University of Alberta

Characterization of Phosphatidylcholine Metabolism in Mouse Hepatocytes after Hepatectomy and in Primary Human Hepatocytes

by

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Abstract

Phosphatidylcholine (PC) is the major component of mammalian membranes and the induction of PC biosynthesis has been shown to be an essential step in cell proliferation in various cell lines. In the liver, PC biosynthesis is regulated by two pathways: the CDP-choline and the phosphatidylethanolamine *N*-methyltransferase (PEMT) pathways. The major enzymes that regulate these two pathways are CTP:phosphocholine cytidylyltransferase (CT)α and PEMT, respectively. In our first study, we elucidated the role of PC biosynthesis during proliferation by monitoring liver regeneration after 70% partial hepatectomy (PH) in mice lacking hepatic CTα (LCTα$^{-/-}$ mice). In the liver, CDP-choline is the major pathway of PC biosynthesis. To our surprise, liver re-growth, DNA synthesis, and PC mass after surgery were not impaired in LCTα$^{-/-}$ mice despite reduced total PC synthesis. Furthermore, PC synthesis in control mice was not induced after PH. Thus, we concluded that CTα is not essential for hepatocyte proliferation i*n vivo* and that basal hepatic PC biosynthesis is sufficient to sustain regeneration after PH. In the second study, we assessed hepatic PC to phosphatidylethanolamine ratio (PC/PE) as a predictor of non-alcoholic fatty liver disease (NAFLD) and post-operative complications after major hepatectomy. LCTα$^{-/-}$ mice, PEMT-deficient mice (*Pemt*$^{-/-}$ mice), and their respective controls were fed a high fat diet to induce various degrees of NAFLD, before PH was performed. We found significant correlation of decreased PC/PE (pre-surgery) with the progression of NAFLD and decreased survival rate after PH.

Additionally, dietary choline supplementation increased hepatic PC/PE in *Pemt*$^{-/-}$ mice with NAFLD, decreased inflammation, and increased survival rate after partial hepatectomy. Thus, choline supplementation may serve as a potential therapy to prevent the progression of NAFLD and to improve post-operative outcome after liver surgery. Finally, we characterized some fundamental aspects of lipid and lipoprotein metabolism in primary human hepatocytes. In particular, we evaluated the synthesis of phospholipids in relation to lipoprotein metabolism. Overall, this work has contributed to the understanding of important processes required for liver regeneration as well as discovering the similarities and differences in lipid and lipoprotein metabolism in primary human hepatocytes compared to hepatoma cell lines and rodent primary hepatocytes.

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Abbreviations

Adv	adenovirus
AdoHcy	*S*-adenosylhomocysteine
AdoMet	*S*-adenosylmethionine
Apo	apolipoprotein
ATP	adenosine triphosphate
BrdU	5-bromo -2'-deoxyuridine
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CDP	cytidine diphosphate
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CK	choline kinase
CPT	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CT	CTP:phosphocholine cytidylyltransferase
DG	diacylglycerol
DTT	dithiothreitol
EGF	epidermal growth factor
ER	endoplasmic reticulum
FBS	fetal bovine serum

FPLC	fast protein liquid chromatography
g	gram
GFP	green fluorescent protein
h	hour
H&E	hematoxylin and eosin
HDL	high density lipoprotein
HF	high fat
HF-CS	high fat with choline supplementation
HGF	hepatocyte growth factor
IL	interleukin
kDa	kilodalton
LCTα	liver specific CTα
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
MAM	mitochondria-associated membrane
MDR	multiple-drug resistant protein
min	minute
mL	millilitre
MMP	matrix metalloproteinase
mRNA	messenger RNA

mTOR	mammalian target of rapamycin
NA	nicotinic acid, niacin
NAFLD	non-alcoholic fatty liver disease
NAM	nicotinamide
NASH	non-alcoholic steatohepatitis
NF-κB	nuclear factor kappa light chain enhancer of activated B cells
PBS	phosphate buffered saline
PC	phosphatidylcholine
PC/PE	phosphatidylcholine to phosphatidylethanolamine ratio
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine *N*-methyltransferase
PEPCK	phosphoenolpyruvate carboxylkinase
PDME	phosphatidyldimethylethanolamine
PH	70% partial hepatectomy
PMME	phosphatidylmonomethylethanolamine
PPAR α	peroxisome proliferator-activated receptor α
PVDF	polyvinylidine fluoride

RPMI	Roswell Park Memorial Institute
ROS	reactive oxygen species
s	second
SDS-PAGE	sodium dodecyl sulphate-polyacrylamine gel electrophoresis
Sp1	specificity protein 1
SR-B1	scavenger receptor class B1
SREBP	sterol regulatory element-binding protein
STAT	signal transducer and activator of transcription
SOCS	suppressor of cytokine signalling
TIMP	tissue inhibitor of matrix metalloproteinase
TG	triacylglycerol
TGH	triacylglycerol hydrolase
TLC	thin layer chromatography
TNF	tumor necrosis factor
T-TBS	tween-tris buffered saline
UCP	uncoupling protein
µL	microlitre
VLDL	very low density lipoprotein

CHAPTER 1: Introduction

1.1 Phosphatidylcholine structure

Phosphatidylcholine (PC) was first described in 1847 as a component of egg yolk and named lecithin after *lekithos*, the Greek equivalent for egg yolk [1]. Soon after, the basic structure of lecithin was identified as 2 fatty acid chains esterified to a glycerol backbone, and a choline group attached to the third hydroxyl group by a phosphodiester bond (Figure 1.1) [2, 3]. Like all phospholipids, PC is amphipathic: it contains a hydrophilic (phosphocholine headgroup) and a hydrophobic domain (fatty acyl chains) [4]. More than 20 different molecular species of PC have been detected in mammalian cells and its diversity has been attributed to the variety of fatty acids attached to the glycerol backbone [5]. Saturated fatty acids (e.g. palmitate, C16:0) are most often found in the *sn*-1 position, while the *sn*-2 position is typically occupied by polyunsaturated fatty acids (e.g. arachidonic acid, C20:4) [5, 6].

1.2 Biological functions of PC

1.2.1 PC and biological membranes

In eukaryotes, PC is the primary building block of cellular membranes [4]. Due to its cylindrical shape and amphipathic nature, PC spontaneously organizes into membrane bilayers [7]. Therefore, PC is an ideal structural component of biological membranes. PC content affects physiological properties of the membrane, such as fluidity, integrity, and curvature [8, 9]. The lipid environment has also been shown to affect membrane protein function [10, 11]. As such, the distribution of PC varies among cell types, organelles, and even between the monolayers of the membrane bilayer [8].

1.2.2 PC and bile

Bile is produced in the liver and facilitates the digestion and absorption of lipids and fat soluble vitamins [12]. The components of bile include bile acids,

cholesterol, and phospholipids. Of total biliary phospholipid content, PC makes up 95% [13]. The secretion of PC into bile in humans is facilitated by multiple-drug resistance protein (MDR) 3, a PC-specific transport protein which transports PC across the hepatic cannicular membrane [14]. Genetic mutation in the MDR3 gene has been linked to intrahepatic and gallbladder cholesterol cholelithiasis (gallstones) [15]. Cholesterol gallstones develop when the concentration of biliary cholesterol exceed that of bile and phosphatidylcholine concentrations required to solubilize cholesterol [16]. Mutation in the MDR3 gene has also been associated with progressive familial intrahepatic cholestasis 3, a disease characterized by persistent blockage of the bile ducts (cholestasis) that progresses to irreversible liver damage and failure [17]. .

1.2.3 PC and lung surfactant

Pulmonary surfactant is essential for lung function by reducing the surface tension at the air-liquid interface and preventing alveolar collapse [18]. Surfactant is secreted by alveolar type II epithelial cells as a lipoprotein-like complex, of which dipalmitoylphosphatidylcholine comprises 80% of the total lipid content. The importance of PC in lung surfactant is demonstrated in mice in which the major pathway for PC synthesis was disrupted in alveolar type II epithelial cells [19]. These mice develop respiratory failure at birth due to decreased levels of dipalmitoylphosphatidylcholine.

1.2.4 PC and cellular signalling

PC in the plasma membrane can be hydrolyzed by several phospholipases in response to hormones, cytokines, growth factors, *etc* [20] (Figure 1.2). For example, phospholipase A_2 hydrolysis of PC produces lyso-PC and arachidonic acid [21]. Arachidonic acid is a precursor of eiconasoids which are important inflammatory mediators. Phospholipase C and D hydrolyze PC to generate diacylglycerol (DG) and phosphatidic acid, respectively [20, 22]. Both DG and

phosphatidic acid have been shown to regulate cellular events such as proliferation, differentiation, membrane vesicle trafficking, and cytoskeleton reorganization.

In addition, PC serves as a substrate for sphingomyelin synthase, which transfers the phosphocholine from PC to ceramide [23]. This reaction produces sphingomyelin and releases DG. The hydrolysis of sphingomyelin can be stimulated by extracellular factors to produce ceramide, which plays a major role in apoptosis and cell division.

1.2.5 PC and Lipoproteins

Lipoproteins enable the transport of hydrophobic lipids through the body *via* the circulatory system [4]. The major components of lipoproteins are phospholipids, neutral lipids and proteins (apolipoproteins). Structurally, a lipoprotein particle consists of a monolayer of phospholipids, cholesterol, and apolipoproteins surrounding a hydrophobic core of triacylglycerol (TG) and cholesteryl ester (CE). Different combinations of proteins and lipids produce several classes of lipoproteins which vary in density (Table 1.1). For example, very low density lipoproteins (VLDL) which are mostly composed of TG are more buoyant than low density lipoproteins (LDL). High density lipoproteins (HDL) are denser than LDL because they contain more proteins than CE. In addition, VLDLs is also much larger in size than LDL, which in turn are larger than HDL.

The relative phospholipid content of lipoproteins increases with density and is directly related to the surface area (or size) of the lipoprotein particle [4]. By far, PC is the major phospholipid component of lipoproteins (60-80%) [24]. Other phospholipid components include sphingomyelin, lyso-PC, phosphatidylethanolamine (PE), phosphatidylserine, and phosphatidylinositol.

1.3 PC biosynthesis

In mammalian cells, PC is produced endogenously *via* 2 distinct pathways [25] (Figure 1.3). The major pathway for PC synthesis is the cytidine diphosphate (CDP)-choline pathway which was first described by Eugene Kennedy and co-workers in the 1950s. The CDP-choline pathway uses dietary choline to synthesize PC *via* 3 enzymatic reactions and is present in all nucleated cells.

In the initial reaction, choline kinase catalyzes the adenosine triphosphate (ATP)-dependent phosphorylation of choline, forming phosphocholine and the by-product adenosine diphosphate. In the second reaction, CTP:phosphocholine cytidylyltransferase (CT) catalyzes the formation of CDP-choline and phosphate from phosphocholine and cytidine triphosphate. In the last reaction, CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) catalyzes the production of PC *via* the condensation of CDP-choline with DG, releasing cytidine monophosphate.

The alternative pathway for PC biosynthesis occurs only in the liver and contributes 30% of total hepatic PC synthesis (CDP-choline pathway provides the remaining 70%) [25]. In this pathway, phosphatidylethanolamine *N*-methyltransferase (PEMT) catalyzes the conversion of PE to PC *via* 3 sequential methylation reactions.

1.4 PC biosynthesis: the CDP-choline pathway

1.4.1 Choline

The importance of dietary choline was first reported by Charles Best in 1932 [26]. Only in 1998 was choline categorized as an essential nutrient by the Food and Nutrition Board of the Institute of Health in the United States of America [27]. The synthesis of PC *via* the CDP-choline pathway is the primary fate of dietary choline [28]. Excess choline can also be irreversibly oxidized to form betaine in the mitochondria of the liver and the kidneys [29] (Figure 1.4).

Betaine acts as a methyl group donor in the homocysteine-methionine methylation pathway, which is important for protein synthesis and transmethylation reactions [30]. Lastly, choline is the precursor of the neurotransmitter acetylcholine and therefore important for neuronal function [31].

The *de novo* synthesis of choline involves the methylation of PE to PC (PEMT pathway) and the subsequent release of the choline moiety [25]. However, the majority of choline is obtained through the diet as free or esterified choline (e.g. PC) [32]. PC is primarily absorbed as lyso-PC in the gut [33] and free choline is absorbed in the upper small intestine (jejunum) [34, 35]. Because choline is positively charged, active transport is required for choline to cross cellular membranes [36]. Three modes of choline transport have been identified: 1) facilitated diffusion, 2) low affinity, sodium-independent transporter, and 3) high affinity, sodium dependent transporter. The uptake of choline *via* facilitated diffusion was described in erythrocytes and is driven by the choline concentration gradient [37]. The high affinity transport of choline is coupled to the biosynthesis of acetylcholine and is primarily found in neuronal tissues [38]. The low affinity choline transporter is ubiquitously expressed and primarily imports choline for phospholipid synthesis [36].

1.4.2 Choline deficiency

The recommended Adequate Intake for choline is 550 mg/day and 425 mg/day for adult males and females respectively [39]. However, a study has found that 90% or more of the population does not meet the recommended choline intake and could be choline deficient [40]. Specific population groups at risk for choline deficiency include pregnant and lactating women, infants, cirrhosis patients, and patients receiving long-term parenteral nutrition [41].

In adults, the major phenotype of choline deficiency is accumulation of TG (fat) in the liver [42-44]. Fatty liver (steatosis) is usually benign but can sometimes lead to more serious liver dysfunction [45].

As a source of methyl donors and a precursor for acetylcholine, choline also has a profound effect on early brain development. Insufficient maternal choline intake has been linked to decreased neurogenesis and angiogenesis in the fetal brain and impaired hipocampal (memory) function in the offspring latter in life [46-48]. In addition, rodent and human studies show females on a low choline diet have an increased risk of giving birth to babies with neural tube defects [49, 50].

Other diseases linked to choline deficiency include skeletal muscle damage [51, 52] and renal dysfunction [53].

