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

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Abstract

We examined whether cardiolipin (CL) synthesis was required to support cholesterol (CH) production from palmitate in Hela cells. Knock down of human cardiolipin synthase-1 (hCLS1) in Hela cells has been shown to reduce CL synthesis (Choi et al., 2007). Hela cells stably expressing shRNA for hCLS1 and mock control cells were incubated for 16 h with [¹⁴C(U)]palmitate bound to albumin (1:1 molar ratio) in the absence or presence of serum. Knock down of hCLS1 in Hela cells resulted in a reduction in [¹⁴C(U)]palmitate incorporation into CL and CH. This reduction in [¹⁴C(U)]palmitate incorporation into CH was most pronounced during incubation under serum-free conditions. The reduction in [¹⁴C(U)]palmitate incorporation into CH was not due to alterations in total uptake of [¹⁴C(U)]palmitate into cells or altered palmitate metabolism since [¹⁴C(U)]palmitate incorporation into phosphatidylcholine the major [¹⁴C(U)]palmitate containing lipid, and its immediate precursor 1,2-diacyl-*sn*-glycerol, was unaffected by hCLS1 knock down. In addition, knock down 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 knock down 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 stress conditions.

Introduction

CL was the first polyglycerolphospholipid discovered and was first isolated from beef heart by Mary Pangborn [reviewed in (Hostetler, 1982)]. Cardiolipin (CL) is both synthesized and localized exclusively within mammalian mitochondria (Daum, Heidorn, & Paltauf, 1986; Hostetler, 1982). CL is localized to both inner and outer mitochondrial membranes and

within contact sites in mammalian cells (Nicolay, Rojo, Wallimann, Demel, & Hovius, 1990). CL is required for the reconstituted activity of a number of key mitochondrial enzymes involved in cellular oxidative metabolism [reviewed in (Chicco & Sparagna, 2007; Hatch, 1998; Hoch, 1992)]. CL anchors cytochrome *c* to the inner mitochondrial membrane (Tuominen, Wallace, & Kinnunen, 2002) and may play a key role in cytochrome *c* release and apoptosis (McMillin & Dowhan, 2002; Ostrander, Sparagna, Amoscato, McMillin, & Dowhan, 2001; Ott, Robertson, Gogvadze, Zhivotovsky, & Orrenius, 2002). CL also plays an essential role in mitochondrial biogenesis (Schlame, Rua, & Greenberg, 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, Mileykovskaya, & Dowhan, 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 cytidinediphosphate-1,2-diacyl-*sn*-glycerol synthetase (CDS). There are two isoforms of this enzyme in mammalian tissues CDS-1 and CDS-2 (Halford, Dulai, Daw, Fitzgibbon, & Hunt, 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, van den Bosch, & van Deenen, 1972). The human CL gene (hCLS1) was recently cloned by four independent laboratories (Chen, Zhang, & Shi, 2006; Houtkooper et al., 2006; Lu et al., 2006; Choi et al. 2007).

De novo CH biosynthesis occurs in all nucleated cells and is up regulated in response to low intracellular sterol concentrations (Vance & Vance, 2002). 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is the major rate limiting step in *de novo* cholesterol synthesis, (Brown & Goldstein, 1999). HMG-CoA reductase activity in cells is regulated by sterols, primarily at the level of expression of HMGR mRNA (Goldstein & Brown, 1990). It has been estimated that approximately two thirds of all cellular CH is from *de novo* biosynthesis (Endo & 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 & Vance, 2002).

Evidence suggests there may be a link between CL and CH in the human body. The mitochondrial enzyme, cytochrome P-450_{scc} (or CYP11A1) is responsible for the oxidative side chain cleavage of CH. It was found that this enzyme has a CL binding site which can enhance the enzyme-substrate interaction (Lambeth, 1981; Pember, Powell, & Lambeth, 1983). Furthermore, human diseases such as BTHS (Kelley et al., 1991) and Tangier's Disease (Fobker et al., 2001) have reported altered metabolism of both CL and CH. More recently, CL has been identified as one of the first lipids to be altered in a rat model of diabetes (Han et al., 2007), often a consequence of metabolic X syndrome, which has been linked to altered CH metabolism (Holvoet, Lee, Steffes, Gross, & Jacobs, 2008). However, it was unknown if CL synthesis was required to support CH biosynthesis. Here we show that

knock down 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 stress conditions.

