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The dynamics of cardiolipin synthesis post-mitochondrial fusion

Fred Y. Xu [a], Heidi McBride [b], Devrim Acehan [c], Frédéric M. Vaz [d], Riekelt H. Houtkooper [e], Raymond M. Lee [f], Michael A. Mowat [g], Grant M. Hatch [a,g,h,*]

[a] Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Canada R3E 0T6
[b] Ottawa Health Institute, University of Ottawa, Ontario, Canada
[c] New York University School of Medicine, Skirball Institute, NY, USA
[d] Academic Medical Center, Laboratory Genetic Metabolic Diseases, Amsterdam, The Netherlands
[e] Laboratory for Integrative and Systems Physiology, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland
[f] Virginia Commonwealth University, Richmond, VA 23298, USA
[g] Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, Canada
[h] Center for Research and Treatment of Atherosclerosis, Internal Medicine, University of Manitoba, Winnipeg, Canada

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ABSTRACT

Alteration in mitochondrial fusion may regulate mitochondrial metabolism. Since the phospholipid cardiolipin (CL) is required for function of the mitochondrial respiratory chain, we examined the dynamics of CL synthesis in growing Hela cells immediately after and 12 h post-fusion. Cells were transiently transfected with Mfn-2, to promote fusion, or Mfn-2 expressing an inactive GTPase for 24 h and *de novo* CL biosynthesis was examined immediately after or 12 h post-fusion. Western blot analysis confirmed elevated Mfn-2 expression and electron microscopic analysis revealed that Hela cell mitochondrial structure was normal immediately after and 12 h post-fusion. Cells expressing Mfn-2 exhibited reduced CL *de novo* biosynthesis from [1,3-^{3}H]glycerol immediately after fusion and this was due to a decrease in phosphatidylglycerol phosphate synthase (PGPS) activity and its mRNA expression. In contrast, 12 h post-mitochondrial fusion cells expressing Mfn-2 exhibited increased CL *de novo* biosynthesis from [1,3-^{3}H]glycerol and this was due to an increase in PGPS activity and its mRNA expression. Cells expressing Mfn-2 with an inactive GTPase activity did not exhibit alterations in CL *de novo* biosynthesis immediately after or 12 h post-fusion. The Mfn-2 mediated alterations in CL *de novo* biosynthesis were not accompanied by alterations in CL or monolysoCL mass. [1-^{14}C]Oleate incorporation into CL was elevated at 12 h post-fusion indicating increased CL resynthesis. The reason for the increased CL resynthesis was an increased mRNA expression of tafazzin, a mitochondrial CL resynthesis enzyme. Ceramide-induced expression of PGPS in Hela cells or in CHO cells did not alter expression of Mfn-2 indicating that Mfn-2 expression is independent of altered CL synthesis mediated by elevated PGPS. In addition, Mfn-2 expression was not altered in Hela cells expressing phospholipid scramblase-3 or a disrupted scramblase indicating that proper CL localization within mitochondria is not essential for Mfn-2 expression. The results suggest that immediately post-mitochondrial fusion CL *de novo* biosynthesis is "slowed down" and then 12 h post-fusion it is "upregulated". The implications of this are discussed.

1. Introduction

Cardiolipin (CL) is a major mitochondrial membrane phospholipid involved in mitochondrial function [reviewed in 1–6]. Reduced CL is one of the underlying biochemical defects for Barth syndrome, a rare X-linked genetic disease [reviewed in 7–9]. Mammalian CL is synthesized *de novo* via the cytidine-5′-diphosphate-1,2-diacylglycerol (CDP-DG) pathway [10]. In this pathway, phosphatidic acid is converted to CDP-DG catalyzed by cytidine diphosphate-diacylglycerol synthetase (CDS) [11]. CDP-DG is then condensed with glycerol-3-phosphate to form phosphatidylglycerolphosphate (PGP) catalyzed by PGP synthase (PGPS), the committed step of CL *de novo* biosynthesis. PGP does not accumulate in mammalian tissues and is rapidly converted to phosphatidylglycerol (PG) via a highly active PGP phosphatase. In the final step of the pathway, PG is condensed with another molecule of CDP-DG to form CL catalyzed by CL synthase (CLS) [12]. Several studies have indicated that CL *de novo* biosynthesis in mammalian cells may be controlled at the level of PGPS. For example, during rat liver regeneration the CL pool is increased via elevated PGPS mRNA expression and activity [13]. In mutant Chinese hamster ovary (CHO) cells defective in PGPS at the restrictive temperature, the CL pool is reduced [14]. In addition, *de novo* synthesis of mammalian CL may be controlled by PGPS activity at the level of the transcription factor PPARα

* Corresponding author. Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, 753 McDermot Avenue, Winnipeg, Manitoba, Canada R3E 0T6. Tel.: +1 204 789 3405; fax: +1 204 789 3932.
E-mail address: hatchgm@ms.umanitoba.ca (G.M. Hatch).

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and thyroid hormone [15,16]. Recently we showed that N-acetylsphingosine (C_2-Cer) stimulated *de novo* CL synthesis in H9c2 cardiac myoblasts and in CHO cells via elevated expression of PGPS mRNA and its activity [17,18]. The Dlc-2 (Stard13) RhoGap gene, when inactivated by retroviral insertion or RNAi knockdown, prevented C_2-Cer induction of PGPS indicating that Rho signaling normally suppresses induction of PGPS [18].

Mitochondrial fusion is an active and dynamic process which is required for growth and maintenance of mitochondria [reviewed in 19–21].The presence of mitofusin (Mfn) is a strict requirement for the mitochondrial fusion process to occur [22–24]. Mutations in the mitochondrial GTPase mitofusin-2 (Mfn-2) cause Charcot–Marie–Tooth neuropathy type 2A, a common yet heterogeneous inherited neuropathy [25]. Mfn is required to link opposing mitochondrial outer membranes together via antiparallel dimerization of a coiled-coil domain [26]. The GTPase domain of Mfn is required for this fusion process [24,26]. The mechanism through which Mfn interacts with phospholipids of the outer mitochondrial membrane, such as CL, to mediate mitochondrial fusion is beginning to emerge. A recent study indicated that mitochondrial phospholipase D targeted to the external face of mitochondria promoted transmitochondrial membrane adherence in an Mfn-dependent manner by hydrolyzing CL to generate PA [27]. CL is localized to both inner and outer mitochondrial membranes [28,29]. Localization of CL within these membranes is mediated in part by phospholipid scramblase-3 (PLS3) [30]. Recently, we showed that altered CL localization mediated by disruption or elevation of PLS-3 expression stimulated CL *de novo* biosynthesis [31]. It is likely that maintenance of CL *de novo* synthesis is important during and after the mitochondrial fusion process. The dynamics of CL *de novo* biosynthesis post-mitochondrial fusion had never been examined. In this study, we show that immediately post-Mfn-2 mediated mitochondrial fusion in Hela cells CL *de novo* biosynthesis is "slowed down" and then 12 h post-fusion it is "upregulated". However, CL synthesis or proper localization of CL within the mitochondria is not a requirement for Mfn-2 expression.