1.4.3 Choline kinase

Choline kinase (CK) catalyzes the initial step of the CDP-choline pathway; however, it is not considered the rate limiting step in the CDP-choline pathway [25]. In mammals, 3 isoforms of CK are expressed and they are encoded by 2 separate genes (*chkα, chkβ)* [54-56]. Alternative splicing of *chkα* mRNA gives rise to the CKα1 and CKα2 isoform, while *chkβ* encodes CKβ. The activity of CK requires the formation of either homo- or hetero-dimers [57, 58]. In most tissues, the active form of CK exists predominantly as α/β heterodimers and α/α or β/β homodimers constitute the remaining CK activity [58]. Interestingly, when CK activity is induced by carbon tetrachloride, there is a shift to the formation of α/α homodimer [58]. This could suggest that CK activity might be regulated by altering the combinations of CK isoforms. Furthermore, CK activity can also be regulated transcriptionally *via* association of transcription factor c-jun to a distal AP-1 element of the *chkα* gene [59]. C*hkα* expression was also induced through

the binding of HIF-1 to the hypoxia response element within the *chkα* promoter [60]. As of yet, *chkβ* has not been shown to be regulated transcriptionally [59].

Both CKα and CKβ are expressed ubiquitously in mouse tissues [58]. Mice deficient in CKα or CKβ have been generated and provide clues to the specific function of each isoform. Deletion of CKα is lethal after embryonic day 3.5, which suggests CKα is involved in embryogenesis [61]. The role of CKα in cell growth is further recapitulated by the observation that CKα expression and activity are increased in several human cancer cells lines as well as tumor tissues [62-65]. Currently, the functional difference between CKα1 and CKα2 is unknown [66].

Mice with a spontaneous deletion of *chkβ* develop hindlimb muscular dystrophy and neonatal forelimb bone deformity [67, 68]. Wu *et al* showed that CKβ is the major isoform in the skeletal muscle of the hind legs [68]. As such, deletion of *chkβ* gene resulted in reduced PC levels only in the hindlimb skeletal muscle, leading to muscular dysfunction. Mutation of *chkβ* gene has also been linked to 4 Japanese patients diagnosed with congenital muscular dystrophy [69]. The mechanism by which *Ckβ*-deficient mice develop forelimb bone deformation is still under investigation.

1.4.4 CTP:phosphocholine cytidylyltransferase

a) Characterization of CTP:phosphocholine cytidylyltransferase

CTP: phosphocholine cytidylyltransferase (CT) catalyzes the rate limiting step in the CDP-choline pathway [70, 71]. Three isoforms of CT are expressed in mammals and are encoded by 2 distinct genes (*Pyct1a, Pyct1b*) [72, 73]. The *Pyct1a* gene was first cloned and characterized by Tang *et al* [72]. It spans 40 kb on chromosome 16 and has 2 transcription start sites. The proximal promoter lacks TATA or CAAT boxes but contains GC-rich elements typically found in the

promoters of housekeeping genes [72]. In all nucleated cells, the product of *Pcyt1*, CTα, is the predominant isoform.

The Pcyt1b gene is located on the X chromosome [73]. *Pcy1b* expression is low in most tissues, but it is highly expressed in the brain and the reproductive organs. *Pcyt1b* encodes 2 isoforms: CTβ1 and CTβ2 in humans, and CTβ2 and CTβ3 in mice. In mouse, the expression of the CTβ2 and CTβ3 is driven by two different promoters [73]. The expression pattern of CTβ2 and CTβ3 was found to be independent of each other during embryonic and postnatal brain development [74]. Both pieces of evidence suggest that the transcription of the 2 isoforms may be differentially regulated.

All CT isoforms are composed of a core catalytic domain, followed by a membrane binding domain, and a C-terminal phosphorylation domain [75]. (Figure 1.5) CTα also has an N-terminal nuclear localization signal that is not present in CTβ1 and CTβ2. CTβ1 and β2 differ in their C-termini: CTβ1 lacks the phosphorylation domain that is present in both CTβ2 and CTα.

CT forms homodimers in the cell and exists in 2 distinct pools [76, 77]. The major regulatory mechanism of CT activity involves the reversible translocation of the protein from the cytosol (inactive pool) to a phospholipid monolayer or bilayer (active pool). The binding of CT to the membrane involves the electrostatic adsorption of CT to the membrane, followed by the intercalation of the lipid binding domain (amphipathic alpha helix) into the nonpolar core of the membrane [78]. The association is believed to relieve the inhibitory restraint in the catalytic domain of the enzyme, thereby activating the enzyme.

b) Regulation of CT activity

The translocation of CTα to the membrane, and hence CTα activation, is regulated by lipids. Diacylglycerol [79], fatty acids [80], oxysterols [81] and low levels of PC [82] have all been found to stimulate the association of CT to membranes. Alternatively, high PC levels inhibit the association of CTα to the membrane in a negative feedback regulation of CTα activity and PC biosynthesis [83]. This proposed mechanism allows for the rapid modulation of PC biosynthesis in response to the availability of lipid precursors and alterations in membrane composition [25].

CTα activity is also regulated by phosphorylation[75]. CTα contains 16 serine residues in its C-terminal domain which can all be phosphorylated. Mutation of all 16 serines to alanines does not impair CTα translocation [84]. However, Shiratori *et al* demonstrated that the activity of membrane-associated CTα could be further increased with the dephosphorylated enzyme [85]. Furthermore, Mallampalli and colleagues showed that CTα activity could be inhibited by the phosphorylation of Ser315 by ERK (p42 kinase) [86]. Thus, phosphorylation of CTα is believed to modulate the activity of the enzyme.

Since the lipid-binding domain of CTβ is very similar to CTα, CTβ activity is also thought to be regulated by the translocation of the enzyme between the cytosol and the membrane [87]. However, very little has been published about this process.

CTα activity is also regulated at the transcriptional level. Specific protein 1 (Sp1) has been identified as the major activator of CTα transcription [88, 89]. Sp1 is phosphorylated by cyclin-dependent kinase-2 and phosphorylated Sp1 binds within the proximal promoter; activating the transcription of the CT gene [90]. Other transcription factors which have been shown to enhance CTα expression include Ets-1 (erythroblastosis virus E26 oncogene homolog -1), TEF-4 (transcription enhancer factor 4), Rb (Retinoblastoma) and E2F [91-93].

Alternatively, Net and Sp1 inhibitory complex are repressors of CTα gene expression [91, 94, 95]. Interestingly, the key transcription factors involved in energy metabolism such as sterol regulatory element-binding proteins, liver X receptors, and peroxisome proliferator-activated receptors, have not been shown to regulate CTα transcription [87]. Instead, the regulation of *Pcyt1a* expression is linked to cell cycle, cell growth, and differentiation [87].

c) Subcellular localization of CT

Recently, the majority of inactive CTα has been localized to the nucleus (instead of the cytosol) in many cultured and primary cells [96]. The presence of CT in the nucleus has been shown to be important for nucleoplasmic reticulum proliferation and/or defining the architecture of the nuclear membrane [97]. In terms of PC biosynthesis, the functional significance of nuclear CTα is still unclear since all other steps in the CDP-choline pathway are cytosolic (e.g. CK) or endoplasmic reticulum (ER)-associated (e.g. CPT). However, there is evidence to suggest that CTα may be exported out of the nucleus into cytoplasm in response to increased cellular demand for PC (e.g. during cell division) [98].

Using drosophila S2 cells, Krahmer *et al* recently demonstrated that CT also translocates from the nucleus onto cytosolic lipid droplets upon fatty acid stimulation [99]. They showed CT-dependent PC production was necessary for stabilizing the formation of many small lipid droplets [99, 100]. Cells lacking CT formed large, lipolysis-resistant lipid droplets and accumulated TG.

All isoforms of CTβ are cytosolic since they lack a nuclear localization signal [101].

1.4.5 Physiological importance of CT

The generation of CT-deficient mice has provided great insight into the biological functions of each isoform. Global deletion of *Pcyt1a* resulted in

embryonic lethality following fertilization day 3.5 [102]. This is most likely due to the importance of CTα in cell growth and differentiation. Therefore, the Cre-lox system was employed to disrupt CT in specific tissues. Deletion of CTα in type 2 lung epithelial cells confirmed the importance of CT in lung surfactant production [19]. These mice developed severe respiratory failure at birth due to decreased synthesis and secretion of dipalmitoylphosphatidylcholine. CTα was also found to be important for macrophage function [103, 104]. Disruption of CTα in macrophages increased sensitivity of the cell to cholesterol induced apoptosis and reduced secretion of cytokines such as tumor necrosis factor (TNF) α and interleukin (IL)- 6 [103, 104].

As one would have predicted, deletion of hepatic CTα altered lipoprotein homeostasis [105, 106]. Mice with loss of hepatic CTα have reduced plasma levels of HDL and VLDL due to impaired secretion [105, 106]. Liver CTα deficient ($LCT\alpha^{-/-}$) mice also developed steatosis, (an accumulation of TG in the liver) when fed a chow diet. The accumulation of hepatic TG is believed to be the result of decreased VLDL secretion due to an insufficient supply of hepatic PC [107]. When fed a high fat (HF) diet, $LCT\alpha^{-/-}$mice developed non-alcoholic steatohepatitis (NASH) [108] which has been linked to the development of cirrhosis and hepatocarcinoma [45].

Generation of CTβ2-deficient mice revealed a role of CTβ2 in gonadal development [109]. These animals had reduced fertility due to impaired maturation of ovarian follicles and defective testicular spermatogenesis [109]. Despite the high expression levels of CTβ2 in the brain and the reported importance of CTβ2 in neuronal outgrowth and axon branching [110, 111], CTβ2 deficient mice have no overt defect in brain development or morphology [109]. However, possible defects in learning, memory, and motor coordination have not yet been reported in these animals.

1.4.6 CDP-choline:1,2-diacylglycerol cholinephosphotransferase

Cholinephosphotransferase (CPT) catalyzes the final step in the CDP-choline pathway [112]. The highest specific activity of CPT was found in the liver, intestine, and adipose (8 - 9 ηmol/min/mg), followed by the brain, lung, kidney, heart and skeletal muscle (1.1 - 8 ηmol/min/mg) [113]. CPT is known to be an integral membrane protein; however, the membrane localization of CPT is still unclear [114]. From cell fractionation studies, the majority of CPT activity was found to associate with the ER, but CPT was also detected on the Golgi, mitochondria, and nuclear membranes. The difficulty in purifying CPT to homogeneity has hindered the development of CPT antibodies and thus subcellular localization studies with immunofluoresence or electron microscopy have not been forthcoming [115]. However, 2 human CPT cDNAs were recently identified based on sequence homology to the yeast CTP cDNA for choline/ethanolaminephosphotransferase and CDP-choline phosphotransferase [116, 117]. Therefore, antibodies could potentially be generated based on predicted amino acid sequences to address the subcellular localization of CPT.

In most studies, CPT is in excess within the cell, which suggests that the amount of CPT does not limit PC biosynthesis [25]. However, decreased availability of DG has been shown to impair PC biosynthesis in hepatocytes due to lack of substrate for CPT activity [118].

1.5 PC biosynthesis: the PEMT pathway

1.5.1 Identification of PEMT

It was Bremer and Greenberg who first demonstrated that PE can be methylated by *S*-adenosylmethionine (AdoMet) to produced PC and *S*-adenosylhomocysteine (AdoHcy) [119-121]. They showed that the production of PC occurred *via* 3 sequential methylation reactions and generated the

intermediates phosphatidylmonomethylethanolamine (PMME) and phosphatidyldimethylethanolamine (PDME) [119-121]. Since there was no accumulation of PMME or PDME when rat hepatic microsomes were incubated with [Me-^{14}C] AdoMet, the initial methylation reaction was predicted to be rate-limiting [121].

By comparing the incorporation of radiolabelled glycerol and methionine into PC in primary rat hepatocytes, Sundler and Akesson estimated that 20% - 40% of hepatic PC biosynthesis could be attributed to the formation of PC from PE [70]. Currently, it is commonly accepted that in liver, 30% of newly synthesized PC is due to the methylation of PE [25]. The remaining 70% is generated by the CDP-choline pathway [25].

The protein responsible for the generation of PC from the methylation of PE was identified to be phosphatidylethanolamine *N*-methyltransferase (PEMT). It was first purified by Ridgway and Vance in 1987 from rat liver homogenates [122]. The authors determined the PEMT protein to be 18 kDa in size [122]. Kinetic analysis showed that the specific activity of PEMT for PE, PMME, and PDME was 0.63, 8.59, 3.75 µmol/min/mg protein respectively, which confirmed that the first methylation of PE is the rate limiting step [122]. Further analysis also revealed that PE, PMME, and PDME all bind to a single active site [123]. This suggests that once PE occupies the active site, the reaction intermediates do not diffuse from the active site, but are preferentially methylated until PC is produced [123].

The cDNA of PEMT was cloned 5 years after the purification of PEMT, from a rat liver library using oligonucleotide probes based on the N-terminal sequence of PEMT purified from rats [124]. The 913 nucleotide cDNA encoded a 199 amino acid protein with a predicted molecular weight of 22 kDa [124]. Subsequently, the human and mouse PEMT genes were also identified [125,

126]. Similar to the rat, the open reading for the mouse and human PEMT cDNA encoded a 199 amino acid protein with a molecular weight of 22 kDa [125, 126]. The mouse and human PEMT protein also share 93.5% and 80.4% homology, respectively, with the rat protein, which suggests a conservation of PEMT among mammals [125, 126]. The human *Pemt* gene was localized to chromosome 17p11.2 and the mouse *Pemt* was mapped to chromosome 11 [125, 126]

1.5.2 Tissue distribution of PEMT

The liver is the only organ with significant PEMT activity. Initial studies by Bremer and Greenberg showed that various tissues (e.g. testes, muscle, heart, brain) contained less than 6% of the activity measured in the liver [121]. The liver specific expression of PEMT was later confirmed when the PEMT protein was only detected in the livers of rats [124]. Likewise, analysis of human tissues showed that the liver is the major tissue where PEMT is expressed by mRNA analysis [127]. Recently, PEMT protein and mRNA have been detected in 3T3-L1 mouse embryonic fibrobasts following adipocyte differentiation and mouse white adipose tissue, albeit at much lower levels when compared to the liver [128, 129].

1.5.3 Subcellular localization of PEMT

Earlier studies detected PEMT activity in the microsomal fraction of liver homogenates [121, 130]. Subcellular fractionation later confirmed that PEMT is located in the ER and a fraction of the ER that is associated with the mitochondria called the mitochondria associated membrane (MAM) [124, 131, 132]. MAM is a region of the ER characterized by a significant concentration of lipid biosynthetic activity [132, 133].

Interestingly, the antibody raised against the last 12 amino acids of rat PEMT only detected the protein in MAM but not the ER fraction [124]. This led

to the identification of 2 possible PEMT varients: PEMT1 is the ER-associated protein and PEMT2 is localized to the MAM [124]. The origin and physiological relevance of the 2 PEMT varients are not known. However, the differential localization of the 2 PEMTs may only be confined to rodents, since human PEMT was detectable in the ER and the MAM with the same antibody [131].