Materials and Methods

Materials

[¹⁴C(U)]Palmitate was obtained from Perkin-Elmer, Woodbridge, Ontario Dupont, Mississauga, Ontario, Canada. DL-3-[glutaryl-3-¹⁴C] hydroxy-3-methylglutaryl Coenzyme A was obtained from American Radiolabeled Chemicals, Inc, St. Louis, USA. DMEM, fetal bovine serum (FBS) and antibiotics were products of Canadian Life Technologies (GIBCO), Burlington, Ontario, Canada. Lipid standards were obtained from Serdary Research Laboratories, Englewood Cliffs, New Jersey, USA. Thin layer chromatographic plates (silica gel G, 0.25 mm thickness) were obtained from Fisher Scientific, Winnipeg, Canada. Ecolite scintillant was obtained from ICN Biochemicals, Montreal, Quebec, 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 Company, St. Louis, USA or Fisher Scientific, Winnipeg, Manitoba, Canada.

Cell culture

HeLa cells were obtained from American Type Culture Collection. HeLa cells were transfected with plasmids containing shRNA to human CLS as previously described (Choi et al., 2007). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 100 U Penicillin, 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 containing or deficient in FBS and [¹⁴C(U)]-Palmitic Acid (bound to albumin 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, Lees, & Stanley, 1957). Briefly, after the initial treatment of cells described above, the media was removed and cells washed with 2 ml PBS. Cells were scraped from the plates using a plastic scraper into 2 ml of methanol:H₂O (1:1, v/v). Cells were transferred with a Pasteur pipette to silanated glass tubes, and an aliquot was taken for total radioactivity and protein (25 µl, and 50 µl, respectively) determination. Protein assays were performed with a BioRad's Protein assay kit. Bovine serum albumin (BSA) was used as a standard. An additional 0.5 ml of H₂O 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, v/v) was followed by a second brief mixing as above and then centrifugation at 2,000 rpm for 5 min. Removal of the aqueous phase was followed by drying down of the organic phase with 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, v/v). 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 & McClarty, 1996). Spots corresponding to lipids were visualized with iodine vapor and removed into plastic scintillation vials and radioactivity in the sample 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 down with nitrogen, as fresh samples seemed 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 (RT) was performed immediately prior to polymerase chain reaction (PCR) as part of the same cycler protocol. The cycler protocol for real time PCR on the Eppendorf Mastercycler ep Realplex 2, consisted of a 30 minute RT step at 50°C , followed by a 15 minute Taq activation step at 95°C followed by a 1 minute separation at 95°C and melting curve that increased in temperature incrementally from 60°C to 95°C over the course of 20 minutes, taking fluorescence readings throughout to determine product quality. The primers used for CDS-1, CDS-2 and PGS in Hela cells have been previously described (Hauff, Linda, & Hatch, 2008). The primers used for HMGR and 18s RNA were as follows: HMGR (NCBI ID: NM_000859); Forward, cgggtGGAAGAGACAGGGATAAAC[FAM]G; Reverse, GGGTATCTGTTTCAGCCACTAAGG and 18s rRNA (X03205); Forward CTCGGGCCTGCTTTGAACAC, Reverse, cgggTGCTCTTAGCTGAGTGTCC[FAM]G. The PCR program for HMGR (400 nM) and housekeeping gene 18s rRNA (100 nM) primers ran 50 cycles of separation at 94°C for 15 seconds and annealing/elongation at 60°C for 45 seconds. Fluorescence readings were taken at the end of every elongation step. The changes in gene expression were analyzed on an Eppendorf Mastercycler ep Realplex, software version 1.5.474, and the data presented as mean fold change (2^{-C_t}) in mRNA expression (Livak & Schmittgen, 2001) relative to 18s rRNA, a gene not affected by short term incubation of cells under altered serum conditions (Schmittgen & Zakrajsek, 2000).