2. Materials and methods

2.1. Materials

Hela cells were obtained from American Type Culture Collection. [^{14}C]Glycerol-3-phosphate, [5-^{3}H]cytidine 5′-triphophate, [1,3-^{3}H] glycerol and [1-^{14}C]oleate 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]. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from 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. Antibodies to Mfn-2 (Cat. # M9073) and β-actin (Cat. # 2066) were obtained from Sigma Chemical Company, St. Louis, MO. USA. ECL Western blotting detection system was from Amersham, Canada. 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. Culture, radiolabeling and harvesting of Hela cells

Hela cells were cultured in DMEM supplemented with 10% (v/v) FBS, and 1% (v/v) penicillin/streptomycin (P/S). Cell cultures were maintained at 37 °C in humidified air, containing 5% CO_2 until 80% confluence. Each dish of cells was subcultured at a 1:4 ratio and confluence was usually obtained after 4 days of incubation. Cells were maintained at a logarithmic growth phase for all experiments. Cells were transfected with Mfn-2 plasmid or Mfn-2 expressing an inactive GTPase plasmid (Mfn2RasG12V) for 24 h as previously described [33]. Cells were then incubated for the last 4 h with DMEM in the absence or presence of 0.1 mM [1,3-^{3}H]glycerol (10 μCi/dish). In other experiments, cells were transfected with Mfn-2 or Mfn-2 expressing an inactive GTPase plasmids for 24 h as above and then incubated for 12 h with 0.1 mM [1,3-^{3}H]glycerol (10 μCi/dish) or 0.1 mM [1-^{14}C]oleate (2 μCi/dish) bound to albumin in a 1:1 molar ratio. Subsequent to incubation the medium was removed and the cells washed 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. 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, and 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 and phospholipids were separated by two-dimensional thin-layer chromatography [32]. In some experiments, phospholipid standards were placed onto plates prior to chromatography and radioactivity incorporated into phospholipids was determined in the spots corresponding to these phospholipids [32].

2.3. Preparation of subcellular fractions and assay of enzyme activities

All isolation procedures were performed at 4 °C. Subcellular fractions were prepared from Hela cells transfected with Mfn-2. 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. Mitochondrial phosphatidylglycerolphosphate synthase (PGPS), cardiolipin synthase (CLS) and monolysocardiolipin acyltransferase (MLCL AT) activities were determined as previously described [32,34].

2.4. Quantitative real-time RT-PCR analysis

Amplification of each target cDNA was performed with QuantiTect SYBR Green PCR Master Mix (Qiagen) using a Eppendorff Realplex^2 mastercycler according to the protocols provided. The PCR cycling was programmed as 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s for 40 cycles. PCR primers used were: human PGP synthase forward, 5′-GAC AAC AACGTC GTC TTG AGT G-3′; reverse, 5′-GAA GTC TGC AAT CTC AGC ACA G-3′ and for 18s forward, 5′-CTC GGG CCT GCT TTG AAC AC-3′; reverse, 5′-cgggTGCTCTTAGCTGAGTGTCC[FAM]G-3′. Relative gene expression was determined using the $^{2-\Delta\Delta}$CT method normalized to the 18s levels [35]. Real-time PCR data quality control, $^{2-\Delta\Delta}$CT and analysis of co-variance calculations were run on the SAS program as described [36]. Primers for human CLS (hCLS1), TAZ, ALCAT1, Mfn-2 and pcr conditions have been described previously [31,37,38].

2.5. Western blot analysis

Hela cells were transfected with Mfn-2 plasmid for 24 h as above. The cells were harvested with and homogenized in 10 mM Tris–HCL, 0.25 M sucrose, 2 mM EDTA, 10 mM 2-mecaptoethanol, pH 7.4, by 50 strokes of a tight fitting Dounce A homogenizer. Mitochondrial fractions were obtained as above. Aliquots containing 25 μg of mitochondrial fraction protein were separated by SDS-polyacrilamide gel electrophoresis (SDS-PAGE) on a 7.5% acrylamide gel with molecular weight standards, using a BioRad Mini-Protean® II Dual Slab Cell electrophoresis unit. Protein from the separating gel was blotted onto PVDF membranes for 90 min at 15 V using a BioRad Trans-Blot SD Semi-Dry Transfer Cell. The expression of Mfn-2 was examined by immunoblotting in 10 ml Tris-buffered saline containing 0.1% Tween-20 with anti-Mfn-2 antibody (1:250 dilution) or (40 μl antibody dissolved in 10 ml Tris-buffered saline containing 0.1% Tween-20) at 4C overnight. Subsequently, the membrane was washed and incubated with peroxidase labeled anti-rabbit IgG secondary antibody (1:1000 dilution) for 1 h at room temperature. Expression of β-actin was examined by incubating the PVDF membrane with anti-β-actin antibody under the conditions described [39]. Identification was according to ECL Western blotting analysis system. Protein bands in the membrane were visualized by enhanced chemiluminescence.

2.6. HPLC-MS analysis of CL and MLCL

HPLC-MS analysis of CL and MLCL was performed as previously described [40]. The HPLC system consisted of a Surveyor quaternary gradient pump, a vacuum degasser, a column temperature controller, and an autosampler (Thermo Electron, Waltham, MA, USA). The column temperature was maintained at 25 °C. The lipid extract was injected onto a LiChrospher 2×250-mm silica-60 column, 5 μm particle diameter (Merck, Darmstadt, Germany). The phospholipids were separated from interfering compounds by a linear gradient between solution B (chloroform/methanol, 97:3, v/v) and solution A (methanol/water, 85:15, v/v). Solutions A and B contained 1 and 0.1 ml of 25% (v/v) aqueous ammonia per liter of eluent, respectively. The gradient (0.3 ml/min) was as follows: 0–10 min, 20% A–100% A; 10–12 min, 100% A; 12–12.1 min, 100% A–0% A; and 12.1–17 min, equilibration with 0% A. All gradient steps were linear, and the total analysis time, including the equilibration, was 17 min. A splitter between the HPLC column and the mass spectrometer was used, and 75 μl/min eluent was introduced into the mass spectrometer. A TSQ Quantum AM (Thermo Electron) was used in the negative electrospray ionization mode. Nitrogen was used as the nebulizing gas. The source collision-induced dissociation collision energy was set at 10 V. The spray voltage used was 3600 V, and the capillary temperature was 300 °C. Mass spectra of CL and MLCL molecular species were obtained by continuous scanning from m/z 380 to m/z 1100 with a scan time of 2 s. CL(14:0)$_4$ was used as internal standard.