1.5.4 Topography of PEMT

Bioinformatic analysis of human hepatic PEMT predicted a polytopic membrane protein with 4 transmembrane domains [131] (Figure 1.6). From protease protection analyses, both the N and C termini were revealed to face the cytosol [131]. Bioinformatics and site directed mutagenesis also identified 2 amino acid motifs within PEMT which were important for binding AdoMet [134]. The ^{98}GxG^{100} and the ^{180}EE^{181} motif were mapped to the cytosolic portion of the 3rd and 4th transmembrane helices, respectively, and would allow for the channeling of AdoMet in the cytosol [134]. The targeting of PEMT to the ER is conveyed by the conserved lysine^{197} residue in the C-terminus [135].

1.5.5 Regulation of PEMT Activity

a) Substrate regulation

PEMT activity is regulated by substrate availability [70, 136]. Addition of ethanolamine, monomethylethanolamine, or dimethylethanolamine to primary hepatocytes increased the production PC *via* PE methylation by elevating the cellular levels of PE, PMME, or PDME, respectively [70, 136]. In addition, PEMT activity is regulated by the cellular ratio of AdoMet to AdoHcy. Exogenous addition of L-methionine to primary hepatocytes increased cellular AdoMet levels and the AdoMet/AdoHcy ratio, which doubled the rate of PC formation from PE [136, 137]. Alternatively, homocysteine supplementation and inhibition

of AdoHcy hydrolase lowered AdoMet/AdoHcy ratio and inhibited PEMT activity [137-139].

b) Transcriptional regulation

Until recently, the transcriptional regulation of *Pemt* was not well understood. Previous analysis of the mouse and human *Pemt* gene promoter revealed GC-rich regions and a lack of a TATA box before the transcriptional start site [125, 127]. Several putative binding sites for transcriptional factors were also characterized, including those for hepatic nuclear factor, activating protein 1, yin yang protein 1, Sp1, and estrogen [125, 127, 140]. Recently, Cole *et al* identified Sp1 as a suppressor of *Pemt* expression during the differentiation of 3T3-L1 cells [128]. Sp1 can act as a transcriptional activator (as with *Pcyt1*) or suppressor through the recruitment of histone deacetylases [141-143]. Cole *et al* demonstrated that 1) Sp1 binds to the GC-rich regions of the *Pemt* promoter, 2) increased transcription of *Pemt* following differentiation of 3T3-L1 cells coincided with decreased level of bound Sp1 to the promoter, and 3) over-expression of Sp1 decreased expression of *Pemt* promoter driven luciferase [128]. Furthermore, Cole *et al* showed inhibition of *Pemt* gene expression by tamoxifen, a common breast cancer treatment drug, and this was also regulated by the binding of Sp1 to the *Pemt* promoter [128].

The transcription factors yin yang protein 1 and Sp3 were also shown to bind to the mouse *Pemt* gene promoter [128]. However, neither protein was involved in regulating *Pemt* expression when measured during adipocyte differentiation [128].

1.5.6 Physiological importance of PEMT

An important question is: what is the physiological importance of PEMT since the CDP-choline pathway is present in the liver and all other tissues? To

answer this question, mice deficient in PEMT (*Pemt*$^{-/-}$ mice) were generated [144]. Initial observations determined that *Pemt*$^{-/-}$ mice were fertile and outwardly normal in comparison to wildtype (*Pemt*$^{+/+}$) littermates. However, when *Pemt*$^{-/-}$ mice were fed a choline deficient diet, which attenuates PC biosynthesis *via* the CDP-choline pathway, *Pemt*$^{-/-}$ mice developed liver failure [145]. Additionally, hepatic PEMT activity and protein expression were significantly increased in LCTα$^{-/-}$ mice [105]. Thus, PEMT activity exists as a compensatory pathway to synthesize PC when the CDP-choline pathway is impaired [145].

The generation of *Pemt*$^{-/-}$ mice also elucidated the physiological relevance of the enzyme in hepatic lipid and lipoprotein metabolism. Like CTα, PEMT activity is required for lipoprotein secretion [146]. When fed a HF/high cholesterol diet for 3 weeks, *Pemt*$^{-/-}$ mice showed reduced VLDL secretion (fasted plasma apolipoproteins (apo)B_{100} and TG) as well as decreased circulating levels of HDL and cholesterol [146]. *Pemt*$^{-/-}$ mice also accumulated a high level of hepatic TG when fed the same diet. However, bile secretion was not altered by PEMT deficiency [147]. Under basal conditions, as well as acute or chronic bile salt administrations, both *Pemt*$^{-/-}$ and *Pemt*$^{+/+}$ mice exhibit similar biliary secretion of bile salt and PC.

a) PEMT and atherosclerosis

Atherosclerosis is the primary cause of cardiovascular disease and contributes to a major proportion of all deaths in North America [148-150]. Atherosclerosis is characterized by the accumulation of lipids and fibrous material in the larger arteries which leads to the thickening of the arterial wall and cardiac dysfunction [148, 151]. Risk factors associated with increased occurrences of atherosclerosis include elevated plasma homocysteine [152-154],

increased plasma concentrations of LDL and its precursor VLDL [155], and decreased circulating levels of HDL [156].

PEMT activity has been shown to influence plasma homocysteine concentrations [157-159]. This is because, during PE methylation, 3 molecules of *S*-adenosylhomocysteine are produced and are subsequently hydrolyzed to homocysteine. In mouse models of PEMT deficiency, plasma homocysteine concentration are significantly decreased in comparison to control animals [158, 159]. Furthermore, in LCTα$^{-/-}$ mice, a 2 fold increase in PEMT activity is correlated with elevated plasma homocysteine concentration [157]. Thus, inhibition of PEMT activity may be protective against cardiovascular events by reducing plasma homocysteine levels.

Arterial lesion formation associated with atherosclerosis is initiated with the accumulation of lipoproteins (e.g. VLDL, LDL) and associated lipids within the artery walls [150]. Recently, PEMT deficiency was shown to improve the dyslipidemic plasma profile and reduced arterial plaque formation in 2 well-established mouse models of atherosclerosis: the LDL receptor deficient (*Ldlr*$^{-/-}$) mouse and the apoE null (*apoe*$^{-/-}$) mouse [158, 160].

Pemt$^{-/-}$*/Ldlr*$^{-/-}$ mice displayed an 80% reduction in atherosclerotic lesion size in comparison to *Pemt*$^{-/-}$*/Ldlr*$^{+/+}$ mice after the animals were fed high the fat/high cholesterol diet for 16 weeks [158]. This was accompanied by a significant decrease in apoB-containing lipoprotein particles and plasma TG and CE levels. The reduction in apoB-containing lipoprotein particles was attributed to decreased VLDL secretion and increased catabolism/clearance of VLDL from plasma. Decreased hepatic PC content as a result of PEMT deficiency has previously been shown to decrease VLDL secretion [146, 161]. Zhao *et al* also demonstrated that the VLDL particles secreted by PEMT deficient mice have a decreased ratio of PC to PE, which increased their catabolism/clearance from the

plasma [158]. Phospholipids on the surface of the lipoprotein provide contact sites for lipases, lipid transport proteins, and cell surface receptors. Therefore, altered PC and/or PE content on the VLDL surface may change the presentation of apolipoproteins such that lipolysis or binding to cell surface receptors is increased [158, 162].

Pemt$^{-/-}$*/apoe*$^{-/-}$ mice also display an improved atherogenic lipoprotein profile (45% and 24% decrease in TG and CE respectively), reduced arterial plaque formation (30%), and improved cardiac function in comparison to *Pemt*$^{-/-}$*/apoe*$^{+/+}$ mice after 1 year on a chow diet [160]. Specifically, PEMT deficiency prevented the development of lipotoxic cardiomyopathy as result of decreased TG and CE in the circulation and in the heart. Although not examined, decreased VLDL secretion and increased catabolism/clearance of VLDLs from plasma most likely contributed to the hypolipidemic profile in *Pemt*$^{-/-}$*/apoe*$^{-/-}$mice.

Increased HDL levels are correlated with the reduced risk of atherosclerosis and cardiovascular disease [156]. HDL transports excess cholesterol from peripheral tissues to the liver for biliary excretion [163]. This process is known as reverse cholesterol transport. In *Pemt*$^{-/-}$ mice, HDL associated cholesterol and PC is significantly decreased in comparison to wildtype mice [146, 159, 164]. In addition, hepatic uptake of the plasma cholesterol and PC associated with HDL was also increased due to elevated hepatic expression of scavenger receptor Class B, Type 1 (SR-B1) [164]. SR-B1 is an HDL receptor responsible for the selective uptake of CE from HDL [165]. Therefore, PEMT deficiency may protect against atherosclerosis *via* the up-regulation of reverse cholesterol transport [164]. However, in mice with *Ldlr*$^{-/-}$or *apoe*$^{-/-}$ genetic backgrounds, PEMT deficiency does not affect plasma HDL levels, which suggests that increased hepatic uptake of HDL may not be the major mechanism by which PEMT deficiency improves atherosclerosis [166].

In conclusion, the lack of PEMT decreases plasma homocysteine levels, improves dyslipidemia, and attenuates atherosclerotic plaque formation. Therefore, inhibition of PEMT may be a potential pharmacological target for the treatment of atherosclerosis.

b) PEMT and choline deficiency

The PEMT pathway is the only *de novo* source of choline [25]. An up-regulation of *Pemt* gene expression has been observed in rats during long term choline deficiency [167]. In addition, plasma choline and homocysteine levels are increased during pregnancy, which may indicate increased PEMT activity in the maternal liver due to a high fetal demand for choline [168, 169]. Interestingly, an estrogen response element has been characterized in the *Pemt* gene promoter [140]. Estrogen mediated activation of PEMT expression may also explain why most men and postmenopausal women, but only 44% of premenopausal women, fed a low choline diet develop liver and muscle dysfunction [52]. Of the 44% of premenopausal women who developed choline deficiency on a low choline diet, 80% carry a single nucleotide polymorphism in the promoter of the *Pemt* gene (-744G→C), which makes the gene unresponsive to estrogen [170]. Therefore, the PEMT pathway may be evolutionary conserved to provide choline when dietary choline is insufficient (e.g. starvation, pregnancy, lactation) [32, 145]

Pemt$^{-/-}$ mice are a model of choline deficiency. Hepatic choline levels in *Pemt*$^{-/-}$ mice are significantly lower than wildtype controls [171]. *Pemt*$^{-/-}$ mice also require more dietary choline than *Pemt*$^{+/+}$ mice to prevent steatosis, a symptom of choline deficiency [161, 171]. In a study which examined whether the lack of choline or betaine was responsible for hepatic steatosis in *Pemt*$^{-/-}$ and *Pemt*$^{+/+}$ mice, the animals were fed a HF diet supplemented with excess choline or betaine [161]. In hepatocytes, 60% of choline is irreversibly oxidized to

betaine [172]. Dietary supplementation of betaine was also shown to alleviate NAFLD and sucrose –induced steatosis [173, 174]. However, when *Pemt*$^{-/-}$ mice were fed a HF diet, choline supplementation prevented hepatomegaly while betaine supplementation did not [161]. Thus, the development of steatosis in PEMT-deficient mice is likely the result of limited choline available for PC biosynthesis and not related to the role of choline role in the homocysteine-methionine methylation pathway.

c) PEMT and non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) consists of a wide spectrum of hepatic pathologies [175]. Steatosis, characterized by hepatic TG accumulation, is the earliest and most benign form of NAFLD. Steatosis can also develop into more serious pathologies such as non-alcoholic steatohepatitis (NASH; steatosis with inflammation), cirrhosis, and eventually liver failure. It is unclear why some patients progress from steatosis to steatohepatitis while others do not. However, the current consensus is that hepatic steatosis sensitizes the liver to a variety of potential metabolic injuries such as oxidative stress and cytokines which promote inflammation [176, 177].

Steatosis develops in *Pemt*$^{-/-}$ mice fed a HF diet. Similar to hepatic CTα deficiency, the lack of PEMT limits the availability of hepatic PC, which impairs VLDL secretion and causes hepatic TG accumulation [107, 146]. PEMT deficiency has also been associated with the development of NASH. This has been observed in 2 models of diet induced NAFLD: choline deficiency and HF diet.

When *Pemt*$^{-/-}$mice were fed a diet deficient in choline, the animals developed severe liver failure and died within 5 days [145, 178]. In addition to 50% reduction in hepatic PC, histological analysis of *Pemt*$^{-/-}$ mice showed increased hepatic TG levels accompanied by severe inflammation. The causal

relationship between decreased hepatic PC levels and NASH was established when *Pemt*$^{-/-}$ mice were bred with mice lacking MDR2 (mouse ortholog of human MDR3) [178]. When fed the choline deficiency diet, *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$ mice were able to prevent the loss of hepatic PC through bile secretion and impair the drop in hepatic PC levels. As such, *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$mice did not develop steatohepatitis or liver failure and lived for at least 90 days on the choline deficient diet.

Li *et al* proposed that the development of steatohepatitis in *Pemt*$^{-/-}$ mice fed the choline deficient diet was initiated by decreased hepatic PC which resulted in the loss of plasma membrane integrity [179]. The authors showed that the integrity of the plasma membrane was dependent on the ratio of PC to PE (PC/PE). As such, a drastic drop in hepatic PC and PC/PE increased the permeability of the plasma membrane. This may potentially allow cytokines to freely pass into the hepatocytes; thereby initiating the development of steatohepatitis. *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$ mice were able to maintain a hepatic PC/PE of approximately 1.4 when fed the choline deficient diet for 21 days while PC/PE was decreased to 0.8 in *Pemt*$^{-/-}$ mice after only 3 days of choline deficient diet. Unfortunately, inflammatory markers were not measured in *Pemt*$^{-/-}$/*Mdr2*$^{-/-}$ mice or *Pemt*$^{-/-}$ mice fed the choline deficient diet to confirm the correlation between inflammation and PC/PE.

In addition, *Pemt*$^{-/-}$ mice developed severe steatosis and plasma alanine aminotransferase levels (a marker of liver damage) were increased when challenged with a HF diet for 10 weeks [161]. The development of liver damage coincided with decreased hepatic PC levels and a low PC/PE. When *Pemt*$^{-/-}$ mice were placed on the HF supplemented diet with additional choline (4 g choline/kg diet *vs.* 1.3g choline/kg diet in HF diet) for 10 weeks, hepatic PC levels and PC/PE were normalized, which prevented liver damage but not steatosis [161].