Assay of in vitro HMG-CoA reductase

Cells were incubated as above and removed from the plates and suspended on ice in 2 ml homogenizing buffer (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose), followed by homogenization with 2×20 strokes of a Dounce A homogenizer. The homogenate was centrifuged at 4°C , $1,000 \times g$ for 5 min and the supernatant centrifuged at 4°C , $10,000 \times g$ for 15 min. The resulting supernatants were then further centrifuged for 1.5 hours at 4°C , $100,000 \times g$, 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's Protein assay. BSA was used as a standard. *In vitro* HMG-CoA reduction assays were performed as described (Ohashi et al., 2003). Reaction buffer containing, at final concentration, 5 mM β -nicotinamide adenine dinucleotide phosphate reduced, tetrasodium salt (NADPH - made fresh), 10 mM Ethylenediamine-tetraacetic acid (EDTA), 10 mM dithiothreitol (DTT), 100 mM Tris-HCl (pH 7.4), was added to 50 μ g 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 μ Ci/ μ mol), cold HMG-CoA to 110 μ M, and incubation at 37°C for 30 minutes. Addition of HCl to 1N, and incubation for a further 30 minutes at 37°C was required to lactonize the mevalonate formed. Samples were then 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 $R_f = 0.6$ – 0.9 were removed into scintillation vials and radioactivity determined, i.e. [14 C]mevalonate formation.

Statistics

One-way or two-way ANOVA, with Dunnett's or Bonferroni's post tests respectively, were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com and data is reported as the means $\pm$ SEM,. The level of significance was defined as $p < 0.05$.

Results and Discussion

We previously demonstrated that knock-down 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) or mock controls (Mock) 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. Knock down of hCLS1 did not affect expression of CDS-1, CDS-2 or PGS in Hela cells incubated in serum-containing or serum-free medium (Figure 1A–C). As previously demonstrated, (Choi et al., 2007) knock down of hCLS1 reduced hCLS1 mRNA expression approximately 20–40% (data not shown). Thus, knock down 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) or mock controls (Mock) 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 radioactivity incorporated into CL determined. We used 0.1 mM palmitate bound to albumin (1:1 molar ratio) to achieve the labeling, since 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 ($0.68 \pm 0.02 \times 10^7$ dpm/mg) and hCLS ($0.70 \pm 0.04 \times 10^7$ dpm/mg) cells. In addition, total uptake of [14 C(U)]palmitate into growth-arrested (serum-free) cells was unaltered between mock ($0.69 \pm 0.05 \times 10^7$ dpm/mg) and hCLS ($0.67 \pm 0.03 \times 10^7$ dpm/mg) cells. Incorporation of

[$^{14}\text{C}(\text{U})$]palmitate into CL was reduced 42% ($p<0.05$) from 0.07 ± 0.01 % of total dpm to 0.04 ± 0.01 % of total dpm by knock down of hCLS1 in serum-supplemented Hela cells and was reduced 50% ($p<0.05$) from 0.08 ± 0.01 % of total dpm to 0.04 ± 0.01 % of total dpm by knock down of hCLS1 in Hela cells incubated in serum-free conditions. Thus, knock down of hCLS1 reduced CL biosynthesis from [$^{14}\text{C}(\text{U})$]palmitate in Hela cells grown in serum-containing or serum-free medium.

To examine if CL synthesis is required to support CH synthesis from palmitate, Hela cells stably expressing shRNA to hCLS1 (hCLS) or mock controls (Mock) were incubated for 16 h in medium containing [$^{14}\text{C}(\text{U})$]palmitate in the absence or presence of serum and the radioactivity incorporated into CH determined. Serum removal would be expected to upregulate CH biosynthesis (Fogelman, Seager, Edwards, & Popjak, 1977). Serum removal resulted in elevated CH synthesis from [$^{14}\text{C}(\text{U})$]palmitate in mock transfected Hela cells (Figure 2A). Thus, palmitate can be used as a carbon source for CH synthesis in Hela cells. However, knock down of hCLS1 resulted in a 20% decrease ($p<0.05$) and 40% decrease ($p<0.05$) in [$^{14}\text{C}(\text{U})$]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., 2008). Since total uptake of [$^{14}\text{C}(\text{U})$]palmitate into cells was unaltered and reduction in [$^{14}\text{C}(\text{U})$]palmitate incorporation into CH was observed for cells incubated in either the absence or presence of serum, the decrease in [$^{14}\text{C}(\text{U})$]palmitate incorporation into CH was not simply due to a potential growth defect caused by reduced CL synthesis. Thus, knock down of hCLS1 reduces [$^{14}\text{C}(\text{U})$]palmitate utilization for CH biosynthesis in Hela cells grown in serum-containing or serum-free medium. However, the effect was most significant when the cells were grown in the absence of serum, a condition where CH demand is highly up regulated (Fogelman et al., 1977).

