On the mechanism of the increase in cardiolipin biosynthesis and resynthesis in hepatocytes during rat liver regeneration

Jennifer WEBSTER*, Jenny Y. JIANG*, Biao LU*, Fred Y. XU*, William A. TAYLOR*, Mathew MYMIN*, Manna ZHANG†, Gerald Y. MINUK*† and Grant M. HATCH*†‡¹

*Department of Pharmacology and Therapeutics, Center for Research and Treatment of Atherosclerosis, University of Manitoba, Winnipeg, Canada R3E 0T6, †Department of Internal Medicine, Center for Research and Treatment of Atherosclerosis, University of Manitoba, Winnipeg, Canada R3E 0T6, and ‡Department of Biochemistry and Medical Genetics, Center for Research and Treatment of Atherosclerosis, University of Manitoba, Winnipeg, Canada R3E 0T6

CL (cardiolipin) is a major mitochondrial membrane phospholipid important for the regulation of mitochondrial function. We examined CL *de novo* biosynthesis and its resynthesis in isolated rat liver hepatocytes prepared 48 h subsequent to two-thirds PHx (partial hepatectomy). The pool size of CL and its *de novo* biosynthesis from [1,3-³H]glycerol were increased 3.3-fold ($P < 0.05$) and 3.1-fold ($P < 0.05$) respectively in hepatocytes prepared from PHx rats compared with sham-operated controls. The reason for the increased CL biosynthesis was a 65 % increase ($P < 0.05$) in enzymic activity in PGP-S (phosphatidylglycerolphosphate synthase), a key enzyme in *de novo* CL biosynthesis. The increase in PGP-S activity was due to a 3-fold increase ($P < 0.05$) of hepatic PGP-S mRNA expression. The increase in *de novo* CL biosynthesis and pool size corresponded to a 2.3-fold increase ($P < 0.05$) in the amount of [1-¹⁴C]linoleic acid incorporated into

CL of hepatocytes prepared from PHx rats compared with sham-operated controls, indicating an increase in CL resynthesis. The activity of MLCL-AT (monolysocardiolipin acyltransferase), a rate-limiting enzyme of CL resynthesis, was increased by 43 % ($P < 0.05$) in hepatocytes prepared from PHx rats compared with sham-operated controls; this result would explain the increase in [1-¹⁴C]linoleic acid incorporation into CL. The increase in MLCL-AT activity was due to an increase in hepatic MLCL-AT protein expression. The results show that CL *de novo* biosynthesis and its resynthesis are increased during liver regeneration.

Key words: cardiolipin, liver, monolysocardiolipin acyltransferase, partial hepatectomy, phosphatidylglycerolphosphate synthase, phospholipid resynthesis.

INTRODUCTION

CL (cardiolipin), the first polyglycerophospholipid discovered, was isolated from ox heart by Pangborn [1]. In rat liver, CL comprises approx. 7 % of the total phospholipid mass (see [2] for a review). CL is required for the reconstituted activity of a number of key mitochondrial enzymes involved in cellular energy metabolism and may be the 'glue' that holds the respiratory chain together [3,4]. Given the importance of loss of CL [5,6], and accumulation of its immediate metabolic product MLCL (monolysocardiolipin) [7], in mitochondria-mediated apoptosis, it is possible that rapid CL synthesis and/or its resynthesis from MLCL is required in response to proapoptotic stimuli-mediated CL degradation to restore cellular homeostasis and prevent the entry of cells into apoptosis. Hence, increased CL biosynthesis and resynthesis may be a protective mechanism against apoptosis [8,9]. In any event, maintenance of the appropriate content and molecular composition of CL is essential for normal cellular function.