PEMT deficiency has also been associated with the development of steatohepatitis in humans. A single nucleotide polymorphism of *PEMT* (V175M) which results in partial loss of activity occurred more frequently (1.7 fold) in humans with NAFLD than in healthy individuals [180]. In addition, reduced hepatic PC and PC/PE ratio (1.2 *vs.* 2.5 in normal controls) were also measured in patients clinically diagnosed with NASH [179, 181]. Therefore, reduced PEMT activity may be associated with the progression of NAFLD.

d) PEMT, obesity, and insulin resistance

Pemt$^{-/-}$ mice fed a HF diet for 10 weeks are protected from obesity and insulin resistance. When compared to *Pemt*$^{+/+}$ mice, *Pemt*$^{-/-}$ gained less weight, had increased energy expenditure, and maintained normal peripheral insulin sensitivity [161]. However, LCTα$^{-/-}$ mice fed the same HF diet became obese and developed similar glucose intolerance as the control animals. Furthermore, *Pemt*$^{-/-}$ mice gained weight and developed insulin resistance when additional choline (but not betaine) was supplemented to the HF diet. Therefore, the authors concluded that choline deficiency in *Pemt*$^{-/-}$ mice, rather than impaired PC biosynthesis, was responsible for the beneficial physiological effects.

In conclusion, inhibition of PEMT may provide protection against atherosclerosis and diet-induced obesity and insulin resistance. However, the effect of PEMT deficiency on NAFLD needs to be addressed before PEMT can be considered a useful pharmacological target.

1.6 PC synthesis and proliferation

1.6.1 Partial hepatectomy and liver regeneration

The liver is unique in that it has the ability to replace lost or damaged tissue by regeneration [182]. Unlike true regeneration (e.g. regrowth of a limb including skin, bone, muscle), liver regeneration is characterized by

compensatory growth where the remaining liver expands in mass to compensate for lost tissue [182]. This process is not dependent on hepatic progenitor cells; instead all existing liver cell types, including hepatocytes, kupffer cells, stellate cells, endothelial cells, and epithelial cells, undergo cell division to replenish liver mass [182, 183]. And in comparison to normal tissue repair, liver regeneration is not associated with necrosis or massive inflammation, which results in fibrosis (e.g. scar tissue) [184, 185]. Thus, liver regeneration is often used as an *in vivo* model of cellular proliferation.

Injury, ischemia, and hepatectomy can stimulate normally quiescent liver cells into the regenerative process through a series of well-orchestrated and distinct steps of initiation/priming, cell-cycle progression, proliferation, growth and termination [186]. Priming denotes the initial stage of regeneration during which quiescent hepatocytes re-enter the cell cycle (G_0-G1) [187]. In the commonly used mouse liver regeneration model of 70% partial hepatectomy (PH), the priming phase occurs during the initial 4 h of regeneration [187] (Figure 1.7). Hepatocytes enter S-phase at 32 h, with the peak of DNA synthesis occurring 36-40 h after PH [188]. This is followed by the proliferation of the remaining types of hepatic cells [189]. It is estimated that the hepatocytes will undergo 1-2 rounds of cell division within the first 2-3 days after surgery. Within 7-10 days, liver mass is completely restored.

The essential signaling circuitry regulating liver regeneration can be encompassed into 3 categories: *cytokines* which are responsible for hepatocytes priming; *growth factors* which are required for cell cycle progression; and the *metabolic pathways,* which links liver function and proliferation [182].

a) Cytokines

The 2 major cytokines responsible for the initiation of liver regeneration are TNFα and IL-6 (Figure 1.8). Expression of TNFα and IL-6 levels are significantly upregulated after PH and elevated serum levels are detected within 30 min after surgery [190-192]. The importance of TNFα and IL-6 in the regenerative process was also corroborated in studies where TNFα and IL-6 signalling were impaired.

Cressman *et al* reported impaired DNA synthesis in IL-6 deficienct mice after PH, which resulted in delayed liver regrowth [193]. These mice also displayed increased mortality rate as result of hepatic necrosis and liver failure after surgery. Likewise, mice injected with a TNFα antibody prior to PH and TNF receptor 1-deficient mice also displayed delayed DNA synthesis and increased mortality after PH [191, 194, 195]. In a revealing study, impaired liver regeneration in TNF receptor 1-deficient mice was rescued by a pre-operative injection of IL-6 [195]. This finding suggested that the major role of TNFα in liver regeneration is to up-regulate IL-6, which in turn initiates the regenerative process [184].

Current evidence proposes the activation of the cytokine network is initiated by the binding of TNFα to TNFα type 1 receptor on kupffer cells [182, 189]. This leads to the activation of transcriptional factor NF-kB (nuclear factor κ light chain enhancer of activated B-cells), which has been shown to stimulate the expression of IL-6 and TNFα [195, 196]. The secretion of IL-6 and TNFα from kupffer cells 1) further stimulates the inflammatory response in kupffer cells, and 2) facilitates the binding of IL-6 to its receptor on hepatocytes which then activates signal transducer and activator of transcription (STAT)3 [182, 189]. STAT3 is an oncogenic transcription factor that activates the transcription of genes important for proliferation and hepatocyte survival [197]. Furthermore, a target gene of STAT3 is suppressor of cytokine signaling (SOCS)3, which inhibits

STAT3 activity by dephosphorylation [198-200]. As such, STAT3 activation of SOCS3 initiates a feedback loop to prevent the continuous activation of the IL-6/STAT3 signalling pathway [201]. Consistent with the important role of STAT3 in liver regeneration, mice with liver specific deletion of STAT3 experienced a high rate of mortality after PH, although the restoration of hepatic mass was only marginally impaired in surviving mice [202]. In addition, mice lacking SOCS3 showed prolonged STAT3 activation, enhanced hepatocyte proliferation, and accelerated liver weight gain after PH [203].

b) Growth factors

Growth factors regulate the passage of cells from G1 to S phase of the cell cycle; thus, growth factors are important for the progression of liver regeneration [204]. Numerous growth factors have been identified to be essential for this process. Several ligands of epidermal growth factor (EGF) receptor [205], including EGF, transforming growth factor α [206], amphiregulin [207], and heparin binding EGF-like growth factor [208] are upregulated upon PH. Consistent with this observation, phosphorylation (activation) of EGF receptor was observed 60 min after PH in rats [209]. More recently, inhibition of EGF receptor *via* RNA interference and genetic deletion in mice confirmed the critical role of the signaling pathway in proliferation of hepatocytes after PH [210, 211].

Another key mitogen upregulated following PH is hepatocyte growth factor (HGF) [212, 213]. After PH, HGF is cleaved from the extracellular matrix and binds to its receptor, c-Met, on the surface of hepatocytes [204]. Activation of the c-Met signaling pathway stimulates the expression of immediate early genes and DNA synthesis. Correspondingly, conditional knockdown of HGF or c-MET expression inhibited the entry of hepatocytes into S-phase from G1, which led to delayed liver regeneration and increased mortality after PH [214]. This

was also observed in mice with hepatic deletion of c-Met [215, 216]. After PH, these animals displayed a significant delay in cell cycle entry and increased mortality due to liver necrosis and jaundice.

HGF is produced as an inactive single chain protein by non-parenchymal cells in the liver [217]. When inactive HGF is secreted, it binds to the extracellular matrix, which then serves as a potential HGF reservoir. Following PH, the remodeling by matrix metalloproteinases (MMPs) releases HGF from the extracellular matrix and HGF is cleaved to its 2-chain active form by urokinase-type plasminogen activator [218, 219]. The activation of HGF by MMPs following PH was demonstrated in mice lacking tissue inhibitor of MPP (TIMP)-1[220]. Loss of TIMP1 led to increased MMP activity, elevated active HGF concentration, and accelerated cell proliferation following PH. Conversely, when TIMP1 was overexpressed, PH-stimulated liver regeneration was impaired [220].

c) Metabolic pathways

The modulation of the multiple metabolic pathways in the remaining hepatocytes after PH is integral to the successful initiation and completion of liver regeneration[187, 221]. Marked changes occur immediately after PH to ensure a sufficient supply of energy substrates to support not only the demands of rapid proliferation but also essential liver functions. Resection of 70% of the liver mass reduces the major repository of readily available glucose (hepatic glycogen) [222, 223]. As a result, plasma glucose levels fall rapidly after PH, eliciting a starvation-like response. In order to maintain glucose homeostasis, the remaining hepatocytes increase gluconeogenesis and shift from predominant utilization of carbohydrates (glycolysis) to increased utilization of lipids (β-oxidation). Indeed, it has long been recognized that the regenerating liver transiently accumulates intracellular triglyceride (TG) 12-48 h after PH [224]. Furthermore, the chemical and genetic inhibition of hepatic TG accumulation

during PH-stimulated liver regeneration has led to detrimental results [225-227]. The mechanisms underlying this accumulation of TG involve increased lipolysis in the adipose and elevated hepatic uptake of fatty acids [222].

The availability of amino acids has also been shown to regulate the process of regeneration after PH. Rats fed a protein-free diet exhibited impaired cell cycle progression after PH in comparison to rats fed a normal diet [228, 229]. Conversely, mixed amino acid administration or a high protein diet has been shown to increase hepatocyte mitotic activity [230, 231]. The mammalian target of rapamycin (mTOR) is part of a complex which regulates protein translation and cell growth in response to nutrient availability [232]. Therefore, it was unsurprising that the activity of p70 S6 kinase and 4E-BP1, both downstream effectors of mTOR, are modulated to increase protein translation after PH [233, 234]. In addition, the deletion of S6 protein, another effector of mTOR, led to near complete loss of DNA replication after PH [235]. Thus, the initiation of protein translation is a critical control point that integrates nutrient and energy levels with cell proliferative signals.

In summary, several cell cycle checkpoints exist during the regenerative process to ensure division does not continue under inappropriate conditions. Progression through the G_0-G1 transition, and late G1 restriction point are dependent on the presence of appropriate cytokines, growth factors, and metabolites [183, 187]. If the requirements are not met, cells can escape from the cell cycle and revert back to quiescent state. Inability of the remaining hepatocytes to proliferate can lead to severe liver failure and death in the recipient of the PH [221]. However, if all the conditions are met, cell division continues until the original liver mass is restored.

1.6.2 Regulation of PC biosynthesis during cellular proliferation

PC is a crucial component of the plasma membrane and its catabolism generates second messengers (i.e. diacyglycerol, phosphatidic acid, arachidonic acid) which have been implicated in regulation of cell division [236, 237]. As such, the importance of the level of PC and its metabolism during cell proliferation has been extensively studied. Using BAC1 macrophages, Jackowski showed that the metabolism of PC is coordinated with the cell cycle [238]. The G1 phase of the cell cycle is characterized by a high rate of PC degradation and synthesis, a phenomenon related to PC's role in cell signaling. However, PC turnover decreases during S phase, mostly as result of decreased PC degradation. These changes result in the accumulation of cellular PC, most likely in preparation for mitosis.

In cell culture conditions where CDP-choline pathway was impaired (i.e. inactive CT mutant or choline deficient media), proliferation is halted at G1 phase of the cell cycle [239-241]. However, with re-introduction of choline, PC or lyso-PC to the medium, progression into S phase can be restored. CTα gene expression is also enhanced during S phase [242]. This is mediated by the binding of phosphorylated Sp1 to the CTα promoter [243]. Thus, up-regulation of CT activity is required for progression from G1 to S phase of the cell cycle.

Unlike CT activity, PEMT is inversely correlated with rapid proliferation and differentiation [244]. In rodent prenatal livers, PEMT mRNA, protein, and activity are very low [244-246]. Only at birth, when proliferation of fetal hepatocytes decline and differentiation occurs, does PEMT activity and mRNA peak and reach steady levels [128]. PEMT mRNA during adipocyte differentiation follows a similar pattern. In pre-adipocytes, PEMT mRNA was not detectable. However, levels of PEMT mRNA increase steadily following differentiation and peak by day 4.

The CDP-choline and the PEMT pathway were also found to be reciprocally regulated during normal liver regeneration in rats [247]. After PH, CTα mRNA, protein and activity were markedly increased, while PEMT gene expression, protein, and activity were reduced. Since Sp1 activity impaired PEMT activity and stimulated CTα gene expression, Cole *et al* proposed that the increased phosphorylation of Sp1 during growth [248] may co-ordinate the regulation of the 2 pathways of PC biosynthesis [128].

1.7 Thesis Objectives

PC is essential for a variety of physiological functions. In the liver, PC synthesized from both the CDP-choline pathway and the PEMT pathway contribute to hepatic lipid metabolism and lipoprotein secretion. Inhibition of either pathway of PC production in the liver, as demonstrated by LCTα$^{-/-}$ and *Pemt*$^{-/-}$ mice, results in NAFLD. PC is also a major component of cellular membranes. In combination with the role of PC role in generating lipid second messengers, PC metabolism has been implicated in the regulation of cell division.

Thesis objective #1:

Hepatic PC biosynthesis was shown to be regulated during liver regeneration after major hepatectomy in the rat [247, 249]. The induction of CDP-choline pathway was also demonstrated to be important for cell growth in various non-hepatic cell lines [238, 241] . Our first objective was to determine the importance of hepatic CTα during cell proliferation *in vivo*. We evaluated the role of CTα in hepatic cell growth by subjecting LCTα$^{-/-}$ mice and wildtype control mice to 70% partial hepatectomy. We monitored liver regeneration by measuring DNA synthesis and the reconstitution of hepatic mass up to 10 days following surgery. We also determined the effect of hepatic CTα deletion on hepatic lipid levels and lipid synthesis during liver regeneration.

Thesis objectives #2:

NAFLD is linked to obesity and diabetes, which are increasingly prevalent in Western populations [250, 251]. The presence of NAFLD is also positively associated with increased post-operative complications and mortality after liver resection and liver transplantation in humans and rodent models [252-256]. As such, it is now common practice to assess NAFLD before major hepatectomy [257]. Decreased hepatic PC content and reduced PC/PE have previously been linked to NAFLD [179, 223]. Our second objective was to investigate the use of pre-operative hepatic PC/PE as an alternative predictor of survival after PH. We also determine whether improving hepatic PC/PE may be a viable therapy to improve post-hepatectomy outcomes in patients with NAFLD. Using 2 mouse models of deficient PC biosynthesis (LCTα$^{-/-}$ and *Pemt*$^{-/-}$ mice), we first evaluated the correlation between hepatic PC/PE and the degree of NAFLD, which was induced by HF feeding. We then compared the correlation of pre-operative hepatic PC/PE to survival rate after 70% partial hepatectomy in NAFLD animals with the standard method of evaluating NAFLD (histopathological analysis). We also assessed choline supplementation as a possible therapy to improve survival rate after liver resection in *Pemt*$^{-/-}$ mice which have developed NAFLD.