2.7. Other determinations

Protein was determined by the method of Lowry using bovine serum albumin as the standard [41]. Electron micrographs of Hela cells were prepared as previously described [42]. Student's t-test was used for determination of statistical significance. The level of significance was defined as $p<0.05$.

3. Results

Mfn-2 is a key protein involved in mediating mitochondrial fusion but the dynamics of CL *de novo* biosynthesis post-fusion had never been examined. It was previously shown that cells overexpressing Mfn-2 have increased rates of fusion [33]. Following 12–24 h of expression, the mitochondria were seen to clump into a completely fused reticulum, indicating that mitochondrial fission cannot proceed in these cells. Electron microscopy showed that these organelle clusters were connected through an extensive array of fused outer membranes, consistent with uncontrolled fusion between adjacent organelles. In contrast, cells expressing Mfn-2 for 24 h were also clustered, however electron microscopy and fusion assays did not reveal any fusion between the membranes. Therefore we used longer time points when all of the mitochondria appear irreversibly fused as relevant time points for lipid analysis.

To determine if CL *de novo* biosynthesis was regulated post-mitochondrial fusion, Hela cells were transiently transfected with Mfn-2 or Mfn-2 with an inactive GTPase for 24 h to promote mitochondrial fusion and expression of Mfn-2 mRNA examined. Real-time PCR analysis indicated that transfection with Mfn-2 resulted in elevated mRNA expression of Mfn-2 and Mfn-2 with an inactive GTPase (data not shown). Western blot analysis confirmed that Mfn-2 protein expression was elevated in Hela cells transfected with either Mfn-2 or Mfn-2 with an inactive GTPase compared to vector controls (Fig. 1). Since cells were incubated with the plasmid for 24 h mitochondrial fusion should be complete. We performed electron microscopy studies to examine the structure of the mitochondria post-fusion. In cells expressing Mfn-2, mitochondrial size and morphology was indeed comparable to control Hela cells immediately after fusion and at 12 h post-fusion (Fig. 2A, B, C).

To determine if CL biosynthesis was altered immediately post-fusion, Hela cells were transiently transfected with Mfn-2 for 24 h and with [1,3-^{3}H]glycerol for the last 4 h and radioactivity incorporated into CL examined. Incorporation of [1,3-^{3}H]glycerol into CL was reduced 55% ($p<0.01$) in Mfn-2 expressing cells compared to mock control (Fig. 3A). Incorporation of [1,3-^{3}H]glycerol into all other phospholipids examined was unaltered (Fig. 3B). Total uptake of [1,3-^{3}H]glycerol into control cells was $3.4\pm0.3\times10^5$ dpm/mg protein and was unaltered in Mfn-2 expressing cells ($3.9\pm0.3\times10^5$ dpm/mg). Thus, the reduction in [1,3-^{3}H]glycerol incorporated into CL was not due to changes in total uptake of [1,3-^{3}H]glycerol. Incorporation of [1,3-^{3}H]glycerol into CL and other phospholipids was unaltered in Mfn-2 expressing an inactive GTPase compared to mock control (Fig. 3C, D). These data indicate that an active GTPase component of Mfn-2 was essential for attenuation of CL biosynthesis from [1,3-^{3}H]glycerol in Hela cells.

The reason for the reduction in incorporation of [1,3-^{3}H]glycerol into CL in Mfn-2 expressing cells was examined. Hela cells were transiently transfected with Mfn-2 for 24 h and PGPS and CLS enzyme activities determined. CLS activity was unaltered in Mfn-2 expressing cells (Table 1). In contrast, PGPS activity was reduced 30% ($p<0.01$) in Mfn-2 expressing cells compared to mock transfected controls. The reduction in PGPS activity was due to a corresponding reduction in the mRNA expression of PGPS (Fig. 4A). CLS mRNA expression was unaltered (data not shown). Thus, the decrease in [1,3-^{3}H]glycerol incorporation into CL was due to a reduced activity and expression of

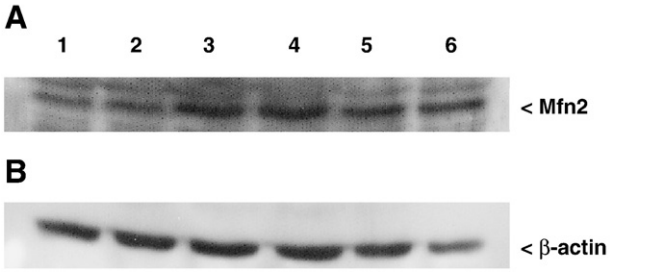

Fig. 1. Western blot analysis of cells expressing Mfn-2. Hela cells were mock transfected (lanes 1 and 2) or transfected with Mfn-2 (lanes 3 and 4) or transfected with Mfn-2 with an inactive GTPase (lanes 5 and 6) for 24 h and Western blot analysis was performed as described in Materials and methods. β-actin was used as a protein loading control. A, Mfn2; B, β-actin. A representative blot is presented.