De novo synthesis of CL in eukaryotic cells occurs through the CDP-DG (CDP 1,2-diacyl-*sn*-glycerol) pathway [10]. PA (phosphatidic acid) is converted into CDP-DG catalysed by CDP-DG synthetase [11]. In the second step of the pathway, CDP-DG condenses with *sn*-glycerol-3-phosphate to form PGP (phosphatidylglycerolphosphate) catalysed by PGP-S (PGP synthase). In several model systems, the level of PGP-S activity may regulate the production of CL [8,12,13]. The PGP formed from PGP-S does

not accumulate in cells and is rapidly converted into PG (phosphatidylglycerol) by PGP phosphatase [11]. In the final step of the pathway, CL is formed from the condensation of PG and CDP-DG catalysed by CL synthase [14].

The high prevalence of the unsaturated fatty acyl molecular species of CL in human and mammalian tissues is well documented [2,15]. In rat liver, evidence was obtained for the presence of a CL resynthesis pathway [16]. From that study it was concluded that MLCL acylation with linoleoyl-CoA (linoleoyl coenzyme A) would provide a potential mechanism for the remodelling of molecular species of newly formed CL. The *in vitro* reacylation of MLCL to CL with linoleoyl-CoA in rat liver mitochondria has been characterized and the enzyme responsible for the acylation of MLCL recently purified from pig liver mitochondria has been characterized [17,18]. Studies in rat heart and liver mitochondria indicate that MLCL-AT (MLCL acyltransferase) may be a rate-limiting enzyme for the molecular remodelling of CL [18,19]. Recently, the presence of a transacylase activity specific for CL remodelling was identified and characterized in rat liver [20].

A previous study has shown that the CL content in whole liver of rats subjected to two-thirds PHx (partial hepatectomy) was restored to preoperative levels by 14 days post-PHx [21]. However, the mechanism for restoration of the CL pool has never been investigated. Nor was it known whether an increased resynthesis of CL accompanied this restoration of CL levels. In the present

Abbreviations used: CDP-DG, CDP 1,2-diacyl-*sn*-glycerol; CL, cardiolipin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MLCL, monolysocardiolipin; MLCL-AT, MLCL acyltransferase; PA, phosphatidic acid; PG, phosphatidylglycerol; PGP, phosphatidylglycerolphosphate; PGP-S, PGP synthase; PHx, partial hepatectomy.

¹ To whom correspondence should be addressed, at Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg (email hatchgm@ms.umanitoba.ca).

study, we utilized the two-thirds PHx model to examine *de novo* CL biosynthesis and resynthesis during hepatocyte regeneration. We show that CL *de novo* biosynthesis is increased in hepatocytes during liver regeneration and that an increase in CL resynthesis coincides with this increase in *de novo* CL biosynthesis.

MATERIALS AND METHODS

Materials

[1-¹⁴C]Linoleoyl-CoA was obtained from American Radio-labeled Chemicals (St. Louis, MO, U.S.A.). [1-¹⁴C]Linoleic acid, [1,3-³H]glycerol, [¹⁴C-U]glycerol-3-phosphate and 1-palmitoyl, 2-[1-¹⁴C]linoleoyl-phosphatidylcholine were obtained from DuPont (Mississauga, ON, Canada). Linoleoyl-CoA was obtained from Serdary Research Laboratories (Englewood Cliffs, NJ, U.S.A.). MLCL [a mixture of 1'(1-acyl-*sn*-glycerol-3-phosphoryl)-3'(1'',2''-diacyl-*sn*-glycerol-3-phosphoryl)glycerol and 1'(1,2-diacyl-*sn*-glycerol-3-phosphoryl)-3'(1''-acyl-*sn*-glycerol-3-phosphoryl)glycerol], produced by phospholipase A₂ hydrolysis of bovine heart CL, was obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Purity of the MLCL substrate was checked by two-dimensional TLC as described in [22]. The fatty acyl molecular species composition of the MLCL substrate was examined as described in [23] and most of it was linoleic acid (90.3 %) and oleic acid (8.6 %). Ecolite scintillation cocktail was obtained from ICN Biochemicals (Costa Mesa, CA, U.S.A.) and thin-layer plates (silica gel 60; 0.25 mm thickness) were obtained from Fisher Scientific (Winnipeg, MB, Canada). A Kodak X-OMAT^{TM} SA film was used for Western-blot analysis. Cell culture media and fetal bovine serum were obtained from Gibco BRL (Rockville, MD, U.S.A.). All other biochemicals were of analytical grade and obtained from either Fisher Scientific (Edmonton, AB, Canada) or Sigma (St. Louis, MO, U.S.A.). Anti-rat β -actin antibody was purchased from Affinity Bioreagents (Golden, CO, U.S.A.). Pig liver MLCL-AT antibody raised in rabbits was used for the determination of MLCL-AT protein expression [18].

