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Regulation of cardiolipin biosynthesis by fatty acid transport protein-1 IN HEK 293 cells

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ABSTRACT

Cardiolipin (CL) is a major phospholipid involved in energy metabolism mammalian mitochondria and fatty acid transport protein-1 (FATP-1) is a fatty acid transport protein that may regulate the intracellular level of fatty acyl-Coenzyme A's. Since fatty acids are required for oxidative phosphorylation via mitochondrial oxidation, we examined the effect of altering FATP-1 levels on CL biosynthesis. HEK-293 mock- and FATP-1 siRNA transfected cells or mock and FATP-1 expressing cells were incubated for 24 h with 0.1 mM oleic acid bound to albumin (1:1 molar ratio) then incubated for 24 h with 0.1 mM [1,3-^{3}H] glycerol and radioactivity incorporated into CL determined. FATP-1 siRNA transfected cells exhibited reduced FATP-1 mRNA and increased incorporation of [1,3-^{3}H]glycerol into CL (2-fold, $p<0.05$) compared to controls indicating elevation in *de novo* CL biosynthesis. The reason for this was an increase in [1,3-^{3}H] glycerol uptake and increase in activity and mRNA expression of the CL biosynthetic enzymes. In contrast, expression of FATP-1 resulted a reduction in incorporation of [1,3-^{3}H]glycerol into CL (65%, $p<0.05$) indicating reduced CL synthesis. [1,3-^{3}H]Glycerol uptake was unaltered whereas activity of cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol synthetase (CDS) and CDS-2 mRNA expression were reduced in FATP-1 expressing cells compared to control. In addition, in vitro CDS activity was reduced by exogenous addition of oleoyl-Coenzyme A. The data indicate that CL *de novo* biosynthesis may be regulated by FATP-1 through CDS-2 expression in HEK 293 cells.

1. Introduction

Cardiolipin (CL) is a polyglycerolphospholipid found in and synthesized exclusively in the mitochondria of mammalian tissues [reviewed in 1–4]. CL is localized to both the inner and outer mitochondrial membranes and within intermembrane contact sites. CL plays an important role in many mitochondria mediated functions. CL is required for the activation of a number of key mitochondrial enzymes, such as cytochrome *c* oxidase and other proteins involved in the electron transport chain, "gluing" the respiratory chain together [1–4,5]. Loss of CL leads to a reduction in mitochondrial respiration [6]. In addition, studies have implicated CL loss in the regulation of mitochondrial-mediated apoptosis and Barth syndrome [reviewed in 7–12]. Thus, the appropriate content of CL is an important requirement for activation of enzymes involved in mitochondrial respiration and in the control of programmed cell death.

In mammalian tissues CL is synthesized *de novo* by the cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol (CDP-DG) pathway [13]. The first step is catalyzed by cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol synthetase (CDS) [14]. CDS catalyzes the conversion of phosphatidic acid (PA) to CDP-DG. In the heart and various mammalian cell lines CDS was shown to be a rate-limiting step of CL biosynthesis [reviewed in 1]. Two CDS enzymes, CDS-1 and CDS-2, have been cloned and characterized in human, mouse, rat and pig [15–21]. In the second step of the CDP-DG pathway CDP-DG is condensed with *sn*-glycerol-3-phosphate to form phosphatidylglycerol (PG) catalyzed by PG phosphate synthase (PGPS) and this is followed by rapid dephosphorylation to PG [14]. In the final step, PG condenses with another CDP-DG molecule to form CL and this is catalyzed by cardiolipin synthase (CLS) [22]. The gene encoding human CLS (hCLS-1) was recently identified and characterized [23–25].

Fatty acids are major components of glycerophospholipids and are required for energy production via beta-oxidation [reviewed in 26]. Fatty acids are taken up into cells either by diffusion due to their solubility in the membrane or by proteins which facilitate their transport [reviewed in 26–29]. Currently three protein families have been implicated in fatty transport, fatty acid translocase/CD 36,

Abbreviations: CL, cardiolipin; PG, phosphatidylglycerol; CDP-DG, cytidine-5′-diphosphate-1,2,-diacyl-*sn*-glycerol; PA, phosphatidic acid; CDS, cytidine-5′-diphosphate-1,2,-diacyl-*sn*-glycerol synthetase; PGPS, phosphatidylglycerolphosphate synthase; CLS, cardiolipin synthase; FATP-1, fatty acid transport protein-1

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plasma membrane fatty acid binding protein and fatty acid transport protein (FATP). Five members of the FATP family have been described in murine cells and a sixth identified from analysis of the human genome [30]. FATP-1 plays a key role in regulating fatty acid transport in mammalian cells. FATP-1 is a bifunctional protein with both transport and long-chain acyl-Coenzyme A synthetase activities [31]. Since fatty acid import into cells plays a key role in fatty acid oxidation and CL is required for oxidative phosphorylation, we examined whether altering the level of fatty acid transport protein in HEK 293 cells could regulate CL biosynthesis. We show that knock down of FATP-1 stimulates and expression of FATP-1 inhibits *de novo* CL synthesis.