Thesis objectives #3:

Primary rodent hepatocytes and hepatoma cell lines are commonly used as model systems to elucidate and study potential drug targets for metabolic diseases such as NAFLD and atherosclerosis . However, if therapies are to be developed, it is essential that our knowledge gained from these systems is translatable to that of the human. Our third objective is to characterize lipid and lipoprotein metabolism in primary human hepatocytes. We examined cell viability and lipoprotein secretion of primary human hepatocytes over 3 days in

culture. We also evaluated phospholipid metabolism in primary human hepatocytes and its relation to lipoprotein secretion.

Table 1.1 Properties of human plasma lipoproteins

The major classes of lipoproteins found in human plasma are characterized by density, size and composition. CE, cholesteryl ester; Chol, cholesterol; HDL, high density lipoprotein; PL, phospholipid; TG, triacylglycerol; VLDL, very low density lipoprotein; LDL, low density lipoprotein. Adapted from Kritchevsky, D., Atherosclerosis and nutrition, Nutr. Int., 2 (1968) 290-297 and Garret and Grisham, Biochemistry, Saunders College Pub (2002).

Table 1.1

			Composition (wt %)				
Lipoprotein	**Density (g/mL)**	**Diameter (nm)**	**Protein**	**PL**	**Chol**	**CE**	**TG**
Chylomicrons	<1.006	100-1000	2	9	1	3	85
VLDL	0.95-1.006	30-80	10	18	7	12	50
LDL	1.006-1.063	18-28	23	20	8	37	10
HDL	1.063-1.210	5-15	55	24	2	15	4

Figure 1.1. Phosphatidylcholine: the molecule

The amphipathic structure of 1-palmitoyl-2-arachidonyl-phosphatidylcholine is shown. Typical fatty acyl-chains at the *sn*-1 and *sn*-2 positions and the choline head group are indicated.

Figure 1.1

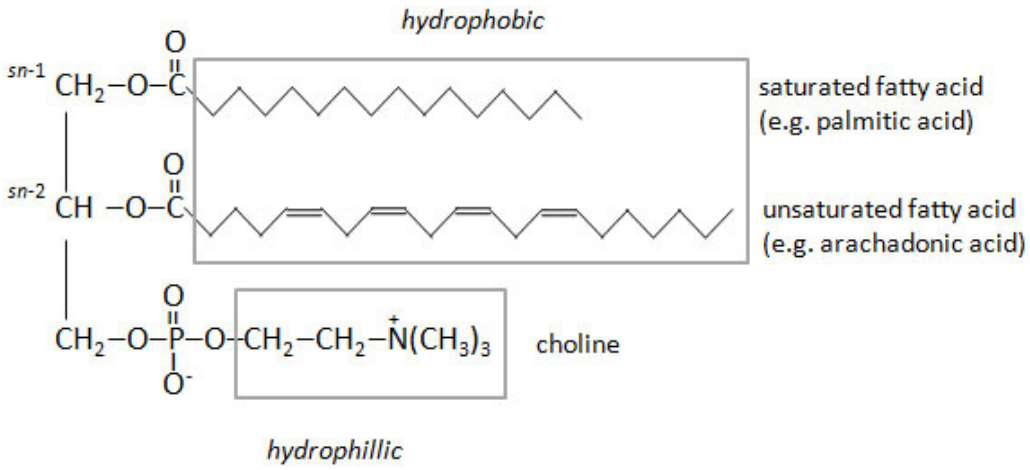

hydrophobic
sn-1
CH2–O–C
O
sn-2
CH –O–C
O
saturated fatty acid
(e.g. palmitic acid)
unsaturated fatty acid
(e.g. arachadonic acid)
CH2–O–P–O–CH2–CH2–N(CH3)3
O-
choline
hydrophillic

Figure 1.2 Signalling molecules derived from phosphatidylcholine

Phosphatidylcholine (PC) in the plasma membrane is hydrolyzed by different phospholipases to generate second messengers. Phospholipase A_2breaks the acyl ester bond at the *sn-2* position of PC to generate lyso-PC and arachidonic acid. Phospholipase C catalyzes the production of phosphocholine and diacylglycerol. Choline and phosphatidic acid are released following the hydrolysis of PC by phospholipase D. And sphingomylinase synthase catalyzes the production of sphingomylin and diacylglycerol *via* the transfer of the phosphocholine head group from PC to ceramide.

Figure 1.2

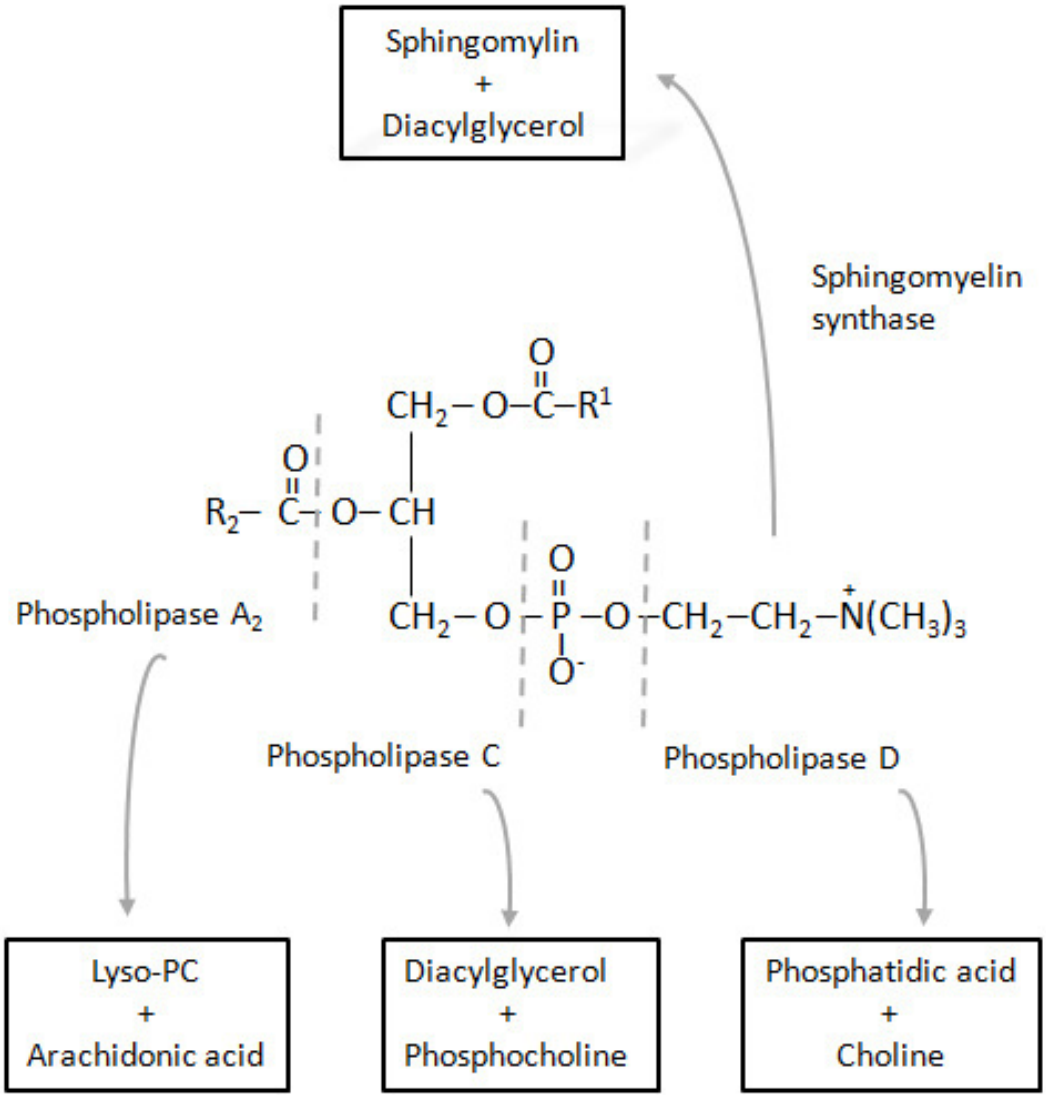

Sphingomylin
+
Diacylglycerol
Sphingomyelin
synthase
Phospholipase A2
Phospholipase C
Phospholipase D
Lyso-PC
+
Arachidonic acid
Diacylglycerol
+
Phosphocholine
Phosphatidic acid
+
Choline

Figure 1.3 The 2 pathways of phosphatidylcholine biosynthesis

The *de novo* synthesis of hepatic phosphatidylcholine (PC) can occur by 2 pathways. The CDP-choline pathway produces PC using choline as the initial substrate. The PEMT pathway generates PC *via* 3 sequential methylation of PE, using *S*-adenosylmethione (AdoMet) as the methyl donor. Other abbreviations include: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AdoHcy, *S*-adenosylhomocystine; CTP, cytidine triphosphate; CDP, cytidinediphosphate; CMP, cytidine monophosphate; DG, diacylglycerol; PDME, phosphodimethylethanolamine; PE, phosphatidylethanolamine; Pi, phosphate; PMME,phosphomonomethylethanolamine.

Figure 1.3

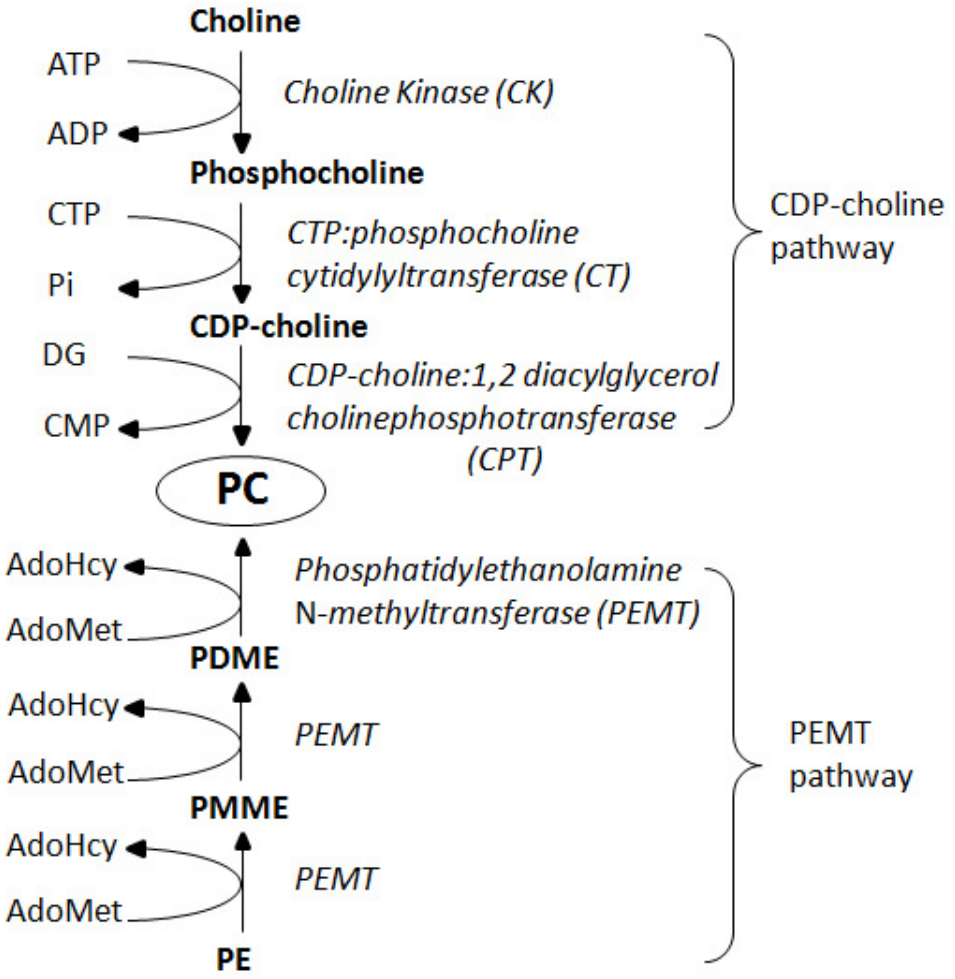

Choline
ATP
ADP
Choline Kinase (CK)
Phosphocholine
CTP
Pi
CTP:phosphocholine cytidylyltransferase (CT)
CDP-choline
DG
CMP
CDP-choline:1,2 diacylglycerol cholinephosphotransferase (CPT)
CDP-choline pathway
PC
AdoHcy
AdoMet
Phosphatidylethanolamine N-methyltransferase (PEMT)
PDME
AdoHcy
AdoMet
PEMT
PMME
AdoHcy
AdoMet
PEMT
PE
PEMT pathway

Figure 1.4 The relationship between choline and methionine metabolism

Abbreviation of enzymes:

BADH:	betaine aldehyde dehydrogenase
BHMT:	betaine-homocysteine methyltransferase
CAT:	choline acetyl transferase
CHDH:	choline dehydrogenase
MAT:	methionine adenosyltransferase
PEMT:	phosphatidylethanolamine *N*-methyltransferase
PLD:	phospholipase D
SAH Hydrolase:	*S*-adenosylhomocystine hydrolase

Abbreviation of molecules:

adoHcy:	*S*-adenosylhomocystine
adoMet:	*S*-adenosylmethionine

Figure 1.4

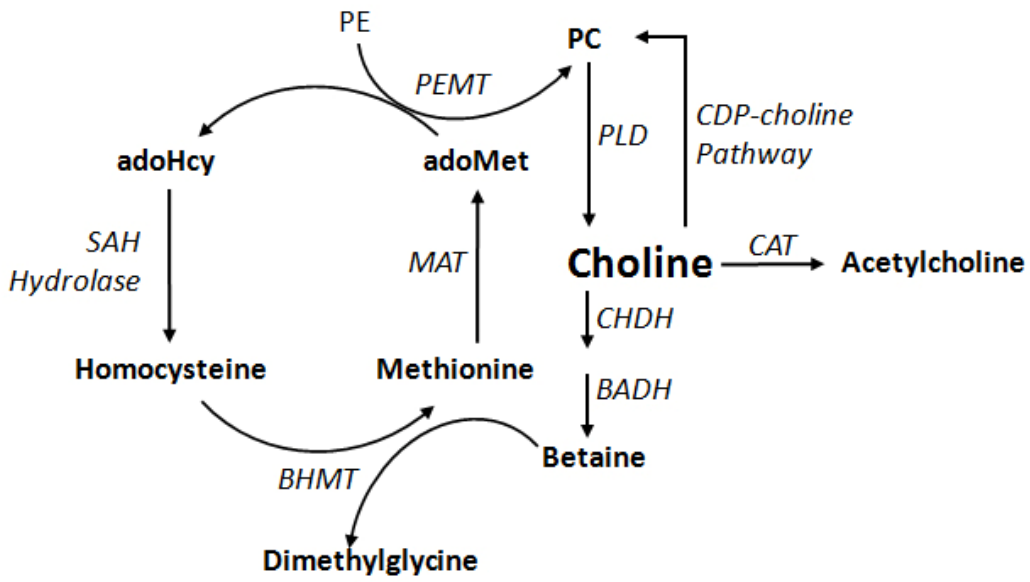

PE
PC
PEMT
PLD
CDP-choline
Pathway
adoHcy
adoMet
SAH
Hydrolase
MAT
Choline
CAT
Acetylcholine
CHDH
Homocysteine
Methionine
BADH
Betaine
BHMT
Dimethylglycine

Figure 1.5 The domain organization of human CTP:phosphocholinecytidylyltransferase α, β2, and β1

CTP:phosphocholine cytidylyltransferase(CT) α contains a N-terminal nuclear localization signal, a catalytical domain, a lipid binding domain, and a C-terminal phosphorylation domain. CTβ2 does not have a nuclear localization signal, but contains a catalytic domain, a lipid binding domain, and an abbreviated C-terminal phosphorylation domain. CTβ1 only has catalytic and lipid binding domains. Adapted from Vance and Vance, Biochemistry of lipids, lipoproteins, and membranes, Elsevier (2002).