Animals

Sprague-Dawley rats (250–300 g) were obtained from Central Animal Care Services, University of Manitoba. Treatment of rats conformed to the guidelines of the Canadian Council on Animal Care. The rats were maintained on rat chow and water *ad libitum* until the day before surgery, when food but not water was withdrawn. All animals were kept in identical housing units on a cycle of 12 h of light and 12 h of darkness. Two-thirds partial hepatectomies were performed between 09:00 h and noon each day as described by Higgins and Anderson [24]. Sham operations, in which appropriate amounts of the liver were exteriorized for the same length of time as rats undergoing PHx, were also performed. Rats were killed by exsanguinations under anesthesia at 48 h after hepatectomy.

Assay of enzymic activities

Since the protein expression of proliferating cell nuclear antigen, a specific marker of liver regeneration, is maximal at 48 h post-PHx in liver (4-fold higher compared with sham-operated controls) [25], activities of the CL biosynthetic enzymes were determined as described in [26] in hepatocytes isolated from 48 h post-PHx rats. Hepatocytes were prepared 48 h post-PHx or from sham-operated animals using the collagenase perfusion method as described in [27], plated on to 6 cm diameter culture dishes (3×10^6 cells/dish) and then incubated overnight in arginine-free

Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal calf serum to select for hepatocytes and allow for full hepatocyte adherence to the dishes. Hepatocytes were then incubated for 24 h in Dulbecco's modified Eagle's medium containing 10 % fetal calf serum. Hepatocytes were then washed with PBS homogenized in buffer A (10 mM Tris/HCl, pH 7.4, 0.25 M sucrose and 0.145 M NaCl) using 30 strokes in a Dounce A homogenizer. The homogenate was centrifuged at 500 g for 5 min and the resulting supernatant centrifuged at 10000 g for 15 min. The resulting pellet was resuspended in buffer A and used as the source of protein for enzymic activities and Western-blot analysis. Lyso-PGP acyltransferase activity was determined as described in [28]. MLCL-AT activity was determined as described in [18]. CL transacylase activity was determined as described in [20].

Radiolabelling of hepatocytes

Hepatocytes were prepared from rat livers 48 h post-PHx or from sham-operated animals and plated on to culture dishes and incubated as described above. In some experiments, cells were incubated for up to 24 h with 0.1 mM [1,3-³H]glycerol (10 μ Ci/dish) or 0.1 mM [1-¹⁴C]linoleic acid (1 μ Ci/dish, bound to BSA in a 1:1 molar ratio). The medium was removed and the cells were washed twice with ice-cold saline and then harvested from the dish with 2 ml of methanol/water (1:1, v/v) for lipid extraction. Phospholipids were then isolated and radioactivity in these determined as described previously [26].

Western-blot analysis

Livers were prepared from two rat livers 48 h post-PHx or two sham-operated animals and mitochondrial fractions prepared as described above. Mitochondrial fractions (50 μ g) were subjected to SDS/PAGE [18]. Protein from the separating gel was blotted on to PVDF membranes and incubated with either preimmune serum or the anti-pig liver MLCL-AT polyclonal antibody (dilution, 1:500 dilution) or anti-rat β -actin antibody as described previously [9,18]. The anti-pig liver antibody was shown to cross-react with the rat liver protein [18]. Identification was according to the ECL® Western blotting analysis system (Amersham Biosciences) using goat anti-rabbit IgG labelled with horseradish peroxidase as the secondary antibody (dilution, 1:2000).