2. Materials and methods

2.1. Materials

[^{14}C]Glycerol-3-phosphate, [5-^{3}H]cytidine-5′-triphophate, and [1,3-^{3}H]glycerol and were obtained from either Dupont, Mississauga, Ontario, or Amersham, Oakville, Ontario, Canada. Phosphatidyl[^{14}C] glycerol was synthesized from [^{14}C]glycerol-3-phosphate as described previously [32]. Human Embryonic Kidney (HEK) 293 cells were obtained from the American Type Culture Collection. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Trizol reagent were obtained from Canadian Life Technologies (GIBCO), Burlington, Ontario, Canada. C8 HEK 293 cells expressing FATP-1 were a generous gift from Dr. David A. Bernlohr, University of Minnesota. Lipid standards were obtained from the 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. Primers were designed and synthesized from Invitrogen, Burlington, Ontario, Canada. Flexitube siRNA was obtained from Qiagen, Cambridge, Massachusetts, USA. All other biochemicals were certified ACS grade and obtained from either Fisher Scientific, Winnipeg, Manitoba, Canada or Sigma Chemical Company, St. Louis, MO. USA.

2.2. Culturing, radiolabeling and harvesting of cells

HEK 293 cells were transfected with FATP-1 subcloned into pcDNA3.0 (C8) or transformed with plasmid control vector (mock) as previously described [33]. Cell lines were maintained on DMEM with 10% FBS and incubated at 37 °C in 5.0% CO_2. Cells expressing FATP-1 were selected with the addition of 400 μg/ml geneticin. In radiolabeling experiments, mock and C8 cell lines were incubated with 0.1 mM oleic acid bound to albumin (1:1 molar ratio) for 24 h and then incubated with 0.1 mM [1,3-^{3}H]glycerol (10 μCi/dish) for 24 h. Subsequent to incubation the medium was removed and the cells washed twice with 2 ml of ice-cold phosphate-buffered saline (PBS). The PBS was removed and 2 ml of methanol:water (1:1 v/v) was added. The cells were harvested using a rubber policeman into test tubes. The mixture was vortexed, and a 50 μl aliquot was taken for protein determination and a 10 μl or 25 μl aliquot taken for determination of total radioactivity. 2 ml of chloroform was added to the tubes followed by 0.5 ml 0.9% NaCl to initiate phase separation. The tubes were vortexed and then centrifuged at 2000 rpm for 10 min (bench top centrifuge), the aqueous phase was removed and 2 ml theoretical upper phase (48 ml methanol, 47 ml 0.9% NaCl, 3 ml chloroform) was added to wash the organic phase. The tubes were vortexed and centrifuged as described above and the aqueous phase removed. The organic phase was dried under a stream of N_2 gas and resuspended in 50 μl of chloroform:methanol (2:1 v/v). A 40 μl aliquot of organic phase was placed onto a thin-layer plate along with phospholipid standards and phospholipids were separated by two-dimensional thin-layer chromatography and radioactivity incorporated into phospholipids determined as described [32]. For determination of phospholipid pool sizes, in some experiments, a 40 μl aliquot of organic phase was placed onto a thin-layer plate in the absence of phospholipid standards and phospholipids were separated by two-dimensional thin-layer chromatography as pool size determined as described [34].

2.3. siRNA transient transfection

Transient transfection of HEK 293 cells followed the Fast-Forward Transfection of Adherent cells with siRNA in 100 mm dishes protocol provided in the HiPerFect Reagent handbook (Qiagen). Cells were seeded onto 100 mm dishes (3.0×10^{6} cells/cm^{2}) in 10 ml of fresh media. Cells were seeded 2 h prior to the addition of siRNA complexes. The FATP-1 siRNA target sequence was 5′-CCC TCT GAA GCT GTT CCT CTA. Complexes of 5 nM FATP-1 siRNA were formed by mixing siRNA with HiPerFect Reagent (Qiagen, Massachusetts, USA) and incubating for 10 min at room temperature. Cells were incubated with these complexes at 37 °C, 5.0% CO_2 for 48 h. Mock treated cells underwent the same transfection process but were incubated with only the HiPerFect reagent. mRNA was isolated with Trizol reagent (GIBCO) to monitor *FATP-1* gene silencing and measure the effectiveness of transfection. Mock and siRNA transfected cells were incubated with 0.1 mM oleic acid bound to albumin (1:1 molar ratio) for 24 h.