Figure 1.5

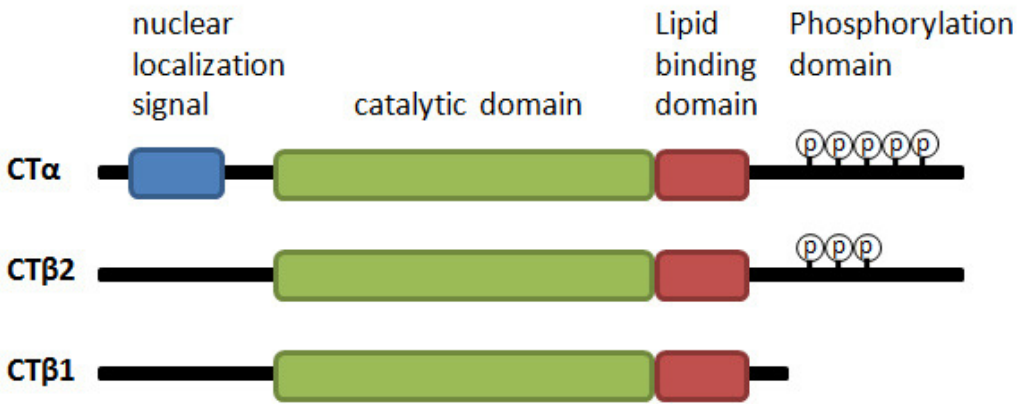

nuclear
localization
signal
catalytic domain
Lipid
binding
domain
Phosphorylation
domain
CTα
CTβ2
CTβ1
p
p
p
p
p
p
p
p
p

Figure 1.6 The topology of human phosphatidylethanolamine *N*-methyltransferase

Phosphatidylethanolamine *N*-methyltransferase (PEMT) contains 4 transmembrane domains which span the endoplasmic reticulum (ER) membrane such that both the N- and C-terminus face the cytosol. The positions of *S*-adenosylmethionine binding sites (GxG, EE) and ER retrieval motif (xxKxx) are indicated. Adapted from Shields *et al*., Biochim. Biophys Acta, (2001) 1532:105-114. and Shields *et al*., JBC, 278 (2003) 2956-2963.

Figure 1.6

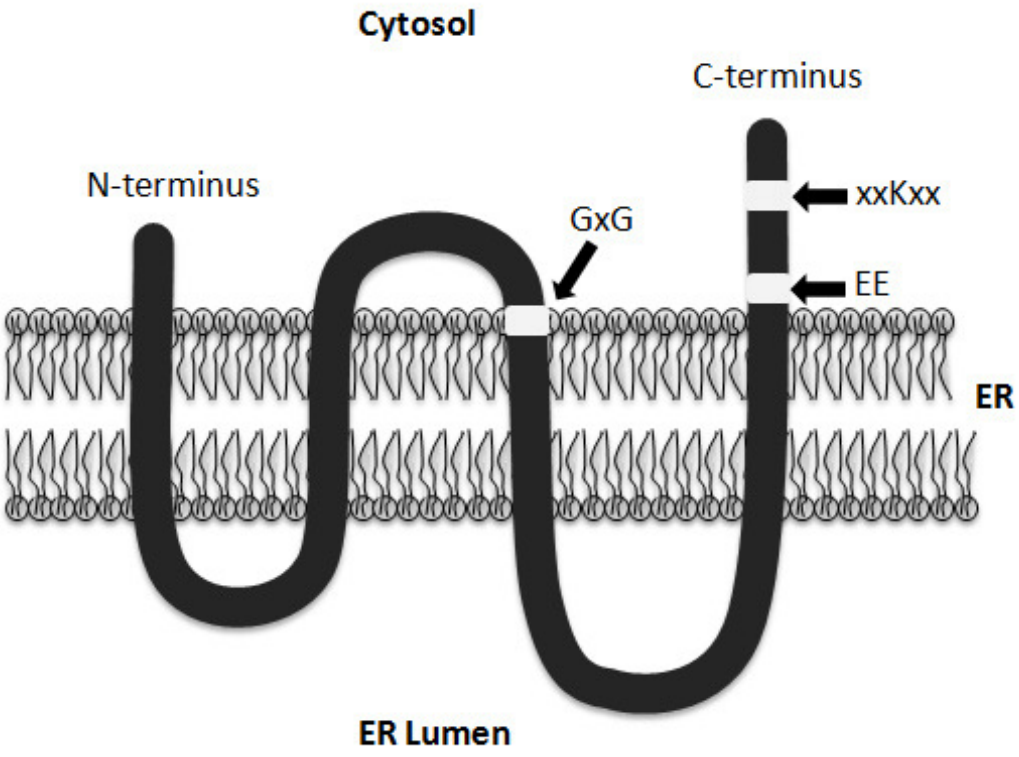


Figure 1.7 The initiation of liver regeneration after 70% partial hepatectomy in mice

Efficient liver regeneration involves the activation of cytokine, growth factor, and metabolic networks for a successful initiation and progression of cell growth. The transient activation of the 3 networks following 70% partial hepatectomy is depicted. Adapted from Fausto *et al*., Hepatology, 43 (2006) S45-S53.

Figure 1.7

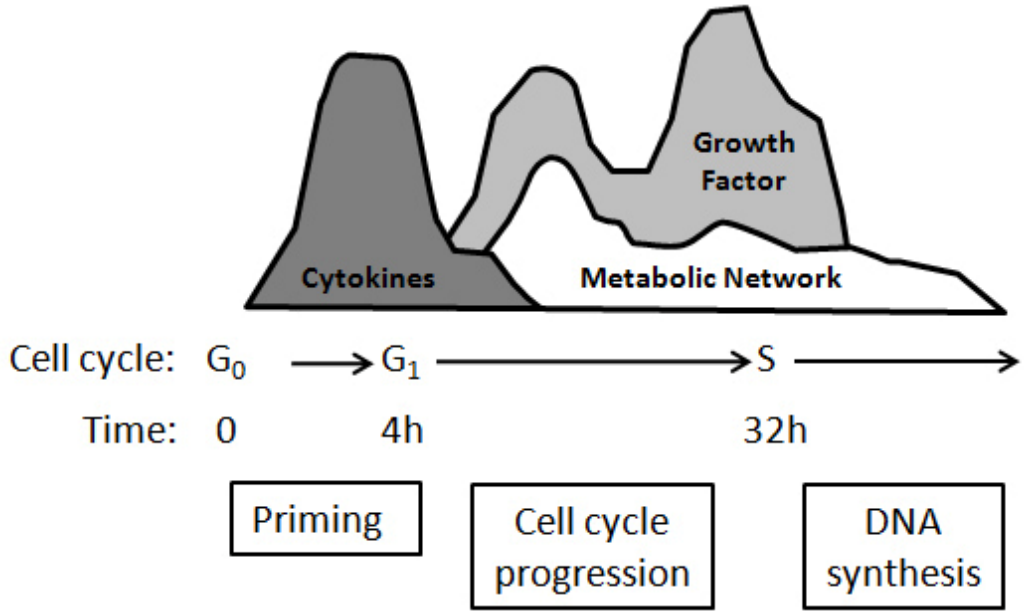
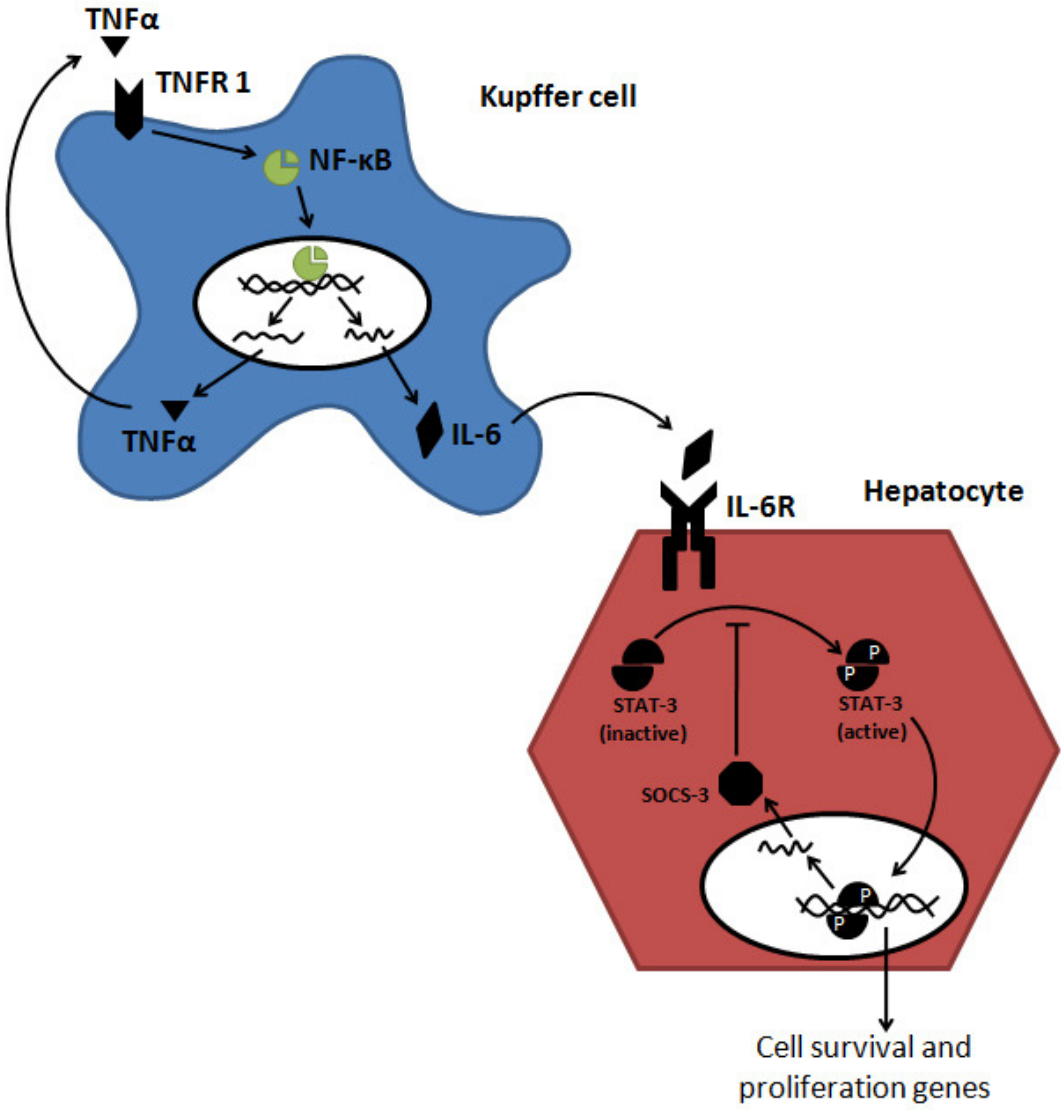

Growth Factor
Cytokines
Metabolic Network
Cell cycle: G0 → G1 → S →
Time: 0 4h 32h
Priming
Cell cycle progression
DNA synthesis

Figure 1.8 Activation of the cytokine network during liver regeneration

Hepatic injury stimulates the binding of tumor necrosis factor (TNF) α to its type 1 receptor (TNFR1) on kupffer cells. This leads to the activation of transcription factor NF-κB, which stimulates the production of interleukin (IL)-6 and TNFα. IL-6 is released into the serum and binds to its receptor (IL-6R) on hepatocytes. This leads to the phosphorylation and subsequent activation of signal transducer and activator of transcription (STAT)-3. Activated STAT-3 translocates to the nucleus where it induces the transcription of multiple genes involved in cell survival and proliferation. STAT3 also activates suppressor of cytokine signalling (SOCS)-3 which inhibits further activation of STAT3. NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells. Adapted from Fausto *et al*. Hepatology, 43 (2006) S45-S53.

Figure 1.8


TNFα
TNFR 1
Kupffer cell
NF-κB
TNFα
IL-6
IL-6R
Hepatocyte
STAT-3
(inactive)
STAT-3
(active)
SOCS-3
P
P
P
P
Cell survival and
proliferation genes

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CHAPTER 2: Impaired PC biosynthesis does not attenuate liver regeneration after 70% partial hepatectomy in hepatic CTP:phosphocholine cytidylyltransferase α-deficient mice

2.1 Introduction

PC is a major component of mammalian cellular membranes and the catabolism of PC generates second messengers (e.g. DG, phosphatidic acid, arachidonic acid), which have been implicated in the regulation of cell division [1, 2]. As such, the importance of PC and its metabolism during cell proliferation has been extensively studied. In cell culture conditions where PC biosynthesis is impaired (e.g. inactive CT mutant, choline deficient media), cell proliferation is halted at G1 phase of the cell cycle [3-5]. However, with re-introduction of choline, PC, or lyso-PC to the medium, progression into S phase is restored. Interestingly, PEMT cannot substitute for CT activity during the progression of the cell cycle. Overexpression of PEMT in cells containing an inactive CT mutant could not rescue the mutant cells from arrested cell division and apoptosis due to insufficient PC content [6, 7]. In addition, DNA synthesis following 70% liver resection in rats was associated with reduced PEMT mRNA, protein and activity [8]. In comparison, CT activity was enhanced. This suggests CDP-choline pathway plays a significant role in cell proliferation.