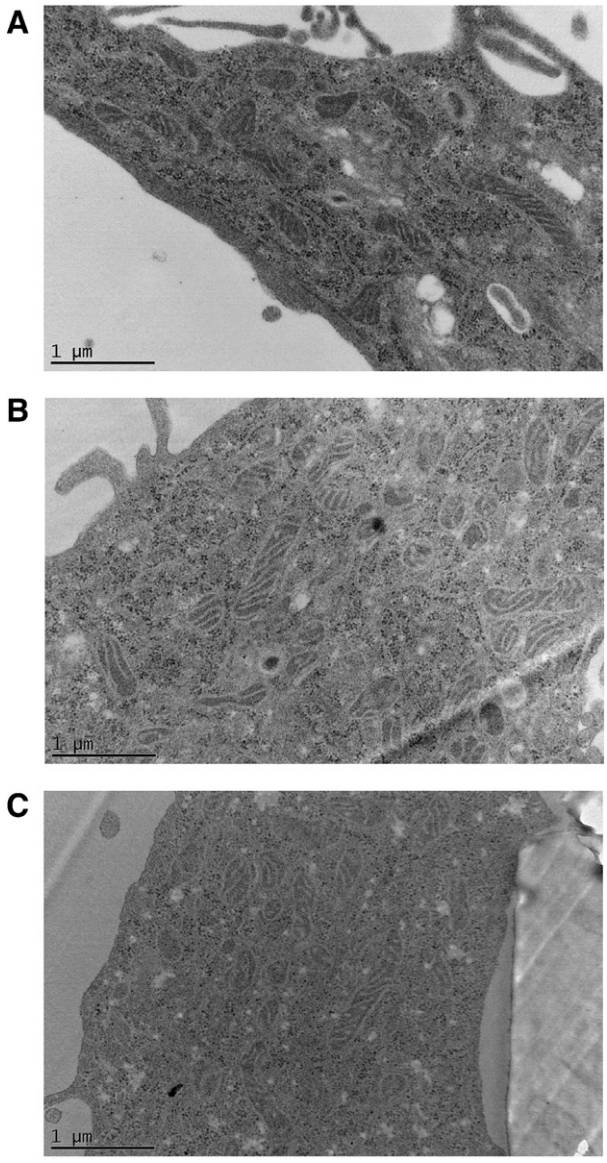

Fig. 2. EM analysis of cells expressing Mfn-2. Hela cells were mock transfected (A) or transfected with Mfn-2 for 24 h (B) or transfected with Mfn-2 for 24 h followed by incubation for a further 12 h (C) and EM analysis was performed as described in Materials and methods. Representative micrographs are presented. Bar is equivalent to 1 micrometer (1 μm).

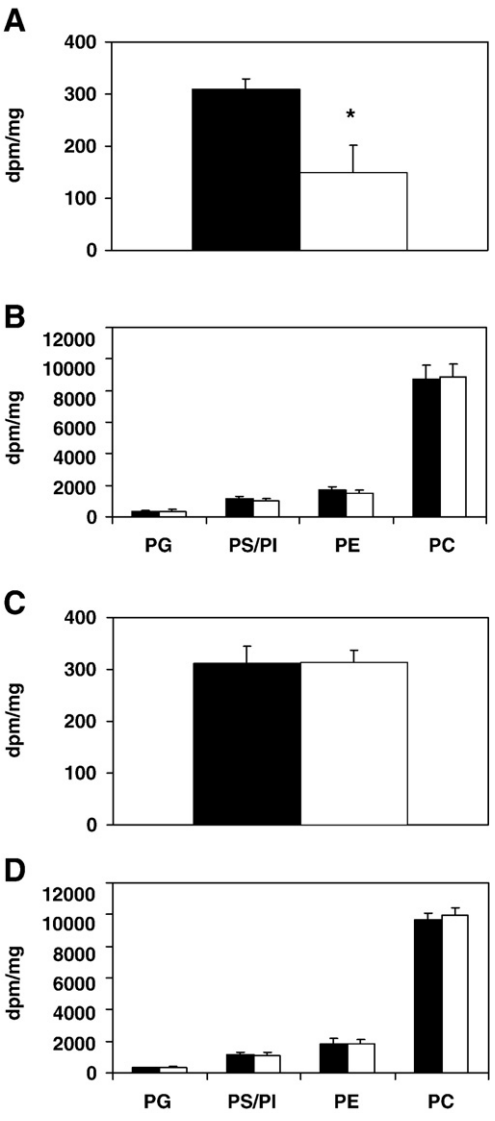

Fig. 3. CL *de novo* biosynthesis from [1,3-^{3}H]glycerol in cells expressing Mfn-2 or Mfn-2 expressing an inactive GTPase incubated for 4 h. Hela cells were mock transfected or transfected for 24 h with Mfn-2 (A, B) or Mfn-2 expressing an inactive GTPase (C, D) and were incubated for the last 4 h with [1,3-^{3}H]glycerol and radioactivity incorporated into CL (A, C), and other phospholipids (B, D) were determined as described in Materials and methods. Closed bars, mock transfected cells; open bars, cells expressing Mfn-2 or Mfn-2 with an inactive GTPase. PG, phosphatidylglycerol; PS/PI, phosphatidylserine/phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine. Data represent the mean ± standard deviation of at least three experiments. *$p<0.05$.

PGPS. Thus, expression of Mfn-2 in Hela cells resulted in decreased CL *de novo* biosynthesis immediately post-fusion.

We next examined if the reduced CL *de novo* biosynthesis immediately post-fusion was associated with reduced levels of CL or its principal metabolite MLCL. Hela cells were transiently transfected with Mfn-2 for 24 h and phospholipid content examined using mass spectrometry analysis. The content of CL and MLCL were unaltered in Mfn-2 expressing cells (Fig. 5A–C).

To determine if CL biosynthesis was altered 12 h after fusion, Hela cells were transiently transfected with Mfn-2 for 24 h then incubated with [1,3-^{3}H]glycerol for 12 h and radioactivity incorporated into CL examined. Surprisingly, [1,3-^{3}H]glycerol incorporated into CL was increased 3-fold ($p<0.03$) (Fig. 6A) and into PG 2-fold ($p<0.02$) (Fig. 6B) in Mfn-2 expressing cells compared to control at 12 h post-transfection. Incorporation of [1,3-^{3}H]glycerol into PC, PE and PS/PI was increased 60% ($p<0.01$), 31% ($p<0.03$) and 45% ($p<0.04$) (Fig. 6C). Total [1,3-^{3}H]glycerol incorporated into control cells was $7.8\pm0.2\times10^5$ dpm/mg protein and was unaltered in Mfn-2 expressing cells ($8.0\pm0.4\times10^5$ dpm/mg). Thus, the elevation in radioactivity incorporated into phospholipids was not due to changes in total uptake of [1,3-^{3}H]glycerol. In Hela cells expressing Mfn-2 with an inactive GTPase there was no alteration in [1,3-^{3}H]glycerol incorporated into all phospholipids at 12 h post-transfection (data not

Table 1
Enzyme activities in cells expressing Mfn-2. Hela cells were transfected with Mfn-2 and mitochondrial fractions prepared immediately or 12 h post-transfection and PGPS, CLS and PLA_2 activities were determined as described in Materials and methods. Data represent the mean ± standard deviation of three experiments. N.D., not determined. All values are expressed as nmol/min/mg protein.

	Control	Immediately post-transfection pmol/min/mg protein	12 h post-transfection
PGPS	197 ± 8	141 ± 6*	273 ± 21*
CLS	3.1 ± 0.3	3.3 ± 0.3	2.9 ± 3
PLA_2	5.3 ± 0.8	N.D.	5.5 ± 0.1

* $p<0.05$.