2.4. Real-time RT-PCR analysis

RNA from oleate-treated mock, FATP-1 knockdown and expressing cells was isolated using Trizol reagent (GIBCO) according to the manufacturer's instructions. Measurement of gene expression by quantitative analysis was carried out using a Mastercycler ep realplex system (Eppendorf). Primers and hybridization probes were synthesized by Invitrogen (Ontario, Canada) and Qiagen (Massachusetts, USA). Quantitative real-time RT-PCR analysis of human cytidine-5′-diphosphate-1,2-diacyl-*sn*-glycerol synthetase-2 (CDS-2), PGPS, CLS, FATP-1 and *β-actin* gene expression was carried out using a Quantitect Probe RT-PCR SYBR Green kit (Qiagen). The sequences of the screening primers were; CDS-2, forward 5′-GTC AGC ATC CCT TTG TCG AT, reverse 5′-CCA AGC AAA CTG ATT CAG CA; PGPS, forward 5′-TCG GCC TCC AGC ACA TTA AG, reverse 5′-AGT CAC TCA GGT TTG CAC CG; CLS, forward 5′-CGA GAG ATG TAA TGT TGA TTG CTG, reverse 5′-CGA ACC GTG GTG TTG GAA GAG TT[FAM]G; FATP-1, forward 5′-CCA CTT GGA TGT CAC CAC TG, reverse 5′-GTG GGA CCC TCC AGT AGA CA; β-actin, forward 5′-AGA AAA TCT GGC ACC ACA AC, reverse 5′-GGG GTG TTG AAG GTC TCA AA. PCR amplification of the housekeeping gene *β-actin* was carried out for each sample as a control for sample loading and to allow normalization among samples. The PCR condition for the each sample was 40 cycles of heating to 95 °C for 15 s, cooling to 60 °C for 30 s and 72 °C for 30 s. A melting curve was taken of each product to determine purity levels. To determine the relative gene expression between mock and FATP-1 expressing cells or mock and FATP-1 knockdown cells ΔΔCt values were calculated. ΔΔCt values were calculated by subtracting the ΔCt (sample) from the ΔCt (control). The β actin control was normalized to 1.0 and each target gene was then normalized following this equation. Results were represented as the mean of three RNA samples from each group.

2.5. Preparation of mitochondrial fractions and enzyme assays

All isolation procedures were preformed at 4 °C. Cells were incubated with 0.1 mM oleic acid bound to albumin (1:1 molar ratio) for 24 h. Cells were harvested using PBS and pipetted into test tubes. The tubes were centrifuged at 500 ×*g* for 10 min and the PBS removed. Addition of 1 ml homogenizing buffer (0.25 M sucrose, 10 mM Tris–HCL, 0.145 M NaCl, pH 7.4) was followed by homogenization by 50 strokes of a tight fitting Dounce A homogenizer. The homogenate was

centrifuged at 1000 ×g for 10 min and the resulting supernatant centrifuged at 12,000 ×g for 15 min. The resulting pellet was resuspended in 0.5 ml of homogenizing buffer by a tight fitting Dounce A homogenizer and used as the source of mitochondrial fraction for assay of mitochondrial enzyme activities. CDS, PGPS and CLS activities were determined as previously described [32].

2.6. Other determinations

Isolation and determination of long-chain acyl-CoA from mitochondrial fractions of HEK 293 mock and C8 cells expressing FATP-1 were previously described [35]. Acyl-CoA content in the samples was determined using gas chromatography and data analyzed by a Shimadzu Chromatopac CR501. Protein was measured by the method of Lowry [36]. In some experiments, equivalent protein amounts of mock and FATP-1 expressing cells were separated by two-dimensional isoelectric focusing/sodium dodecyl sulphate polyacrylamide gel electrophoresis and stained as described [37].

3. Results

Since fatty acid import into cells plays a key role in fatty acid oxidation and CL is required for oxidative phosphorylation, we examined whether altering the level of FATP-1 regulated *de novo* CL biosynthesis. HEK 293 cells were transfected with FATP-1 siRNA for 48 h and FATP-1 mRNA expression was determined by real time-PCR. FATP-1 mRNA expression was significantly knocked down (Fig. 1). Mock and FATP-1 siRNA transfected cells were then incubated with 0.1 mM oleate (bound to albumin 1:1) for 24 h and then with 0.1 mM [1,3-^{3}H]glycerol for 24 h and the radioactivity incorporated into phospholipids determined. Incorporation of [1,3-^{3}H]glycerol into CL was increased 2-fold ($p<0.05$) in FATP-1 siRNA transfected cells compared to mock transfected controls (Table 1). In addition, [1,3-^{3}H]glycerol incorporated into other glycerophospholipids was also increased approximately 2-fold ($p<0.05$). Total uptake of [1,3-^{3}H]glycerol was increased 2-fold ($p<0.05$) in FATP-1 siRNA transfected cells compared to mock transfected controls. These data indicated that knock down of FATP-1 in HEK 293 cells stimulated CL and phospholipid *de novo* synthesis from glycerol at the level of glycerol uptake.