It has previously been shown that whole body deletion of CTα in mouse is lethal at embryonic day E3.5 [9]. However, mice in which the CTα gene is selectively disrupted in the liver (LCTα$^{-/-}$ mice) are viable and fertile [10]. Characterization of LCTα$^{-/-}$ mice show that the animals have an 85% reduction in total hepatic CT activity and 6-25% reduction of hepatic PC mass [10]. The remaining CT activity was attributed to the presence of CTβ2 in hepatocytes as well as CT activity in non-hepatic cells. The livers of LCTα$^{-/-}$ mice also contained fewer but larger hepatocytes, which suggest that hepatocytes lacking CTα may not be able to produce enough PC for normal cell division [10]. Thus, we used LCTα$^{-/-}$ mice to study the requirement of CTα in hepatic cell division *in vivo*.

To elucidate the relationship between CTα and hepatocyte proliferation, we performed 70% partial hepatectomy (PH) on LCTα$^{-/-}$ and LCTα-*floxed* (control) mice. Adult hepatocytes are normally in a quiescent state; however, injury (e.g. liver resection) can trigger the proliferation of the remaining hepatocytes [11]. Thus, partial hepatectomy is a good model to study hepatocyte growth *in vivo*.

To our surprise, liver regeneration and DNA synthesis after PH in LCTα$^{-/-}$ mice were normal in comparison to LCTα-*floxed* mice. Hepatic PC levels in LCTα$^{-/-}$ mice were also comparable to that of LCTα-*floxed* mice during liver regeneration. Similar to previous reports, PC synthesis (measured by the *de novo* synthesis of hepatic PC from [^{3}H]glycerol) in LCTα$^{-/-}$ mice was reduced by ~50% in comparison to control mice. However, total PC synthesis was not increased in LCTα-*floxed* or LCTα$^{-/-}$ mice after PH when compared to its respective sham-operated littermates. This may suggest that the basal rate of PC synthesis in hepatocytes can sustain regeneration after PH even when the level of PC synthesis is reduced by 50%. Alternatively, compensatory mechanisms may provide CTα$^{-/-}$ hepatocytes with the necessary PC for proliferation.

2.2 Method and materials

2.2.1 Materials

All chemicals reagents were from standard commercial sources. Primers were purchased from The Institute of Biomolecular Design at the University of Alberta. [2-^{3}H]glycerol (10 Ci/mmol) was purchased from MP Biomedicals (#27059H). PMME was purchased from Avanti Polar Lipids. *S*-adenosyl-[methyl-^{3}H] methionine was purchased from Amersham. Polymerase chain reaction (PCR) components for genotyping were all purchased from Invitrogen.

2.2.2 Animal care

All animal procedures were performed in accordance with the University of Alberta Animal Policy and Welfare Committee, which adheres to the principles for biomedical research involving animals developed by the council for International Organizations of Medical Sciences. LCT$\alpha^{-/-}$ mice were generated by Jacobs *et al* [10]. A mouse expressing the Cre recombinase gene driven by the liver specific albumin promoter (albumin-Cre) was crossed with a homozygous CTα_{flox} mouse. The resulting pups, which were heterozygous for the CTα_{flox} gene and the albumin-Cre gene, were bred with mice homozygous for CTα_{flox} mice. Mice identified as being homozygous for CTα_{flox} was used as control mice (ie. LCTα-*floxed*). Mice identified as being homozygous for CTα and heterozygous for Cre were used as LCT$\alpha^{-/-}$ mice. The albumin-Cre mouse was a gift from Dr. Mark A. Magnuson (Vanderbilt University). The CTα_{flox} mouse was generated in the lab of Dr. Ira Tabas [12]. In the CTα_{flox} mouse, *lox*P sites flank the 4th and 5th exons of the wildtype CTα gene, which encode a major portion of the CTα catalytic domain.

All animals were exposed to a 12 hour light-dark cycle, had free access to water, and fed *ad libitum* before and after surgery. All animals were fed a rodent chow diet containing 4.5% (w/w) fat, 39% carbohydrate, and 20% protein (LabDiet, #5053). In some experiments, the animals were switched to a high fat diet (BioServ, #F3282) containing 36% (w/w) fat, 36% (w/w) carbohydrate, and 21% (w/w) protein 2 weeks before surgery. These animals were also fed the high fat diet after surgery. All experiments were performed on male mice between the ages of 12-15 weeks. Animals were euthanized by cervical dislocation under anesthesia. In all cases, the mice were not fasted before euthanasia.

2.2.3 Surgical procedures

The animals were subjected to PH using a modified method of Higgins and Anderson [11] [13]. The left lateral, left median, and right median lobes of

the liver were separately isolated, tied off, and excised without disrupting the biliary tract and gallbladder (Figure 2.1). Sham operations consisting of laparotomy and manual manipulation of the liver were also performed. All surgeries were performed between 9am-12pm under isofluorane anesthesia by Dr. Lin-Fu Zhu (University of Alberta).

2.2.4 Histology & staining with hematoxylin and eosin

At time of euthanization, the liver was excised and a portion of the liver was fixed in 10% buffered formalin for at least 24 hours. The fixed livers were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) by the University of Alberta Advanced Microscopy Facility. Liver sections were visualized by a dissecting light microscope (40x objective magnification) and images were captured by the Nikon DXM 1200 camera (University of Alberta Advanced Microscopy Facility).

2.2.5 Calculation for mass of regenerating liver

Immediately after dissection of the liver, the wet liver weight was measured. Regeneration of hepatic mass after PH was calculated as the percentage of sham liver weight. Sham liver weight was obtained from mice of the same genotype that was euthanized at the same time as the animals subjected to hepatectomy.

2.2.6 Incorporation of 5-bromo-2'deoxyuridine

Two hours before sacrifice, LCTα-*floxed* and LCTα$^{-/-}$ mice were injected intraperitonealy with 50 mg/kg of 5-bromo-2'deoxyuridine (BrdU), a synthetic nucleoside that is the analogue of thymidine (Sigma, #B5002). The incorporation of BrdU into DNA was monitored by immunohistochemistry. Embedded sections, which were mounted on slides, were deparaffinized by incubating the slides in xylene for 3 min and rehydrated in decreasing concentrations of ethanol (e.g. slides were submerged in 95%, 75%, 50%, 25% ethanol for 5 min each).

Slides were then boiled in sodium citric acid buffer (10mM sodium citrate, 0.05% Tween, pH 6) for 15 min in the microwave. When the slides were cooled to room temperature, they were incubated in 2N HCl for 30 min at 37°C, and then rinsed 3 times in PBS (pH 7.4). Slides were blocked in 1% bovine serum albumin (BSA) in PBS (10mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH7.4) for 30 minutes and incubated for 2 hours in Alexa Fluor® 488 conjugated anti-BrdU antibody (1:40 dilution in 1% BSA, 0.3% Triton X-100 in PBS) (Molecular Probes #A21303). Slides were rinsed in PBS 3 times for 15 minutes each and fixed in ProLong (Molecular Probes #P7481). Slides were visualized with Leica DM IRE2 fluorescence microscope (Leica Microsystems) using excitation wavelength of 495 ηm. The number of positive BrdU hepatocytes per field was counted. The percent of hepatocytes undergoing DNA synthesis was calculated as the number of positive BrdU hepatocytes divided by the total number of hepatocytes per field (>700 cells). Each group contained 3-6 animals. More than 3 hepatic sections prepared from each animal were visualized.

2.2.7 Enzymatic assays

Mice were euthanized and the liver was excised and flash frozen in liquid N_2. Frozen liver tissue was homogenized with a glass/Teflon homogenizer in homogenizing buffer (50mM Tris-HCl, 150 mM NaCl, 1mM EDTA, 1mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) and then sonicated for 30 s. Protein concentration of the resulting liver homogenate was determined by Bradford assay (Bio-Rad) using BSA as standard according to manufacturer's instructions.

a) CT assay

Total CT activity was measured in liver homogenates by monitoring the conversion of [^{3}H]phosphocholine into CDP-choline [14]. The reaction was carried out at 37°C. 60 µL of liver homogenate (50 µg of protein in deionized

water) and 10 µL of PC:oleate (10 mM PC:oleate in 150 mM NaCl, 50 mM Tris-HCl, pH7.5, 1 mM EDTA, 0.025% NaN_2, 0.5 mM phenylmethylsulfonyl fluoride, 2mM DTT) was added to a 15 mL plastic tube and incubated in 37°C waterbath for 2 min before the start of the assay. The assay was initiated with the addition of 30 µL of the CT assay buffer to the tube and the reaction time of the assay was 20 min. The CT assay buffer contains 17.8 µL of buffer A (324 mM Tris-HCl, 225 mM NaCl, 10mM EDTA, 50 mM magnesium acetate, pH 7.5), 10 µL [^{3}H]phosphocholine (15 mM, 10 µCi/µmol), and 2.2 µL of 135 mM CTP (Sigma #C-1506). The reaction was stopped by the immersion of the assay tube into boiling water for 2 min. Samples were then centrifuged at 2000 rpm for 5 min. [^{3}H]CDP-choline was separated from the [^{3}H]phosphocholine by thin layer chromatography (TLC). 15 µL of the centrifuged reaction mixture and 5 µL of a standards cocktail (80 mg/mL phosphocholine and 15 mg/mL CDP-choline) was applied to plastic-backed silica gel 60 TLC plates and developed in methanol/0.6% NaCl/NH_4OH (10/10/0.9; v/v) for 45 min. Plates were sprayed with 0.1% 2,7-dichlorofluoroscein in methanol while still wet and the CDP-choline band was visualized under UV light (254 ηm). The band corresponding to CDP-choline was scraped into scintillation vials and 500 µL of water and 5 mL of scintillation fluid was added to each vial. The tubes were vortexed and radioactivity was counted the next day. Specific activity of the assay was measured by counting the radioactivity in 30 µl of CT assay buffer. Activity was calculated as:

= [(dpm/assay)*6.7] / [(specific activity)*(time)*(mg protein)]

b) PEMT activity assay

PEMT activity was measured in liver homogenate by following the production of PC from PMME and *S*-adenosyl-[methyl-^{3}H] methionine [15]. 68.7 µl of the sample (50 µg of protein in deionized water) and 51.3 µl of PEMT assay

buffer are mixed in a glassed capped tube. The PEMT assay buffer includes 15 µL of buffer (1.25 M Tris-HCl, pH 9.2), 15 µL of DTT (50 mM), 12 µL of PMME substrate mix (0.375 mg PMME in 20 mM Tris-HCl, pH 9.1, 0.01% EDTA (w/v), 0.002% Triton (w/v)), and 9.3 µL of 1% Triton). The tubes are placed in a 37°C waterbath and each reaction was started with the addition of 30 µL of *S*-adenosyl-[methyl-^{3}H] methionine (1 mM, 315 µCi/mmol). After 30 min, the reaction is stoped with the addition of 2 mL chloroform/methanol (2/1, v/v) and 2 mL 0.5% NaCl. The tubes are vortexed, centrifuged at 2000 rpm for 5 min, and the upper phase was discarded. The lower phase was then washed 3 times with 2 mL of wash buffer (0.5% NaCl/methanol/chloroform, 50/50/4, v/v). 200 µL of the lower phase was then pipette into scintillation vials, dry down under air, and solubilized with 5 mL of scintillation fluid. The scintillation vials were vortexed and the radioactivity was counted immediately. To determine the specific activity of *S*-adenosyl-[methyl-^{3}H] methionine, the radioactivity of 30 µL of *S*-adenosyl-[methyl-^{3}H] methionine was measured. Activity was calculated as:

= [(dpm/assay)*10] / [(specific activity)*(time)*(mg protein/assay)]

2.2.8 Analysis of phospholipids

Hepatic lipids were extracted from liver homogenate by method of Folch *et al* [16]. Briefly, samples (1 mg of protein) were suspended in 1 mL of PBS, to which 4 mL of chloroform/methanol (2/1; v/v) was added. The mixture was vortexed vigourously for 20 s and centrifuged for 10 min at 2000 rpm. The lower organic phase was extracted and dried down under nitrogen. Dried samples were re-suspended in 100 µL of chloroform/methanol (2/1; v/v) and resolved by TLC.

Phospholipids (e.g. PC and PE) were resolved by TLC in the solvent system chloroform/methanol/acetic acid/water (50/30/8/4; v/v) until the solvent front

was half way up a glass backed silicate coated TLC plate [17]. Only 80% of the sample dissolved in chloroform/methanol (2/1 v/v) was loaded onto the TLC plate. Bands corresponding to PC and PE loading standards (Avanti) were visualized by iodine vapor and scraped into glass tubes.

PC and PE were quantified by phosphorus assay [18] from scraped silica. Sodium phosphate (0-200 ηmol) was used as the standard. Standards were first boiled at 180°C until dry. When the standards were cooled to room temperature, 450 µL of 70% perchloric acid was added to each tube containing either unknown sample or standard. The tubes were then heated at 180°C for 60 min with a marble covering the top of each tube to prevent evaporation. After allowing the tubes to cool, the marbles were removed and 2.5 mL of deionized water was added to each tube. Next, 0.5 mL of of ammonium molybydate (2.5% w/v) and 0.5 mL of ascorbic acid (10% w/v) was added sequentially. The tubes were vortex immediately after each addition. The samples were then incubated in hot water (90-95°C) for 15 min to allow for colour development and centrifuged for 5 min at 2000 rpm to pellet the silica. Absorbance of the supernatent was read at 820 ηm. The concentration of PC or PE in each sample was calculated from the standard curve.