RT (reverse transcriptase)-PCR analysis

The cDNA for rat liver PGP-S was amplified with a pair of specific primers synthesized by Invitrogen^{TM} Life Technologies. The rat PGP-S primers used for the RT-PCR experiments were (GenBank® accession no. XM.221142): forward primer, 5'-GCTCATGAAGGGGCAGATAA-3', and reverse primer, 5'-CTGCAACTGCAAGGACACAT-3'. The PCR product length was 508 bp. The rat GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers used for the RT-PCR experiments were: forward primer, 5'-GCTGCCTTCTCTTGTGACAAA-3', and reverse primer, 5'-CACGCCACAGCTTCCAGA-3'. The PCR product length was 616 bp. Livers were prepared from rat livers 48 h post-PHx or from sham-operated animals and total RNA was isolated using the TRIzol® reagent according to the manufacturer's instructions. The RNA pellet was suspended in autoclaved, double-distilled water and quantified by absorbance at 260 nm using the 260:280 nm ratio as an index of purity. The integrity of the RNA was confirmed by denaturing agarose-gel electrophoresis of the isolated RNA sample. The first-strand cDNA from 1 μ g of total RNA was synthesized with 150 units of Moloney-murine-leukaemia virus RT, 25 pmol of random hexamer primer, 20 units of RNase inhibitor, 1 mM dithiothreitol and 10 pmol each of

the four deoxynucleotides, in a total volume of 15 μ l. The reaction mixture was incubated at 37°C for 1 h and the reaction was terminated by boiling the sample at 95°C for 5 min. An aliquot of the resultant cDNA preparation was used directly for each amplification reaction. PCR was performed in 20 μ l of reaction mixtures containing 8 pmol of primer, 8 pmol of each dNTP and 0.4 unit of *Taq* DNA polymerase. The mixture was overlaid with 30 μ l of mineral oil to prevent evaporation and incubated in a PerkinElmer DNA thermal cycler under the following conditions. For the PCR of PGP-S: 26 cycles, denaturation, 30 s at 94°C; annealing, 30 s at 60°C; extension, 2 min at 72°C. For the PCR of GAPDH: 25 cycles, denaturation, 1 min at 94°C; annealing, 1 min at 60°C; extension, 2 min at 72°C. The amplified RT-PCR products were analysed by 1.5% agarose-gel electrophoresis in 1 $\times$ TAE buffer (40 mM Tris acetate and 2 mM sodium EDTA) and visualized by staining with 0.5 μ g/ μ l ethidium bromide. An increase in the level of a specific mRNA is caused by either an increase in its rate of synthesis, a decrease in its rate of degradation or a combination of these two processes. Hence, mRNA stability assays were conducted using actinomycin D as an inhibitor of RNA synthesis. The mRNA levels for GAPDH and PGP-S were determined by RT-PCR at 4 h intervals after the actinomycin treatment. No apparent changes in mRNA degradation were observed within a 24 h period, indicating that PHx did not cause any change in the degradation of PGP-S mRNA.

Other analyses

Proliferating cell nuclear antigen level was measured as described previously [25]. Phospholipid content was determined as described previously [29]. Protein content was determined as described previously [30]. Molecular species of PG from liver homogenates were determined as described in [31]. Student's *t* test was used for the determination of significance. The level of significance was defined as $P < 0.05$.