The pool size of CL and the major phospholipids, phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were determined in mock and FATP-1 siRNA transfected cells. The pool size of CL was increased 2.3-fold ($p<0.05$) in FATP-1 siRNA transfected cells compared to controls (Fig. 2A). However, the overall phospholipid content in the cells did not change as no alterations in the pool size of the major phospholipids PE and PC were observed. Thus, *de novo* CL synthesis was increased in FATP-1 siRNA transfected HEK 293 cells incubated with oleate.

The reason for the increase in CL was examined. The mitochondrial fractions were prepared from mock and FATP-1 siRNA transfected cells and the activities of the enzymes of the CDP-DG pathway determined. CDS, PGPS and CLS activities were increased 23% ($p<0.05$), 21% $p<0.05$) and 71% ($p<0.05$), respectively, in FATP-1 siRNA transfected cells compared to controls (Table 2A). Thus, the increase in CL content in FATP-1 siRNA transfected cells was due to an increase in the activities of the enzymes of the CDP-DG pathway of CL synthesis.

The reason for the increase in enzyme activities of CDS, PGPS and CLS in oleate-treated FATP-1 siRNA transfected cells was examined. Total mRNA was prepared from mock and FATP-1 siRNA transfected cells and expression of CDS-2, PGPS and CLS mRNA were examined. CDS-2, PGPS and CLS mRNA expressions were all elevated in the FATP-1 siRNA transfected cells to the mock transfected cells (Fig. 3A–C). Thus, the content of CL and increased [1,3-^{3}H]glycerol incorporation into CL in oleate-treated FATP-1 siRNA transfected cells was due to an increase in activity and mRNA expression of enzymes of the CDP-DG pathway of CL synthesis.

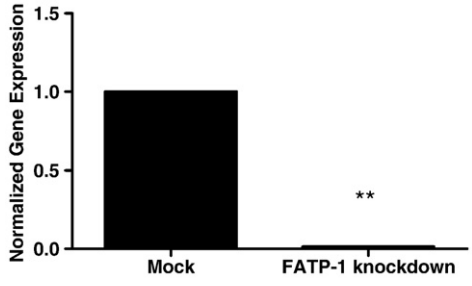

Fig. 1. FATP-1 mRNA expression in mock transfected HEK 293 cells and FATP-1 siRNA transfected cells. Mock and FATP-1 siRNA transfected cells were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and total RNA isolated and expression of FATP-1 and β-actin mRNA determined as described in Materials and methods. Data represents the mean of three dishes.

Table 1
[1,3-^{3}H]Glycerol incorporation into phospholipids in mock and FATP-1 siRNA transfected cells.

Phospholipid	Mock (dpm/mg protein × 10^6)	FATP-1 knockdown
Cardiolipin	1.7 ± 0.4	3.3 ± 0.6*
Phosphatidylglycerol	0.4 ± 0.1	1.1 ± 0.1*
Phosphatidylcholine	17.6 ± 3.9	37.6 ± 2.9*
Phosphatidylethanolamine	6.4 ± 1.5	13.7 ± 1.1*
Phosphatidylserine/inositol	1.1 ± 0.3	2.0 ± 0.1*
Phosphatidic acid	1.1 ± 0.2	2.5 ± 0.4*
Total radioactivity	30.6 ± 0.7	62.1 ± 0.5*

Mock and FATP-1 siRNA transfected cells were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and then incubated with 0.1 mM [1,3-^{3}H]glycerol for 24 h and radioactivity incorporated into phospholipids determined as described in Materials and methods. Data represents the mean ± S.D. of three dishes.
* $p<0.05$.