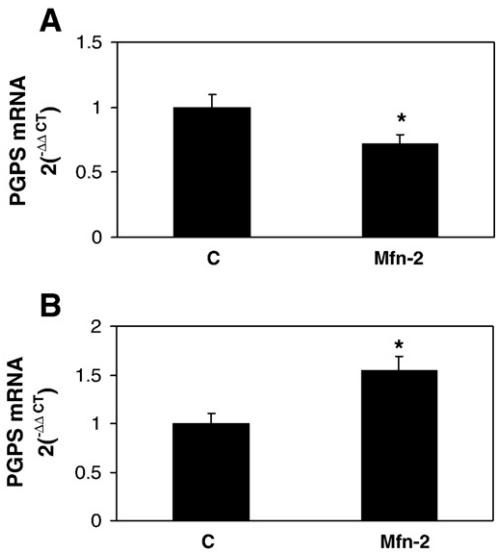

Fig. 4. Expression of PGPS mRNA in cells expressing MFN-2. Hela cells were transfected with Mfn-2 and total RNA isolated and expression of PGPS mRNA was determined immediately after or 12 h post-fusion as described in Materials and methods. A. Immediately after fusion. B. 12 h post-fusion. C. Mock transfected Hela cells; Mfn-2, Hela cells transfected with Mfn-2. Results represent ratio of PGPS to 18s ribosomal RNA. Data represent the mean ± standard deviation of three experiments. $^{*}p<0.05$.

shown), confirming the above observation that an active GTPase component of Mfn-2 is essential for modulation of CL biosynthesis from [1,3-^{3}H]glycerol in Hela cells.

The reason for the increased incorporation of [1,3-^{3}H]glycerol into CL was examined. Hela cells were transiently transfected with Mfn-2 for 24 h and PGPS and CLS enzyme activities determined 12 h post-fusion. CLS activity was unaltered in Mfn-2 expressing cells (Table 1). In contrast, PGPS activity was increased 39% ($p<0.01$) in Mfn-2 expressing cells compared to control. The increase in PGPS activity was due to a corresponding increase in the mRNA expression of PGPS (Fig. 4B). In contrast, CLS mRNA expression was unaltered (data not shown). Thus, the increase in [1,3-^{3}H]glycerol incorporation into CL 12 h post-mitochondrial fusion was due to an increased activity and mRNA expression of PGPS. Together these data indicate that CL *de novo* biosynthesis was reduced immediately after mitochondrial fusion process but is upregulated 12 h post-fusion.

Unsaturated fatty acids enter into CL mainly by remodeling processes [reviewed in 3,7]. The major unsaturated fatty acid found in Hela cells phospholipids is oleate (18:1) comprising almost 50% of the total [44]. We examined if the observed alterations in CL *de novo* biosynthesis were associated with alteration in [1-^{14}C]oleate incorporated into CL. Hela cells were transfected with Mfn-2 for 24 h and then incubated with [1-^{14}C]oleate and radioactivity incorporated into CL determined immediately after or 12 h post-transfection. Incorporation of [1-^{14}C]oleate into all phospholipids were unaltered in Mfn-2 expressing cells compared to control immediately post-fusion (data not shown). In addition, total [1-^{14}C]oleate incorporated into cells was 2.5×10^{5} dpm/mg protein and was unaltered in Mfn-2 expressing cells (2.5×10^{5} dpm/mg). In contrast, incorporation of [1-^{14}C]oleate into CL was increased 66% ($p<0.007$) in Mfn-2 expressing cells compared to control at 12 h post-fusion (Fig. 7A). [1-^{14}C]Oleate incorporated into PG (Fig. 7B) and other phospholipids (Fig. 7C) was unaltered. Total [1-^{14}C] oleate incorporated into cells was 8.6×10^{5} dpm/mg protein and was unaltered in Mfn-2 expressing cells (8.2×10^{5} dpm/mg). Thus, the elevation in radioactivity incorporated into CL was not due to changes in total uptake of [1-^{14}C]oleate in the transfected cells. In Hela cells expressing Mfn-2 with an inactive GTPase there was no alteration in [1-^{14}C]oleate incorporated into phospholipids immediately after or 12 h post-fusion (data not shown). Thus, the increase in [1-^{14}C]oleate incorporation into CL by accompanied the elevation in CL *de novo* biosynthesis 12 h post-mitochondrial fusion.

The reason for the increase in [1-^{14}C]oleate incorporation into CL in MFN-2 expressing cells 12 h post-fusion was examined. Hela cells were transfected with Mfn-2 for 24 h and then mitochondrial PLA_2 and expression/activity of the CL remodeling enzymes TAZ/MLCL AT and expression of the endoplasmic reticulum CL resynthesis enzyme ALCAT1 were examined 12 h post-transfection. Expression of TAZ mRNA was increased 30% ($p<0.05$) 12 h post-fusion (Fig. 8A). In contrast, expression of ALCAT1 mRNA was unaltered (Fig. 8B). MLCL AT activity was unaltered (data not shown). In addition, PLA_2 activity was unaltered in cells transfected with Mfn-2 (Table 1). Thus the increase in CL resynthesis from [1-^{14}C]oleate in Mfn-2 expressing cells 12 h post-fusion was likely due to an increase in expression of the mitochondrial CL resynthesis enzyme TAZ.

We previously demonstrated that N-acetylsphingosine (C_2-Cer) induced PGPS activity and its mRNA expression in H9c2 cardiac myoblast cells and in CHO cells and this lead to elevation in *de novo* CL biosynthesis but that C_2-Cer did not alter PGPS activity or CL biosynthesis in Stard13 mutant CHO E91 cells resistant to C_2-Cer induced apoptosis [17,18]. We tested the hypothesis that forced PGPS expression and elevation of CL synthesis itself would directly alter Mfn-2 mRNA expression. Hela cells were incubated in the absence or presence of 30 μM C_2-Cer for 24 h and the mRNA expression of Mfn-2 determined. PGPS enzyme activity was 197 ± 8 pmol/min/mg protein in Hela cells and was elevated 35% ($^{*}p<0.05$) to 265 ± 12 pmol/min/mg protein in Hela cells treated with C_2-Cer. Mfn-2 mRNA expression was unaltered in cells incubated in the absence or presence of C_2-Cer (Fig. 9A). CHO K1 cells or C_2-Cer resistant CHO E91 cells were then incubated in the absence or presence of 30 μM C_2-Cer for 24 h and the mRNA expression of Mfn-2 determined. PGPS enzyme activity was elevated 35–39% in CHO K1 cells but not in CHO E91 cells as previously described [18]. Interestingly, Mfn-2 mRNA expression was reduced 50% in both CHO K1 and in CHO E91 cells resistant to C_2-Cer induced apoptosis when these cells were incubated with C_2-Cer when compared to controls (Fig. 9B). These data indicate that forced expression of PGPS mRNA and activity and hence elevated CL biosynthesis does not alter nor is linked to the expression of Mfn-2. However, C_2-Cer incubation of CHO cells inhibited Mfn-2 mRNA expression independent of PGPS expression. The data indicate that elevation in CL synthesis does not affect Mfn-2 expression.