Palmitate is a saturated fatty acid which will enter into most phospholipids via *de novo* biosynthesis (Vance & Vance, 2002). It could be argued that general [$^{14}\text{C}(\text{U})$]palmitate metabolism was disrupted by knock down of hCLS1. To address this, Hela cells stably expressing shRNA to hCLS1 (hCLS) or mock controls were incubated 16 h with medium containing [$^{14}\text{C}(\text{U})$]palmitate in the absence or presence of serum and radioactivity incorporated into the major palmitate containing lipid PC and its immediate precursor DG, determined. Serum removal resulted in an elevation in [$^{14}\text{C}(\text{U})$]palmitate incorporation into PC and DG compared to serum supplemented cells, respectively (Figure 2B–2C). Knock down of hCLS1 did not affect incorporation of [$^{14}\text{C}(\text{U})$]palmitate into PC or DG in Hela cells grown in serum-containing or serum-free medium. Thus, the reduction in [$^{14}\text{C}(\text{U})$]palmitate incorporation into CH with knock down 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 [$^{14}\text{C}(\text{U})$]palmitate incorporation into CH with knock down of hCLS1 was examined. 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, microsomal fractions were prepared and HMGR enzyme activity determined. Serum removal resulted in an increase in HMGR activity in both mock and hCLS cells (Figure 3A). Knock down of hCLS1 in Hela cells reduced HMGR *in vitro* activity 46% ($p<0.05$) in the

absence of serum compared to control. The effect was only significant when cells were grown in the absence of serum. Finally, we examined expression of HMGR mRNA in these cells using real time-PCR. Hela cells stably expressing shRNA to hCLS1 (hCLS) or mock controls were incubated in the absence or presence of serum for 16 h total RNA prepared and HMGR mRNA expression determined. Knock down of hCLS1 in Hela cells reduced HMGR mRNA expression 28% ($p < 0.05$) in the absence of serum compared to control (Figure 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 [$^{14}\text{C}(\text{U})$]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.

To examine if knock down of hCLS1 altered CH degradation, Hela cells stably expressing shRNA to hCLS1 (hCLS) or mock controls were incubated for 16 h in the absence or presence of serum and the pool size of CH determined. Knock down of hCLS1 did not affect the CH pool size when cells were incubated in either in the absence or presence of serum (Figure 3C). Thus, knock down of hCLS1 did not alter CH degradation.

Conclusion

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

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Figure 1.

Expression of enzymes of the CDP-DG pathway of CL biosynthesis in Hela cells.

Hela cells stably expressing shRNA to hCLS1 (hCLS) or mock controls (Mock) 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) determined. Data represent the mean $\pm$ SEM (n=3).

Figure 2.

Synthesis of lipids from $[^{14}\text{C}(\text{U})]$ palmitate in Hela cells.

Hela cells stably expressing shRNA to hCLS1 (hCLS) or mock controls (Mock) were incubated for 16 h in medium containing $[^{14}\text{C}(\text{U})]$ palmitate in the absence or presence of serum. Cells were harvested and radioactivity incorporated into CH (A), PC(B) and DG (C) determined. Data represent the mean $\pm$ SEM (n=3), *p<0.05.

Figure 3.

HMGR activity and mRNA expression and CH pool size in Hela cells.

Hela cells stably expressing shRNA to hCLS1 (hCLS) or mock controls were incubated 16 h with medium in the absence or presence of serum, microsomal fractions prepared and HMGR enzyme activity determined (A). Hela cells stably expressing shRNA to hCLS1 (hCLS) or mock controls were incubated 16 h with medium in the absence or presence of serum, total RNA was prepared and mRNA expression of HMGR determined (B). Hela cells stably expressing shRNA to hCLS1 (hCLS) or mock controls were incubated 16 h with medium in the absence or presence of serum and CH pool size determined (C). Data represent the mean $\pm$ SEM (n=3), *p<0.05.