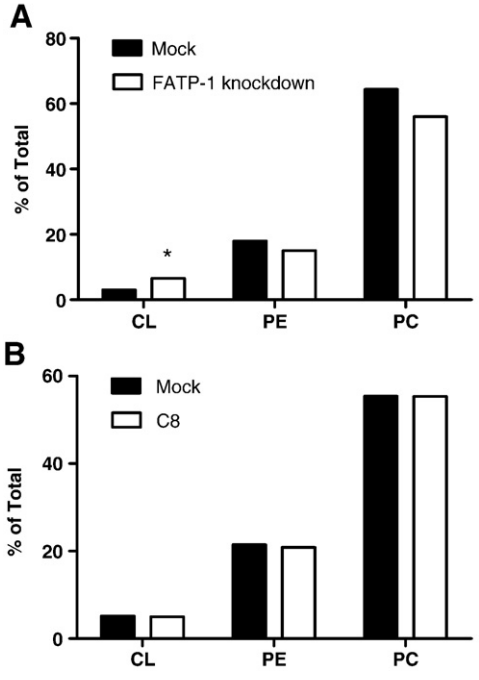

Fig. 2. Pool size of major glycerophospholipids in FATP-1 siRNA transfected cells or FATP-1 expressing C8 cells incubated with oleate. Mock and FATP-1 siRNA transfected cells (A) or mock and FATP-1 expressing C8 cells (B) were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and the pool size of the major glycerophospholipids were determined as described in Materials and methods. Data represents the mean ± S.D. of three dishes and is expressed as a percent of total phospholipid. *$p<0.05$.

Table 2
Enzyme activities in FATP-1 siRNA transfected cells or FATP-1 expressing cells incubated with oleate.

A.		
Enzyme	Mock (pmol/min/mg protein)	FATP-1 knockdown
CDS	23.9 ± 0.1	31.0 ± 0.6*
PGPS	57.1 ± 5.8	198.8 ± 3.7*
CLS	0.7 ± 0.3	2.4 ± 0.6*
B.		
Enzyme	Mock (pmol/min/mg protein)	C8
CDS	41.2 ± 6.1	25.5 ± 2.4*
PGP	162.8 ± 31.5	163.1 ± 6.9
CLS	1.0 ± 0.2	0.9 ± 0.2

Mock and FATP-1 siRNA transfected cells (A) or mock and FATP-1 expressing C8 cells (B) were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and the enzyme activities of the CDP-DG pathway determined as described in Materials and methods. Data represents the mean ± S.D. of three dishes.
* $p<0.05$.

Since FATP-1 knockdown in HEK 293 cells resulted in an increase in CL synthesis we examined if expression of FATP-1 in HEK 293 cells had the corollary effect on CL synthesis. Mock or C8 cells expressing FATP-1 were incubated with 0.1 mM oleate (bound to albumin 1:1) for 24 h and then with 0.1 mM 1,3-^{3}H]glycerol for 24 h and radioactivity incorporation into CL determined. Incorporation of [1,3-^{3}H]glycerol into CL was reduced 65% ($p<0.05$) in C8 cells compared to control (Table 3). In addition, [1,3-^{3}H]glycerol incorporated into PG and PC was decreased. In contrast, total uptake of [1,3-^{3}H]glycerol was unaltered between C8 cells and controls. These data indicated that expression of FATP-1 in HEK 293 cells inhibited CL synthesis from glycerol.

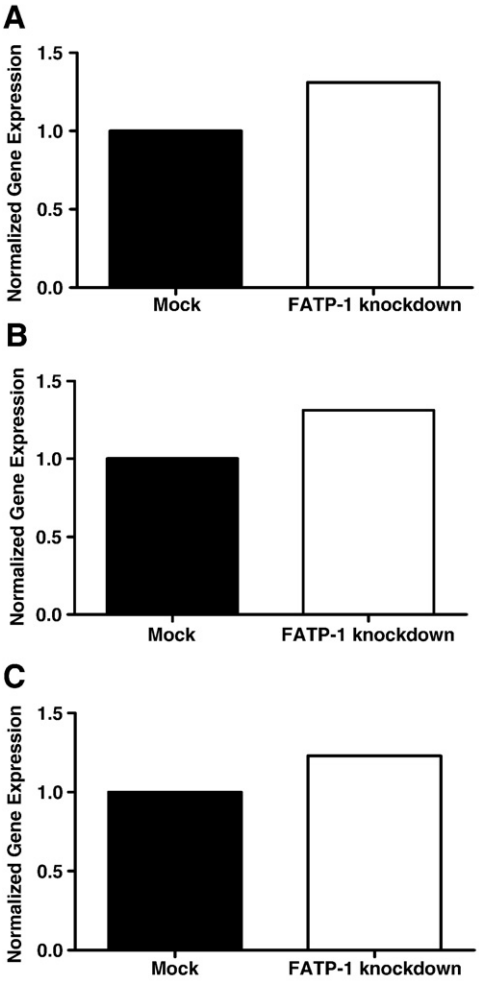

Fig. 3. CDS-2, PGPS and CLS mRNA expression in mock and FATP-1 siRNA transfected cells. Mock and FATP-1 siRNA transfected cells were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and total RNA was isolated and expression of CDS-2 (A), PGPS (B) and CLS (C) mRNA determined as described in Materials and methods. Data represents the mean of three dishes.

