**(41): 38061–38067. PMID:11500520.

Ott, M., Robertson, J.D., Gogvadze, V., Zhivotovsky, B., and Orrenius, S. 2002. Cytochrome *c* release from mitochondria proceeds by a two-step process. Proc. Natl. Acad. Sci. U.S.A. **99**(3): 1259–1263. doi:10.1073/pnas.241655498. PMID:11818574.

Pelletier, G., Li, S., Luu-The, V., Tremblay, Y., Bélanger, A., and Labrie, F. 2001. Immunoelectron microscopic localization of

three key steroidogenic enzymes (cytochrome $P450_{scc}$, 3 β-hydroxysteroid dehydrogenase and cytochrome $P450_{c17}$) in rat adrenal cortex and gonads. J. Endocrinol. **171**(2): 373–383. doi:10.1677/joe.0.1710373. PMID:11691658.

Pember, S.O., Powell, G.L., and Lambeth, J.D. 1983. Cytochrome $P\text{-}450_{scc}$-phospholipid interactions: evidence for a cardiolipin binding site and thermodynamics of enzyme interactions with cardiolipin, cholesterol, and adrenodoxin. J. Biol. Chem. **258**(5): 3198–3206. PMID:6826558.

Pfeiffer, K., Gohil, V., Stuart, R.A., Hunte, C., Brandt, U., Greenberg, M.L., and Schägger, H. 2003. Cardiolipin stabilizes respiratory chain supercomplexes. J. Biol. Chem. **278**(52): 52873–52880. doi:10.1074/jbc.M308366200. PMID:14561769.

Schlame, M., Rua, D., and Greenberg, M.L. 2000. The biosynthesis and functional role of cardiolipin. Prog. Lipid Res. **39**(3): 257–288. doi:10.1016/S0163-7827(00)00005-9. PMID:10799718.

Schmittgen, T.D., and Zakrajsek, B.A. 2000. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J. Biochem. Biophys. Methods, **46**(1-2): 69–81. doi:10.1016/S0165-022X(00)00129-9. PMID: 11086195.

Schwarz, D., Kisselev, P., Wessel, R., Jueptner, O., and Schmid, R.D. 1996. α-Branched 1,2-diacyl phosphatidylcholines as effectors of activity of cytochrome $P450_{SCC}$ (CYP11A1): modeling the structure of the fatty acyl chain region of cardiolipin. J. Biol. Chem. **271**(22): 12840–12846. doi:10.1074/jbc.271.22.12840. PMID:8662703.

Spencer, C.T., Bryant, R.M., Day, J., Gonzalez, I.L., Colan, S.D., Thompson, W.R., et al. 2006. Cardiac and clinical phenotype in Barth syndrome. Pediatrics, **118**(2): e337–e346. doi:10.1542/peds.2005-2667. PMID:16847078.

Tuominen, E.K., Wallace, C.J., and Kinnunen, P.K. 2002. Phospholipid-cytochrome *c* interaction: evidence for the extended lipid anchorage. J. Biol. Chem. **277**(11): 8822–8826. doi:10.1074/jbc.M200056200. PMID:11781329.

Vance, D.E., and Vance, J.E. 2002. Biochemistry of lipids, lipoproteins and membranes. 4th ed. Elsevier Science, Amsterdam.

Zhang, M., Mileykovskaya, E., and Dowhan, W. 2002. Gluing the respiratory chain together: cardiolipin is required for supercomplex formation in the inner mitochondrial membrane. J. Biol. Chem. **277**(46): 43553–43556. doi:10.1074/jbc.C200551200. PMID:12364341.