Phospholipid scramblase-3 (PLS3) is a unique member of the phospholipid transfer protein family and transports CL between inner and outer mitochondrial membranes [30]. We previously showed that CL *de novo* biosynthesis was elevated in Hela cells expressing PLS3 (PLS3) or Hela cells expressing a disrupted PLS3 (F(258)V) [31]. To further examine if elevation in CL synthesis was not directly linked to Mfn-2 expression, we examined if alteration of CL localization within the mitochondria would affect expression of Mfn-2. Mfn-2 mRNA expression was examined in vector, PLS3 or F(258)V Hela cells. Mfn-2 mRNA expression was unaltered between control, PLS3 or F(258)V cells (data not shown). These data further confirm that elevation in CL synthesis is not directly linked to mitochondrial fusion and proper CL localization within mitochondria is not essential for Mfn-2 mRNA expression.

4. Discussion

In this study we examined the dynamics of CL *de novo* biosynthesis immediately after and 12 h post-mitochondrial fusion. The major findings of this paper are 1. CL *de novo* biosynthesis is reduced immediately post-Mfn-2-mediated mitochondrial fusion and this is due to a reduction in the activity and mRNA expression of PGPS, 2. 12 h post-Mfn-2 induced mitochondrial fusion, CL *de novo* biosynthesis is upregulated and this is mediated by an increase in activity and mRNA expression of PGPS, 3. the upregulation in CL *de novo* biosynthesis is

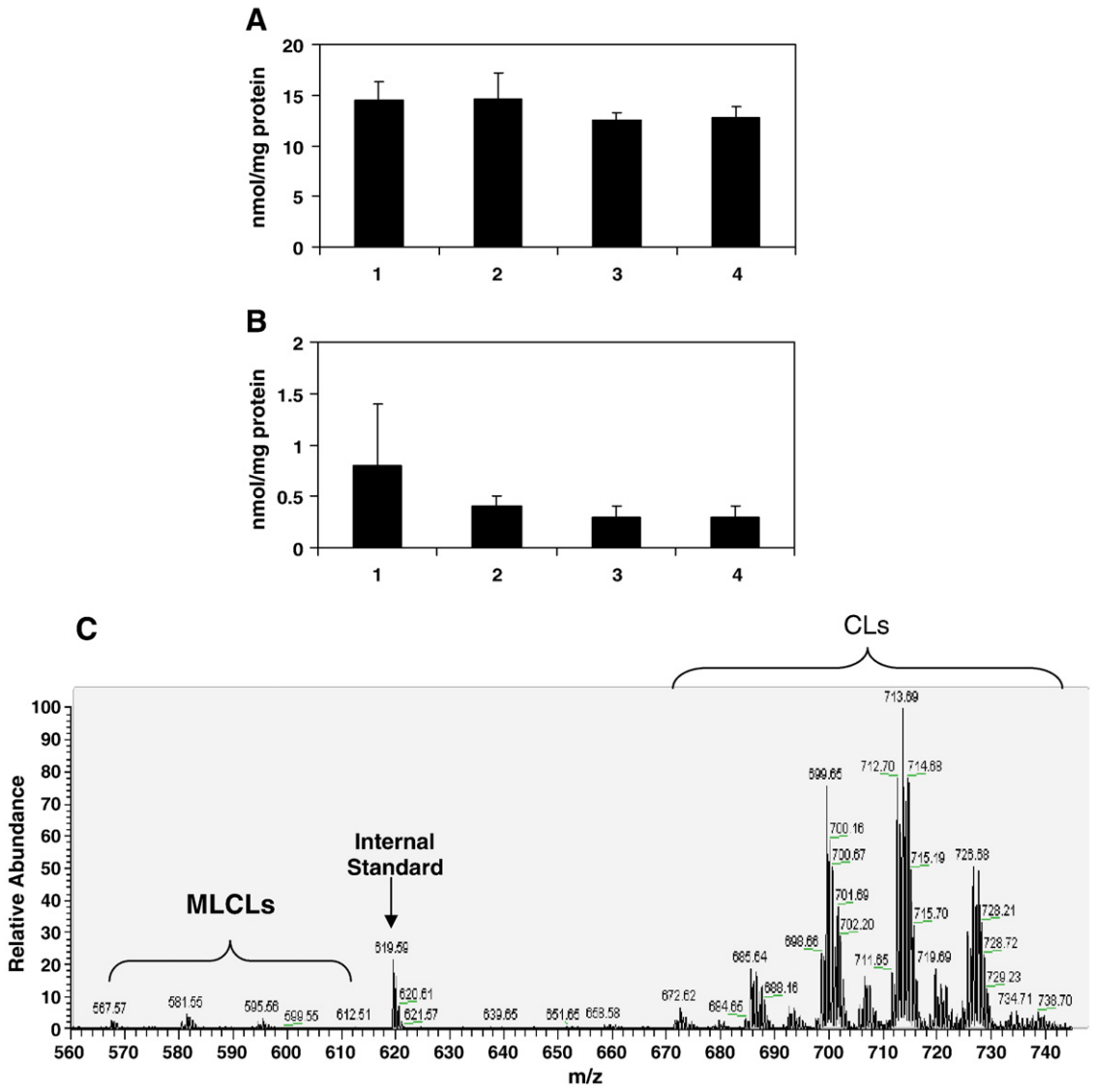

Fig. 5. CL and MLCL content in Hela cells expressing Mfn-2. Hela cells were mock transfected (1, 3) or transfected with Mfn-2 (2, 4) and CL (A) and MLCL (B) were determined by mass spectrometry analysis as described in Materials and methods. C. A representative spectra of CL and MLCL analysis. Data represent the mean ± standard deviation of three experiments.

accompanied by an increase in oleate incorporation into CL at 12 h post-mitochondrial fusion, 4. expression or knock down of PGPS mRNA and enzyme activity does not itself alter expression of Mfn-2, 5. proper localization of CL is not required for expression of Mfn-2, and 6. mitochondrial fusion regulates CL synthesis. The results of the current study provide evidence that CL *de novo* biosynthesis is "slowed down" immediately post-mitochondrial fusion but is then "upregulated" 12 h post-fusion.

Hela cells expressing a functional Mfn-2 exhibited reduced CL *de novo* biosynthesis from [1,3-^{3}H]glycerol and this was due to a decrease in PGPS activity and mRNA expression during fusion. Expression of Mfn-2 did not alter the biosynthesis of other phospholipids from [1,3-^{3}H]glycerol indicating that the effect of Mfn-2 expression was specific to mitochondrial CL. In Hela cells expressing Mfn-2 with an inactive GTPase CL *de novo* biosynthesis was unaltered indicating that the GTPase component of Mfn-2 is critical for regulation of CL biosynthesis by Mfn-2. The results suggest that mitochondrial CL *de novo* biosynthesis is apparently "slowed down" immediately post-fusion. Interestingly, radioactivity incorporated into PG was unaltered even though PGPS activity was decreased in these cells. This is likely due to the rapid movement of [1,3-^{3}H]glycerol from PG to CL in mammalian cells [10]. In contrast, 12 h post-fusion PG and CL *de novo* biosynthesis from [1,3-^{3}H]glycerol were elevated and this was due to a increase in PGPS activity and its mRNA expression indicating that once fusion is completed CL *de novo* biosynthesis is upregulated. The increase in [1,3-^{3}H]glycerol incorporation into the other phospholipids at 12 h post-fusion is likely required to support the increased mitochondrial biogenesis although the level of elevated synthesis was lower than that of mitochondrial CL.