Table 3
[1,3-^{3}H]Glycerol incorporation into phosholipids in C8 cells expressing FATP-1.

Phospholipid	Mock (dpm/mg protein × 10^5)	C8
Cardiolipin	3.5 ± 1.4	1.2 ± 0.4*
Phosphatidylglycerol	2.4 ± 0.2	1.7 ± 0.4*
Phosphatidylcholine	30.7 ± 8.4	12.4 ± 4.4*
Phosphatidylethanolamine	8.8 ± 1.9	5.1 ± 0.9
Phosphatidyserine	0.9 ± 0.2	0.6 ± 0.2
Phosphatidic acid	0.6 ± 0.1	0.4 ± 0.3
Total radioactivity	12.4 ± 0.1	11.1 ± 0.3

HEK 293 mock and C8 cells expressing FATP-1 were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and then incubated with 0.1 mM [1,3-^{3}H] glycerol for 24 h and radioactivity incorporated into phospholipids determined as described in Materials and methods. Data represents the mean ± S.D. of three dishes.
* $p<0.05$.

The pool size of CL and the major phospholipids in FATP-1 expressing cells were examined. The pool size of CL and the other major phospholipids (PE and PC) were unaltered between vector and C8 cells treated with oleate (Fig. 2B). Thus, *de novo* CL biosynthesis from [1,3-^{3}H]glycerol was decreased in C8 cells expressing FATP-1 incubated with oleate but this did not affect the CL pool size. Since the pool size of phospholipids was unaltered we examined if FATP-1 expression altered protein expression in these cells. Equivalent protein amounts from mock and C8 cells were separated by two-dimensional isoelectric focusing/sodium dodecyl sulphate polyacrylamide gel electrophoresis and stained as described in Materials and methods. As seen in Fig. 4, expression of FATP-1 cells altered the protein expression profile compared to control. As expected the level of FATP-1 protein was elevated (closed arrow B). In contrast, the level of other several proteins (open arrows in B) was reduced. Thus, FATP-1 expression reduced the expression of several proteins in HEK 293 cells and this may, in part, have accounted for the lowered [1,3-^{3}H]glycerol incorporation into CL.

The reason for the decrease in [1,3-^{3}H]glycerol incorporation into CL in C8 cells was then examined. Mitochondrial fractions were isolated from mock and C8 cells and CL biosynthetic enzyme activities determined. CDS activity was reduced 38% ($p<0.05$) in C8 cells compared to controls (Table 2B). PGPS and CLS activities were unaltered between control and C8 cells. The reason for the decrease in CDS activity in C8 cells was examined. Mock and C8 cells were incubated with 0.1 mM oleate for 24 h and total RNA isolated and CDS-2 mRNA expression determined. CDS-2 mRNA expression was decreased in C8 cells compared to controls (Fig. 5). FATP-1 exhibits acyl-Coenzyme A synthetase activity [31] and expression of FATP-1 in C8 cells was shown to elevate intracellular fatty acid levels [33]. A previous study had indicated that CDS enzyme activity was inhibited by oleic acid in isolated lymph node lymphocytes [39]. Mitochondrial fractions from HEK 293 cells were prepared and CDS enzyme activity determined in the absence or presence of various concentrations of oleoyl-Coenzyme A. CDS in vitro activity was reduced in the presence of 1 μM or greater concentrations of oleoyl-Coenzyme A (Fig. 6A). This data indicated that the reduction in the CDS enzyme activity seen in C8 cells expressing FATP-1 might also be in part due to increases in fatty acyl-Coenzyme A in these cells. Next, long-chain acyl-Coenzyme A levels were determined in control and C8 cells. Long-chain acyl-Coenzyme As were significantly increased 44% ($p<0.05$) in C8 cells