RESULTS

CL *de novo* biosynthesis and resynthesis are increased in hepatocytes during liver regeneration

CL *de novo* biosynthesis and its resynthesis were examined in hepatocytes prepared from regenerating liver. As demonstrated previously by our group, the protein expression of proliferating cell nuclear antigen, a specific marker of liver regeneration, was maximal (4-fold higher compared with sham-operated controls) in liver at 48 h post-PHx (results not shown), indicating active regeneration [25]. Rats were either sham-operated or subjected to PHx, hepatocytes were isolated 48 h later and then incubated with [1,3-³H]glycerol for up to 24 h and the radioactivity incorporated into phospholipids of the CDP-DG pathway was determined. As seen in Figure 1(A), [1,3-³H]glycerol incorporated into PG increased with time period of incubation for up to 8 h and then decreased by 24 h in hepatocytes prepared from PHx rats compared with sham-operated controls. Incorporation of [1,3-³H]glycerol into CL increased with time period of incubation in hepatocytes prepared from PHx rats compared with sham-operated controls (Figure 1B). In addition, radioactivity incorporated into PA was low (50–100 d.p.m./mg of protein) and could not be accurately determined. Thus CL synthesis from [1,3-³H]glycerol was increased in hepatocytes during liver regeneration.

The pool sizes of phospholipids of the CDP-DG pathway were examined. The pool sizes of CL and PG were increased 2.3-fold ($P < 0.05$) and 3.8-fold ($P < 0.05$) respectively in hepatocytes prepared from 48 h PHx rats compared with sham-operated controls

Figure 1 Incorporation of [1,3-³H]glycerol into CLs and PGP in rat liver hepatocytes from PHx animals

Rats were either sham-operated (●) or subjected to PHx (○) and hepatocytes were isolated 48 h later, incubated for up to 24 h with 0.1 mM [1,3-³H]glycerol, and the radioactivity incorporated into PG (A) and CL (B) was determined as described in the Materials and methods section. Results are expressed as the means $\pm$ S.D. for three animals.

Table 1 Pool sizes of phospholipids of the CDP-DG pathway in hepatocytes prepared from PHx rats

Rats were either sham-operated or subjected to PHx, hepatocytes were isolated 48 h later and the pool sizes of phospholipids of the CDP-DG pathway were determined. Results represent the means $\pm$ S.D. for three separate sets of hepatocytes.

Phospholipid	Pool size (nmol of P _i /mg of protein)	
	Sham	PHx
CL	4.8 $\pm$ 0.5	15.7 $\pm$ 1.7*
PG	0.7 $\pm$ 0.1	3.4 $\pm$ 0.3*
PA	0.3 $\pm$ 0.1	0.5 $\pm$ 0.1

* $P < 0.05$.

(Table 1). The total lipid phospholipid pool was 164 nmol/mg of protein in hepatocytes prepared from sham-operated rats and was 174 nmol/mg of protein in hepatocytes prepared from PHx rats. Thus the CL and PG masses were increased in hepatocytes prepared from PHx rats.

The fatty acyl composition of CL from rat liver is unique in that it contains mainly mono- and diunsaturated chains with 16 or 18 carbons, with a relatively homogeneous distribution of double bonds and carbon numbers among the four acyl chains [15]. Since the fatty acid composition of CL was unaltered in whole liver preparations from rats undergoing liver regeneration [32], we examined the major molecular species of liver PG in sham-operated rats and rats subjected to PHx. The molecular species

Table 2 Incorporation of labelled glycerol-3-phosphate into molecular species of PG in livers of PHx rats

Rats were either sham-operated or subjected to PHx; livers were isolated 48 h later and mitochondrial fractions were prepared and the species pattern of PG was determined. Results represent the average of two determinations and did not differ by more than 15 %.

Molecular species	Percentage of total	
	Sham	PHx
16:0-18:1	24	26
16:0-16:0	18	20
16:0-18:2	17	14
18:1-18:2	9	7
16:0-20:4	8	8
16:0-16:1	7	7
18:0-18:2	6	5
Others	11	13

Figure 2 Incorporation of [1-¹⁴C]linoleate into CL in rat liver hepatocytes from PHx animals

Rats were either sham-operated (●) or subjected to PHx (○), hepatocytes were isolated 48 h later and then incubated for up to 24 h with 0.1 mM [1-¹⁴C]linoleate bound to albumin, and the radioactivity incorporated into CL was determined as described in the Materials and methods section. Results are expressed as the means $\pm$ S.D. for three animals.

of liver PG was unaltered between sham-operated and PHx rats (Table 2). The results for the sham-operated animals were essentially similar to those reported previously [31]. The small differences between the two studies may be attributed to the use of Wistar rats in [31] versus Sprague–Dawley rats used in the present study.