The PGPS and CLS enzymes have little fatty acyl substrate specificity for precursors of CL [3]. Thus, *de novo* synthesized CL must be rapidly remodeled to obtain the specific fatty acyl molecular composition of CL seen in the mitochondria. Hela cell CL is highly enriched with oleate [43]. Cells expressing functional Mfn-2 exhibited increased [1-^{14}C]oleate incorporation into CL at 12 h but this was unaltered during fusion. Although a portion of [1-^{14}C]oleate incorporation into CL may have come from increased primary biosynthesis, the increased [1-^{14}C]oleate incorporation into CL 12 h post-fusion in Hela cells expressing Mfn-2 was accompanied by an increased expression of the mitochondrial CL resynthesis enzyme TAZ. In contrast, [1-^{14}C]oleate incorporation into CL was unaltered during and 12 h post-fusion in Hela cells expressing

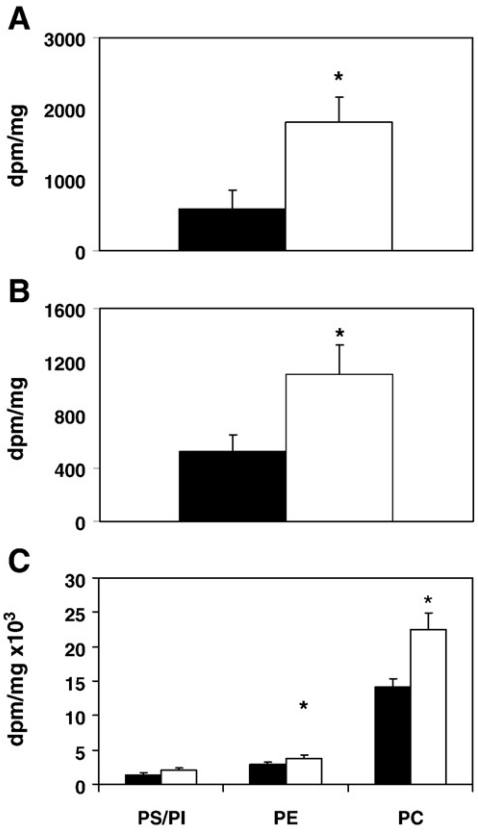

Fig. 6. CL *de novo* biosynthesis from [1,3-^{3}H]glycerol in cells expressing Mfn-2 incubated for 12 h. Hela cells were mock transfected or transfected with Mfn-2 and then incubated for 12 h with [1,3-^{3}H]glycerol and radioactivity incorporated into CL (A), PG (B) and other phospholipids (C) was determined as described in Materials and methods. Closed bars, mock transfected cells; open bars, cells expressing Mfn-2. Data represent the mean ± standard deviation of three experiments. $^*p<0.05$.

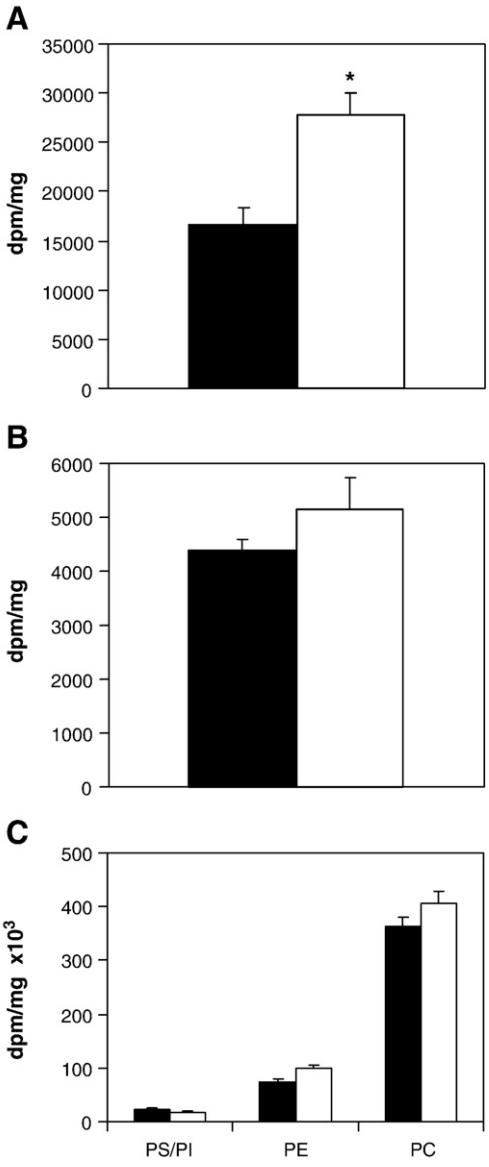

Fig. 7. CL synthesis from [1-^{14}C]oleate in cells expressing MFN-2. Hela cells were transfected with Mfn-2 for 24 h and then incubated for 12 h with [1-^{14}C]oleate and radioactivity incorporated into CL (A), PG (B) and PS/PI, PE and PC (C) was determined as described in Materials and methods. A. Data represent the mean ± standard deviation of three experiments. $^*p<0.05$.

Mfn-2 with an inactive GTPase. Thus, a functional GTPase component was required for [1-^{14}C]oleate incorporation into CL 12 h post-fusion. Since CL *de novo* biosynthesis was slowed down during fusion it would not be expected that [1-^{14}C]oleate entrance into CL via remodeling would be altered in Mfn-2 expressing cells. In contrast, an increase in [1-^{14}C]oleate incorporation into CL might be expected to accompany the elevation in *de novo* CL biosynthesis seen 12 h post-fusion in Mfn-2 expressing cells.