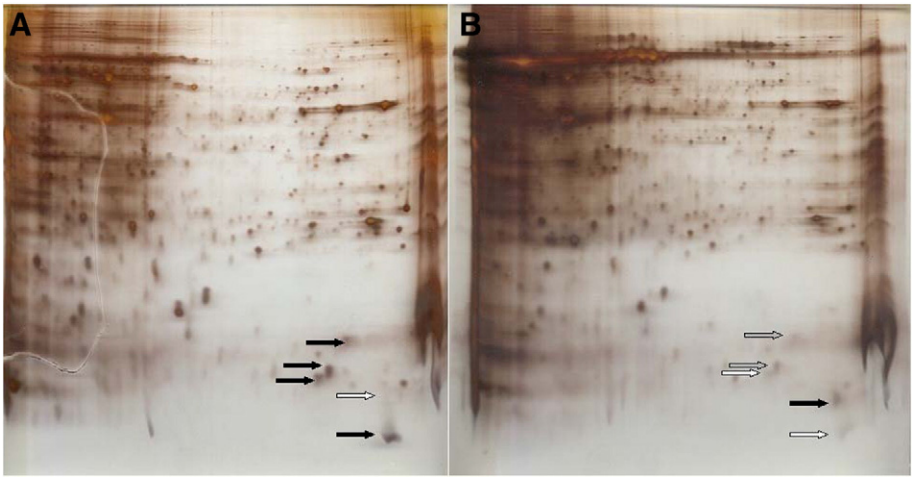

Fig. 4. Protein expression profile in FATP-1 expressing cells. Equivalent amounts of protein lysates from mock (A) and C8 (B) cells were separated by two-dimensional isoelectric focusing/sodium dodecyl sulphate polyacrylamide gel electrophoresis and stained as described in Materials and methods. Closed arrows in B indicate a protein whose level was elevated; open arrows in B indicate proteins in which the level was reduced. A representative gel is depicted.

compared to control (Fig. 6B). The elevated long-chain acyl-Coenzyme A levels observed in C8 cells were consistent with a previous study in rats in which supplementation with a Tallow diet resulted in elevated liver acyl-Coenzyme A levels [38]. Thus, the decreased CL synthesis in oleate-treated C8 cells could be due to reduction in CDS enzyme activity, CDS-2 mRNA expression as well as elevation in intracellular acyl-Coenzyme A.

4. Discussion

The objective of this study was to examine if altering the level of FATP-1 in HEK 293 cells could regulate CL biosynthesis. Incorporation of [1,3-^{3}H]glycerol into CL was increased when FATP-1 was knocked down in HEK 293 cells compared to control. In addition, CL mass was elevated 2-fold when FATP-1 was knocked down in these cells. The reason for the increased CL content was likely an increase in [1,3-^{3}H]glycerol uptake and utilization for CL synthesis and an increase in activity and expression of the CL biosynthetic enzymes. The alteration in CL synthesis was reversed in FATP-1 expressing cells. In this case, [1,3-^{3}H]glycerol incorporation into CL was reduced in cells expressing FATP-1 compared to control even though [1,3-^{3}H]glycerol uptake was unaltered. The reason for the decrease in [1,3-^{3}H]glycerol incorporation into CL was a reduction in CDS enzyme activity and CDS-2 mRNA expression. Interestingly, the content of CL was unaltered in FATP-1 expressing cells compared to controls. Although *de novo* CL synthesis from [1,3-^{3}H]glycerol was lower in these cells expressing FATP-1, it was not surprising that there was no alteration in CL mass since normal turnover of CL is slow, in a matter of days, in mammalian cells [reviewed in 2]. The above observations indicate that CL synthesis is specifically modulated by FATP-1 knock down or expression.

Knock down of FATP-1 increased whereas FATP-1 expression did not affect PGPS and CLS activities. CDS is a rate-limiting step of CL biosynthesis in the CDP-DG pathway of mammalian cells [32]. CDS activity was increased by FATP-1 knockdown and decreased by FATP-1 expression in HEK 293 cells. These alterations in CDS enzyme activity by alteration in FATP-1 expression suggest that the modulation of CL biosynthesis by FATP-1 expression is likely mediated through CDS. The reason for the increased CDS activity in FATP-1 siRNA transfected cells was an increase in CDS-2 mRNA

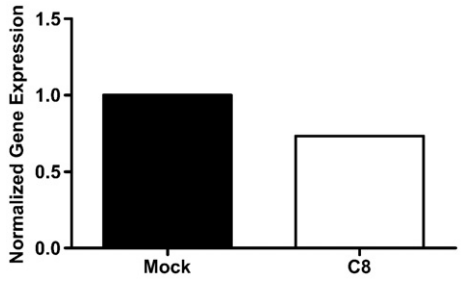

Fig. 5. CDS-2 mRNA expression in mock and C8 cells expressing FATP-1. Mock and C8 cells were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and total RNA isolated and expression of CDS-2 mRNA determined as described in Materials and methods. Data represents the mean of three dishes.