The resynthesis of CL was examined. Hepatocytes were prepared as above and then incubated with [1-¹⁴C]linoleic acid for up to 24 h and radioactivity incorporated into phospholipids of the CDP-DG pathway was determined. As seen in Figure 2, the amount of [1-¹⁴C]linoleic acid incorporated into CL increased with time period of incubation, 2.3-fold ($P < 0.05$) by 24 h, in hepatocytes prepared from PHx rats compared with sham-operated controls. Radioactivity incorporated into PA and PG was low (50–100 d.p.m./mg of protein) and could not be accurately determined. Thus CL resynthesis from MLCL was increased in hepatocytes during liver regeneration.

PGP-S and MLCL-AT activities and their expression are increased in hepatocytes during rat liver regeneration

The reason for the increased incorporation of [1,3-³H]glycerol and [1-¹⁴C]linoleic acid into CL during liver regeneration was exam-

Table 3 Activities of enzymes of CL biosynthesis and resynthesis in hepatocytes prepared from PHx rats

Rats were either sham-operated or subjected to PHx, hepatocytes were isolated 48 h later and the activities of the enzymes of CL biosynthesis and resynthesis were determined. Results represent the means $\pm$ S.D. for three separate sets of hepatocytes.

Enzyme [pmol $\cdot$ min ⁻¹ $\cdot$ (mg of protein) ⁻¹]	Sham	PHx
CDP-DG synthetase	1.4 $\pm$ 0.2	1.3 $\pm$ 0.2
PGP-S	157 $\pm$ 27	259 $\pm$ 32*
CL synthase	2.4 $\pm$ 0.4	2.4 $\pm$ 0.5
MLCL-AT	804 $\pm$ 60	1149 $\pm$ 41*

* $P < 0.05$.

Figure 3 Validity of mRNA quantification for rat liver PGP-S

Under the RT–PCR conditions used, the levels of the PCR products were dependent on the amount of templates used in the reaction. At least a 10-fold difference in cDNA levels of PGP-S was detected.

ined. Rats were either sham-operated or subjected to PHx, hepatocytes were isolated 48 h later, mitochondrial fractions were prepared and the activities of the enzymes involved in CL biosynthesis and resynthesis were determined. As seen in Table 3, the activities of PGP-S and MLCL-AT were increased by 65 % ($P < 0.05$) and 43 % ($P < 0.05$) respectively in mitochondrial fractions of hepatocytes from rats subjected to PHx compared with sham-operated controls. Surprisingly, mitochondrial CDP-DG synthetase and CL synthase activities were unaltered in hepatocytes from rats subjected to PHx compared with sham-operated controls. Hepatocyte mitochondrial lyso-PGP acyltransferase activity was 0.55 ± 0.12 nmol $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹ in sham-operated controls and was unaltered in hepatocytes from rats subjected to PHx (0.53 ± 0.09 nmol $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹). CL transacylase activity was 1.0 ± 0.2 pmol $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹ in hepatocyte mitochondria prepared from sham-operated controls and was unaltered in hepatocytes from rats subjected to PHx [1.0 ± 0.2 pmol $\cdot$ min⁻¹ $\cdot$ (mg of protein)⁻¹].