Does altered PGPS expression and CL *de novo* biosynthesis itself regulate Mfn-2 expression? We tested this directly by examining Mfn-2 mRNA expression in C_2-Cer-treated Hela cells which exhibited elevated PGPS activity. Since elevation of PGPS activity mediated by C_2-Cer did not affect Mfn-2 mRNA expression in Hela cells, it can be concluded that elevated PGPS activity and hence CL biosynthesis itself does not control Mfn-2 expression in Hela cells. In Hela cells, C_2-Cer was previously shown to stimulate mitochondrial network fragmentation linked to endoplasmic reticulum calcium release and cell death [44]. A more recent study in isolated cardiac myocytes showed that downregulation of Mfn-2 levels to 75% of control, mediated by RNA knock down, resulted in increased mitochondrial fission in isolated cardiac myocytes [45]. However, this did not enhance C_2-Cer induced mitochondrial fragmentation. In the current study, C_2-Cer addition to both CHO K1 and CHO E91 cells resistant to C_2-Cer-mediated apoptosis exhibited decreased Mfn-2 mRNA expression. Since E91 cells show resistance to C_2-Cer induced apoptosis [18], it is likely that the effects of C_2-Cer on mitochondrial-mediated apoptosis are not directly linked to mitochondrial fusion. Taken together, the results of these studies and our current study support the notion that the C_2-Cer-mediated fragmentation of mitochondria may not involve Mfn-2 directly.

Does altered CL localization affect Mfn-2 expression? We tested this directly by examining mitochondrial fusion in Hela cells expressing PLS3 (PLS3) or an inactive PLS3 (F258V). PLS3 expression was shown to increase CL localization on the outer mitochondrial membrane whereas expression of inactive PLS3 resulted in CL accumulation on the inner mitochondrial membrane [30]. Expression of Mfn-2 in PLS3 cells and in F258V cells expressing an inactive PLS3 was unaltered compared to control Hela cells indicating that expression of Mfn-2 is independent of mitochondrial localization of CL. We previously showed that CL biosynthesis was elevated in PLS3 Hela cells expressing PLS3 or Hela F (258)V cells expressing a disrupted PLS3 [31]. That study and our current study suggest that the mitochondrial fusion process may be regulated independently of CL *de novo* biosynthesis.

Why is CL synthesis altered post-mitochondrial fusion? It is possible that during mitochondrial fusion CL biosynthesis is “slowed down” in order to allow for uninterrupted fusion of individual mitochondria to occur. This may be related to the energy price of disruption and bending of membrane lipid bilayers during bilayer fusion [46,47]. Alternatively, it

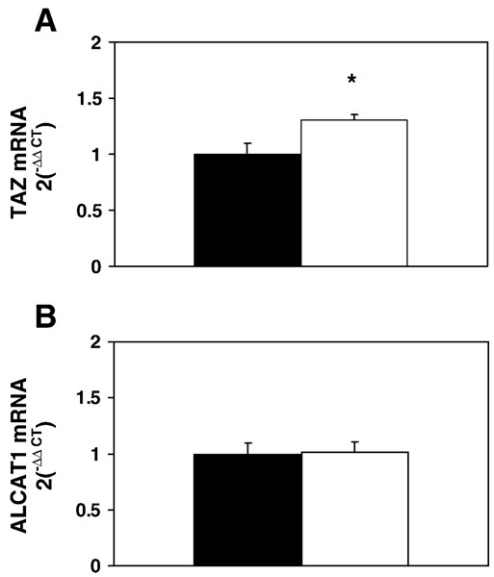

Fig. 8. Expression of TAZ and ALCAT1 mRNA in cells expressing MFN-2. Hela cells were transfected with Mfn-2 and total RNA isolated 12 h post-fusion and mRNA expression of TAZ (A) and ALCAT1 (B) was determined as described in Materials and methods. Data represent the mean ± standard deviation of three experiments. $^{*}p<0.05$.

is possible that Mfn-2 could promote short term cessation of mitochondrial biogenesis. We recently showed that loss of CL on the outer mitochondrial membrane by disruption of PLS3 resulted in elevated CL biosynthesis in Hela cells likely in an attempt to restore CL on the outer surface of the outer mitochondrial membrane [31]. It is possible that CL *de novo* biosynthesis is upregulated 12 h post-fusion in order to replace CL lost from the outer mitochondrial membrane during Mfn-mediated phospholipase D hydrolysis of CL on the outer membrane [27]. However, MS analysis of cells immediately post-fusion did not reveal any change in the level CL nor its metabolite MLCL indicating that it is not a change in the CL or MLCL pool size per se that results in the stimulation of CL biosynthesis. In addition, the PA levels were low and

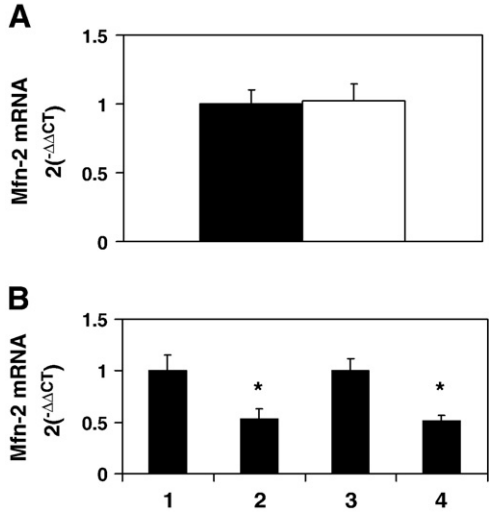

Fig. 9. Mfn-2 expression in Hela cells and in CHO K1 and CHO E91 cells treated with C_2-Cer. A. Hela cells were incubated in the absence (closed bar) or presence (open bar) of 30 μM C_2-Cer for 24 h and mRNA expression of Mfn-2 was determined as described in Materials and methods. B. CHO K1 (1, 2) or E91 (3, 4) cells were incubated in the absence (1, 3) or presence (2, 4) of 30 μM C_2-Cer for 24 h and mRNA expression of Mfn-2 was determined as described in Materials and methods. Results represent ratio of Mfn-2 to 18s ribosomal RNA. Data represent the mean ± standard deviation of three experiments. $^{*}p<0.05$.

were unaltered in Mfn-2 treated cells (data not shown). Moreover, EM analysis indicated that Hela cell mitochondria appeared morphologically normal immediately after and 12 h post-fusion. Finally, new synthesis of all phospholipids from [1,3-^{3}H]glycerol or [1-^{14}C]oleate was elevated 12 h post-fusion. We suggest that the upregulation of CL biosynthesis observed 12 h post-fusion in Hela cells may be required to restore the normal rates of CL biosynthesis. In addition, it appears that mitochondrial fusion may regulate cellular phospholipid biosynthesis from [1,3-^{3}H]glycerol and [1-^{14}C]oleate precursors.

The data presented here have shown that active Mfn-2 signals for increased mitochondrial biogenesis, leading to the upregulation of lipid synthesis pathways. This is consistent with the emerging evidence that Mfn-2, unlike Mfn-1, appears to have signaling capabilities that link the health of the mitochondria with their biogenesis. We believe that this is an important new finding that provides compelling new evidence to implicate the fusion machinery with the control of mitochondrial numbers. Future work will uncover the signaling intermediates that link Mfn-2 to the upregulation of lipid biogenesis.

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