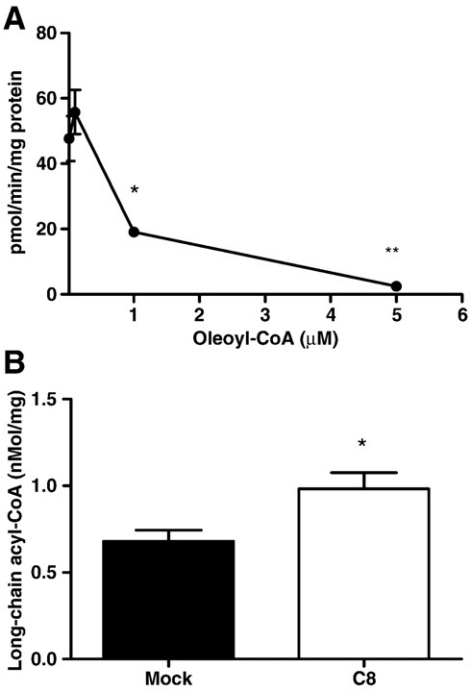

Fig. 6. CDS activity in 293 cells in the presence of various concentrations of oleoyl-Coenzyme A. (A) Mitochondrial fractions were prepared from HEK 293 cells and then incubated with 0 μM or 0.01 μM or 0.1 μM or 1 μM or 5 μM oleoyl-Coenzyme A and CDS activity determined as described in Materials and methods. (B) Long-chain acyl-Coenzyme A were determined in mock and C8 incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h as described in Materials and methods. Data represents the mean ± S.D. of three dishes. *$p<0.05$, ** $p<0.01$.

expression. In addition, CDS-2 mRNA expression was reduced in FATP-1 expressing cells that exhibited a reduction in CDS activity. CDS-2 mRNA expression is widespread throughout the body with highest expression in the heart, brain and kidney [16–20]. In contrast, CDS-1 mRNA expression is low in most tissues throughout the body outside of the retina [16–20]. The above data suggest that CL biosynthesis may be modulated by FATP-1 expression through CDS activity and CDS-2 mRNA expression.

How does FATP-1 expression modulate CL biosynthesis through CDS? Although we did not measure CDS-2 protein in these cells due to a lack of an available antibody, the level of several proteins were reduced in FATP-1 expressing cells. It is possible that CDS-2 may be one of these proteins and lowered CDS-2 protein may have accounted for the reduced CDS enzyme activity in FATP-1 expressing cells. In addition to its fatty acid transport activity, FATP-1 is an acyl-Coenzyme A synthetase that couples fatty acid synthesis to acyl-Coenzyme A production [31]. CDS enzyme activity was shown to be inhibited by oleic acid in isolated hog lymph node lymphocytes [39]. We observed a reduction in CDS activity with addition of exogenous oleoyl-Coenzyme A indicating that elevation in fatty acid, previously observed in HEK 293 cells expressing FATP-1 [33], may inhibit CDS enzyme activity in these cells. The free cytosolic concentration of acyl-Coenzyme A esters is in the low nanomolar range, and unlikely exceeds 0.2 μM under the most extreme conditions [40]. Thus, it is likely that under normal physiological conditions CDS activity may not be regulated by cytosolic acyl-Coenzyme A levels. However, FATP-1 has been shown to collaborate and interact with carnitine palmitoyl-transferase-1 (CPT1), which imports long-chain fatty acids into the mitochondria [41]. This interaction would allow an increase in long-chain acyl-Coenzyme A by FATP-1 acyl-Coenzyme A synthetase in the cytosol to reflect an increase in acyl-Coenzyme A within the mitochondria in C8 cells expressing FATP-1. Thus, the decrease in CDS activity observed in FATP-1 expressing cells could be due in part to an oleoyl-Coenzyme A accumulation within the mitochondria of these cells. CDS activity was shown to be regulated by the composition of PA [42]. For example, in rat brain membrane fractions a 20% reduction in CDS activity was observed when 1-stearoyl-2-oleoyl-PA was replaced with 1,2-dioleoyl-PA. Thus, increasing the oleate concentration of the PA substrate may alter CDS activity through substrate composition. We did not observe significant changes in the fatty acyl composition of PA in our experiments (data not shown). Thus, regulation of CDS activity through PA also seems unlikely. It is well documented that fatty acids have a significant effect on RNA abundance of genes encoding proteins involved in fatty acid metabolism in mammals [43]. Fatty acids may increase the expression of fatty acid transport proteins including carnitine palmitoyl transferase-1 and FATP [44]. Hepatocyte nuclear factor-4α (HNF-4α) is a member of a transcription factor family involved in hepatocyte differentiation and cellular metabolism [45,46]. HNF-4α is broadly expressed with very high mRNA expression in the kidney [47]. Expression of HNF-4α may be regulated by binding of long-chain acyl-Coenzyme A's and coexpression of acyl-Coenzyme A synthetase potentiated activation of HNF-4α [48]. Transcription factor binding analysis of the *CDS* gene using PROMO predicts a potential HNF-4α binding site. Since FATP-1 is an acyl-Coenzyme A synthetase [31] alteration in expression of FATP-1 could potentially modulate HNF-4α activation and hence CDS expression. This will form the basis for future studies on the nature of regulation of CDS expression mediated by FATP-1. In summary, altering FATP-1 levels in HEK 293 cells appears to modulate CL synthesis via regulation through CDS activity and expression.

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