Rats were then either sham-operated or subjected to PHx, mRNA was prepared from the livers 48 h later and PGP-S mRNA level was determined. Under the RT–PCR conditions employed, the level of the PCR product was dependent on the amount of templates employed in the reaction (Figure 3). The mRNA level of PGP-S was increased by more than 3-fold ($P < 0.05$) in livers prepared from rats subjected to PHx compared with sham-operated controls (Figure 4). Mitochondrial fractions were then prepared from sham-operated or PHx rats and the protein expression

Figure 4 PGP-S mRNA expression in rat livers from PHx animals

Rats were either sham-operated or subjected to PHx, livers were isolated 48 h later, mRNA was prepared and PGP-S mRNA levels were determined as described in the Materials and methods section. (A) Five left lanes, PGP-S mRNA expression in livers prepared from five different sham-operated animals; five right lanes, PGP-S mRNA expression in livers prepared from five different animals subjected to PHx. (B) The intensity of the bands relative to GAPDH is plotted.

Figure 5 MLCL-AT protein expression in rat livers from PHx animals

Rats were either sham-operated or subjected to PHx, livers were isolated 48 h later, mitochondrial fractions were prepared and MLCL-AT protein expression was determined as described in the Materials and methods section. (A) A representative blot. Lanes 1 and 2, MLCL-AT protein expression in livers prepared from two different sham-operated animals; lanes 3 and 4, MLCL-AT protein expression in livers prepared from two different animals subjected to PHx. (B) The intensity of the bands relative to β -actin is plotted in arbitrary units.

of MLCL-AT was determined by Western-blot analysis. Expression of MLCL-AT protein was increased in mitochondrial fractions of hepatocytes prepared from rats subjected to PHx compared with sham-operated controls (Figure 5). Thus the increased incorporation of $[1,3\text{-}^3\text{H}]$ glycerol and $[1\text{-}^{14}\text{C}]$ linoleic acid into CL during liver regeneration from rats subjected to PHx was due to an increase in PGP-S and MLCL-AT activities and their expression.

DISCUSSION

The objective of the present study was to investigate the reason for the increase in CL during liver regeneration. Our results indicate that (i) CL content and its *de novo* biosynthesis and resynthesis are increased during rat liver regeneration post-PHx, (ii) the reason for the increase in *de novo* CL biosynthesis is an increase in the activity and mRNA expression of PGP-S, a key enzyme in

de novo CL biosynthesis [8,12,13], and (iii) the reason for the increase in CL resynthesis is an increase in the activity and protein expression of MLCL-AT, a key enzyme of CL resynthesis [17–19].

The CL content in hepatocytes prepared from PHx rats was increased by more than 3-fold compared with sham-operated controls, implying increased hepatic CL synthesis during liver regeneration. Interestingly, the increase in the pool size of CL was higher relative to the increase in the total phospholipid pool when expressed in terms of $(\text{mg of protein})^{-1}$. These results are consistent with the observation of a different rate of restoration of CL compared with other phospholipids during rat liver regeneration [21]. $[1,3\text{-}^3\text{H}]$ Glycerol incorporation into CL was increased in hepatocytes prepared from PHx rats, indicating an increase in the *de novo* CL biosynthesis during liver regeneration. The observation of increased PGP-S activity and its mRNA expression in hepatocytes and liver prepared from PHx rats provided a potential mechanism for this increase in *de novo* CL biosynthesis during liver regeneration.

Interestingly, CDP-DG synthetase and CL synthase activities were not altered in hepatocytes prepared from PHx rats. In yeast, the expression of CDP-DG synthetase and CL synthase activities were regulated by growth [33,34]. However, in contrast with PGP-S, neither of these enzymes was regulated by inositol starvation, indicating a differential pattern of regulation of these enzymes during the yeast growth phase. Treatment of rats with thyroxine was shown to stimulate rat liver and heart CL synthase activities and rat heart PGP-S activities [13,35]. However, rat heart CDP-DG synthetase activity was unaltered by thyroxine treatment [13]. PGP-S activity was increased both in clofibrate-treated H9c2 cells and in the hearts of clofibrate-fed mice [9]. In contrast, CL synthase activities were unaltered in these rodent models. In addition, CDP-DG synthetase activity and mRNA expression were increased only in clofibrate-fed mice [9]. In a mutant Chinese-hamster ovary cell line defective in PG synthesis, PG and CL were decreased, and this attenuated cell growth and function of the electron-transport chain [12]. When the Chinese-hamster ovary cell mutant was transfected with the cDNA of PGP-S, the PG and CL levels and mitochondrial function were restored [36]. The above studies indicate that a complex regulation of the *de novo* CL biosynthetic enzymes exists in eukaryotes and implicate PGP-S as a key player in CL biosynthesis in non-pathophysiological or drug-induced cellular growth and proliferation events in mammalian tissues.

CL from rat liver contains mainly tetra-linoleoyl species [15]. Resynthesis of rat liver CL appears to be essential to obtain the observed enrichment of CL with linoleate, since the CL synthase had no selective preference for various fatty acid-specific species of CDP-DG [37]. In addition, the species pattern of the CL precursors PA, CDP-DG and PG in rat liver were similar enough to imply that the enzymes of the CL biosynthetic pathway are not molecular species-selective [31]. In support of the above studies is the observation that the molecular species composition of PG, the immediate phospholipid precursor of CL, was unaltered in livers from PHx rats compared with sham-operated controls.

Rat liver mitochondrial CL may also be remodelled by a deacylation–reacylation cycle in which newly synthesized CL is rapidly deacylated to MLCL and then reacylated back to CL with linoleoyl-CoA [16,17]. $[1\text{-}^{14}\text{C}]$ Linoleate incorporation into CL was increased in hepatocytes prepared from PHx rats, indicating an increase in CL resynthesis. We recently purified the pig liver MLCL-AT to homogeneity and produced a polyclonal antibody to the protein that cross-reacts with the enzyme from rat liver mitochondria [18]. The enzyme utilized various unsaturated acyl-CoAs to a similar extent and had >10-fold higher specificity

for unsaturated fatty acyl-CoAs compared with saturated fatty acyl-CoAs [17,18]. In regenerating livers of PHx rats, increase in partitioning of long chain fatty acyl-CoA towards acylglycerol synthesis has been observed [38]. In the present study, lyso-PGP acyltransferase activity and [$1\text{-}^{14}\text{C}$]linoleate incorporation into PG were unaltered in hepatocytes prepared from PHx rats compared with sham-operated controls. Thus the increase in [$1\text{-}^{14}\text{C}$]linoleate incorporation into CL was not due to an increase in [$1\text{-}^{14}\text{C}$]linoleate incorporation into its immediate precursor PG. The fact that CL transacylase activity was low compared with MLCL-AT activity coupled with an observed increase in MLCL-AT activity and protein expression in PHx animals could explain why there was an increase in [$1\text{-}^{14}\text{C}$]linoleate incorporation into CL in the hepatocyte radiolabelling experiments. The content of CL was shown to double during normal liver growth from birth to adulthood in the rat and the fatty acid composition was altered, with a net enrichment of 18:2 [39,40]. In addition, the fatty acid composition of CL was not changed in whole liver preparations from rats undergoing liver regeneration [32]. Thus the increased hepatic MLCL-AT activity observed in PHx could be a compensatory mechanism to maintain the high unsaturated fatty acid content of the newly synthesized CL molecule.

During the revision of this paper, the presence of another MLCL-AT activity was demonstrated [41]. The activity of this recombinant enzyme was similar to rat liver mitochondrial MLCL-AT in that it exhibited specificity for unsaturated fatty acyl-CoAs. However, the recombinant enzyme recognized both MLCL and dilyso-CL as substrates. Although the recombinant enzyme was localized to the endoplasmic reticulum, it is possible that it contributes to mitochondrial CL resynthesis. In summary, the observation of increased mitochondrial CL biosynthesis and its resynthesis from MLCL during liver regeneration in rats support the notion that new CL biosynthesis and its subsequent resynthesis may be a coupled process in mammalian cells.

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