2.2.9 Analysis of neutral lipids

TG, cholesterol, and CE were quantified by gas-liquid chromatography. Liver homogenate (0.5 mg of protein in 0.2 mL) was digested in 2 mL of phospholipase C (2 units in 17.5 mM Tris, 10 mM CaCl2, pH 7.3) and 2 mL of diethyl ether for 2 h at 30°C. Tridecanoin (2ug) in 1 mL chloroform was then added as the internal standard. Lipids were extracted with 6 mL chloroform/methanol (2/1; v/v). The organic phase is passed through a plugged pasteur pipette containing anhydrous Na_2SO_4 and dried under N_2. Dried lipids were then dissolved in 100 µl of Sylon BFT (Supelco) to protect any free hydroxyl

and carboxy residues as its trimethylsilyl ether/ester derivatives. Sylon BFT contains N,O-bis (trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (99/1; v/v). The solvent was evaporated under N_2, the sample redissolved in 100 µl of hexane and quantified by gas-lipid chromatography (Agilent Technologies, 6890 series equipped with a flame ionization detector) [19]. Each sample was injected into an Agilent high performance capillary column (HF-5, 15m x 0.32 x 0.25 µm). The oven temperature was raised from°C 170 to 290°C at 20°C and then to 340°C at 10°C/min. Helium was used as a carrier gas (87 cm/s) with a constant flow rate of 4.5 mL/min.

2.2.10 *In vivo* incorporation of [^{3}H]glycerol into hepatic lipids

Mice were injected intraperitonealy with [^{3}H]glycerol (0.5µCi/g body weight) 34 hours after surgery. Mice were sacrificed 1 h after injection and the livers were dissected and snap frozen in liquid N_2. Lipids were extracted from 1 mg of liver homogenate according Folch *et al* [16]. Phospholipids and neutral lipids were separated by one dimensional 2 system TLC. Phospholipids were first separated by running the solvent chloroform/methanol/acetic acid/water (50/30/8/4; v/v) half way up the plate. The plates were allowed to dry briefly. Next, the neutral lipids were separated by running the plates in heptanes/diisopropyl ether/acetic acid (60/40/4; v/v) until the solvent system reaches the top. Lipids were visualized with iodine vapor and bands corresponding to PC, PE, and TG and scraped into scintillation vials containing 5 mL of scintillation fluid. Radioactivity was measured the next morning. Total radioactivity was counted from 10 µl of total liver homogenate.

2.2.11 Immunoblot analysis

Liver homogenates (40 µg protein) were heated for 10 min at 95°C in a modified Laemmli buffer (50mM Tris, 10% glycerol (v/v), 5% SDS (w/v), 1% 2-meraptoethanol (v/v), 0.02% bromophenol blue (w/v)). Samples were

electrophoresed on a 12 % SDS-polyacrylamide gel in 25 mM Tris-HCl, 192 mM glycine, and 0.1% SDS buffer and the proteins were transferred to polyvinylidine fluoride (PVDF) membranes by electroblotting in 25 mM Tris-HCl, 192 mM glycine, 10% (v/v) methanol for 2 hours at 100 V. Following the transfer, the membranes were then blocked for 1 hour in 5% skim milk in T-TBS (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) and incubated in rabbit anti-PCNA antibody (1:200 dilution with 5% skim milk in T-TBS; Abcam #2426-1) overnight at 4°C. Membranes were then washed 4x15 min in T-TBS. Next, membranes were incubated in goat anti-rabbit secondary antibody (1:2000 dilution in 5% skim milk in T-TBS). Again, membranes were washed in T-TBS for 4x15 min. Finally, proteins were detected using enhanced chemiluminescence system (Amersham Biosciences) according to the manufacturer's instructions.

2.2.12 Genotyping of LCTα$^{-/-}$ mice

DNA was isolated from mouse tail using the DNeasy Blood and Tissue Kit according to the manufacturer's instructions (Qiagen) and suspended in TE buffer (10mM Tris-HCl, pH 8, 1 mM EDTA). The primers used amplify the CT gene are: forward primer (5'-TCTTTGCTTGCATCAGAGCC-3') and reverse primer (5'-GAAGTAGGCACTGAACTTAGG-3'). The primers used to amplify the Cre gene are: forward (5'- CCCAAGAAGAAGAGGAAGGTGTCC-3') and reverse (5'-CCCAGAAATGCCAGATTACG-3').

Each PCR reaction contains genomic DNA (150 ηg in 5 µL sterile deionized water), Mg^{+2} free PCR buffer (2.5 µL of 10x solution), dNTPs (0.2 µL of 25 mM stock), Mg^{+2} (1.25 µL of 50 mM stock), forward primer (0.625 µL of 10 µM stock), reverse primer (0.625 µL of 10 µM stock), sterile deionized water (14.7 µL) and Platinum Taq (0.1 µL). The program used to amplify each gene was as follows:

1) Initial melt: 94°C for 2 min

2) denaturing step: 94°C for 1 min

3) annealing step: 62°C for 1 min

4) extention step: 72°C for 1 min

5) cycling: repeat steps 2 to 4 for another 22 cycles

7) holding step: 4°C for forever

The amplified products of the PCR was analyzed by agarose gel electrophoresis. The products were separated on a 1.5% agarose gel in TE buffer, at 100 V for 20 min. The estimated product of CT and Cre is 300 basepairs and 600 basepairs, respectively.

2.2.13 Statistical analysis

All data are represented as mean ± S.E. One or two way ANOVA was used in all comparisons. In all tests, $p<0.05$ was considered significant.

2.3 Results

2.3.1 Rate of liver regeneration after PH in LCTα-*floxed* mice

PH was performed in LCTα-*floxed* mice and regeneration of hepatic mass was monitored up to 10 days following surgery. We show in LCTα-*floxed* mice, 54% of original liver weight is regenerated 2 days after PH. And 85% of the original liver is regenerated 6 days after PH (Figure 2.2).

2.3.2 Liver regeneration is not decreased after PH in LCTα$^{-/-}$ mice

To elucidate the role of CTα in hepatic cell division *in vivo*, we monitored liver regeneration in LCTα$^{-/-}$ mice and LCTα-*floxed* littermates after PH. In mice, maximal rate of DNA synthesis occurs between 36 h and 44 h following PH [20, 21]. Therefore, we measured DNA synthesis 38 h after PH by *in vivo*

incorporation of BrdU into DNA and protein expression of proliferating nuclear cell antigen (PCNA). PCNA is a cofactor of DNA polymerase δ and its expression is enhanced during DNA synthesis [22]. Surprisingly, we did not observe any differences in DNA synthesis between LCTα$^{-/-}$ and LCTα-*floxed* mice after PH (Figure 2.3). Similarly, we did not observe any difference in liver weight between LCTα$^{-/-}$ and LCTα-*floxed* mice 38 h and 192 h (8 days) after PH. This suggests the lack of CTα does not impair hepatic cell division when proliferation is stimulated after liver resection.

2.3.3 Phospholipid levels are normal in LCTα$^{-/-}$ mice after PH

We next determine the consequence of hepatic CTα deletion on phospholipid content during liver regeneration. We measured hepatic PC and PE content after PH in LCTα-*floxed* and LCTα$^{-/-}$ mice. Coincident with normal liver regeneration, hepatic PC content in LCTα$^{-/-}$ mice was similar to LCTα-*floxed* mice after PH (Figure 2.4A). The level of PE (Figure 2.4B) and the molar ratio of PC to PE (Figure 2.4C) were also not changed after PH. This suggests LCTα$^{-/-}$ mice were able to maintain hepatic membrane phospholipid levels during regeneration despite the lack of hepatic CTα.

2.3.4 Hepatic TG level is increased in LCTα$^{-/-}$ mice after PH

Hepatic TG is known to accumulate transiently in the liver after PH to provide energy and building materials necessary during regeneration [23]. Immediately after PH, lipolysis is stimulated in adipocytes, releasing fatty acids into blood and increasing hepatic fatty acid uptake and TG synthesis in the liver [23, 24]. Likewise, we observed an accumulation of lipid droplets in LCTα-*floxed* and LCTα$^{-/-}$ livers sampled after PH (Figure 2.5). The lipid droplets were most abundant 38 h after PH and disappears 192 h after PH. Similarly, quantification of hepatic neutral lipids show TG levels increase following PH in LCTα-*floxed* and LCTα$^{-/-}$ mice (Figure 2.6A). Interestingly, there is a 3-fold increase in hepatic TG

in LCTα$^{-/-}$ mice in comparison to LCTα-*floxed* mice 38 h after PH. There was no difference in hepatic cholesterol ester (Figure 2.6B) or cholesterol (Figure 2.6C) between LCTα-*floxed* and LCTα$^{-/-}$ mice after sham or PH.

2.3.5 PC synthesis is not enhanced during liver regeneration in LCTα-floxed and LCTα$^{-/-}$ mice

In the liver, PC can also be synthesized *via* pathways regulated by PEMT and CTβ2 in addition to CTα regulated CDP-choline pathway. To determine if LCTα$^{-/-}$ mice maintained PC during liver regeneration *via* the enhancement of PEMT and/or CTβ2 activity, we measured the *in vitro* activity of total CT and PEMT after PH. We show the remaining CT activity (e.g. CT β2) is not increased after PH (*vs.* sham) in LCTα$^{-/-}$ mice (Figure 2.7A). Furthermore, PEMT activity in LCTα-*floxed* and LCTα$^{-/-}$ mice is decreased after PH in comparison to sham to similar degrees (Figure 2.7B). This suggests that CTβ2 and PEMT are not enhanced in LCTα$^{-/-}$ mice during liver regeneration to maintain hepatic PC concentration.

We also measured the *de novo* synthesis of hepatic phospholipids in LCTα-*floxed* and LCTα$^{-/-}$ mice by the incorporation of [^{3}H]glycerol into hepatic PC and PE after sham and PH. The uptake of [^{3}H]glycerol by the resected liver was increased in comparison to sham operated livers (Figure 2.8A); therefore we controlled for the increased cellular uptake by dividing [^{3}H]PC by total radiolabel in the liver. The incorporation of ^{3}H-glycerol into PE was not affected in LCTα-*floxed* and LCTα$^{-/-}$ mice after sham or PH (Figure 2.8B). Similar to previous reports, total PC production in LCTα$^{-/-}$ mice was reduced by 50% in comparison to control animal after sham (Figure 2.8C). Moreover, [^{3}H]glycerol incorporation into PC was unaffected 34 h after PH in LCTα$^{-/-}$ mice in comparison to sham. In corroboration with our *in vitro* data, this suggests the maintenance of hepatic PC

concentration in LCTα$^{-/-}$ mice during liver regeneration was not due to the enhancement of the remaining pathways of PC biosynthesis.

2.3.6 TG synthesis after PH in LCTα-floxed and LCTα$^{-/-}$ mice

To understand why there is a 3 fold increased in TG in LCTα$^{-/-}$ mice in comparison to LCTα-*floxed* mice, we measured the *de novo* synthesis of hepatic TG following PH *via* the incorporation of [^{3}H]glycerol into hepatic TG. The incorporation of [^{3}H]glycerol into hepatic TG after PH suggests that TG synthesis is similarly increased in LCTα-*floxed* and LCTα$^{-/-}$ mice in comparison to sham (Figure 2.8D). Therefore, the greater accumulation of TG in LCTα$^{-/-}$ mice was not due to higher rates of TG synthesis.

2.3.7 High fat diet decreases PC and increases TG levels in the livers of LCTα$^{-/-}$ mice

We have demonstrated that liver regeneration is not impaired in LCT*α*$^{-/-}$ mice after PH. This could be because hepatic PC content is maintained before and after PH despite a 50% reduction in total PC production. We wondered if liver regenerative process will still proceed normally in LCTα$^{-/-}$ mice if PC levels were reduced. To limit the availability of cellular PC, we placed LCTα$^{-/-}$ mice on a high fat (HF) diet for 2 weeks. After the HF challenge, LCTα$^{-/-}$ mice showed a significant decrease in hepatic PC content in comparison to LCTα-*floxed* mice (Figure 2.9A); however, hepatic PE content was not affected (Figure 2.9B). As such PC/PE was significantly decreased in comparison to control mice (Figure 2.9C). LCTα$^{-/-}$ mice also accumulated higher levels of TG in comparison to LCTα-*floxed* mice (Figure 2.9D). However, we did not observe a difference in liver size between LCTα$^{-/-}$ mice and LCT-floxed mice. The percent of liver *vs.* body weight in LCTα-*floxed* and LCTα$^{-/-}$ mice was 4.54 ±0.26 % *vs.* 5.01±0.54 %, respectively. There was also no change in hepatic CE (Figure 2.9E) or cholesterol (Figure 2.9F).

2.3.8 HF diet decreases survival but does not affect liver regeneration after PH in LCTα$^{-/-}$ mice

We have demonstrated that liver regeneration is not impaired in LCT$\alpha^{-/-}$ mice after PH and hepatic PC content is maintained before and after PH despite a 50% reduction in [^{3}H]glycerol incorporation into PC. To determine if the liver regenerative process will still proceed normally in a model of PC deficiency, we performed PH in LCTα$^{-/-}$ and control mice after a high fat (HF) challenge. LCTα$^{-/-}$ mice have previously been shown to develop reduced hepatic PC/PE ratio when fed a HF diet for 2 weeks. Therefore, we performed PH in LCTα$^{-/-}$and control mice after a 2 week HF challenge.

Post-surgery (48 h), hepatic PC level in LCTα$^{-/-}$ mice was still reduced when compared to LCTα-*floxed* mice (Figure 2.9A). However, liver regeneration as measured by liver weight was comparable between LCTα$^{-/-}$ mice and LCTα-*floxed* mice (Figure 2.10A). Interestingly, we did observed a 17% decrease in survival rate in HF-fed LCTα$^{-/-}$ mice *vs.* their control littermates 24 hours after surgery (Figure 2.10B). There was no change in PE level and PC/PE after PH in LCTα$^{-/-}$ mice when compared to sham operated mice of the same genotype. However, TG and CE levels in LCTα$^{-/-}$ mice did significantly increase after PH.

2.4 Discussion

2.4.1 Hepatic CTα is not essential for hepatocytes proliferation after PH

We hypothesized that the deletion of CTα would impair hepatocyte proliferation after PH because the formation of membrane PC during cell growth was shown to be dependent on CTα in cultured cells and during mouse embryogenesis. Furthermore, the livers of LCTα$^{-/-}$ mice also contained fewer but larger hepatocytes, which suggest that hepatocytes lacking CTα may have impaired cell division as result of decreased PC production. Surprisingly, we

found the LCTα$^{-/-}$ mice were able to undergo liver regeneration at a rate comparable to control mice which suggests CTα is not essential for hepatocyte proliferation. This is coincident with recent *in vivo* studies in which CTα was deleted specifically in lung [25] and macrophages [12]. Deletion of CTα in mice lung epithelial cells did not impair lung development during embryogenesis but did result in severe respiratory failure at birth [25]. The authors suggest pulmonary CTα is not essential for the proliferation and differentiation of lung epithelials but is required for synthesis and secretion of PC in lung surfactant. Similarly, macrophages deficient in CTα were viable and developed normally [12]. However, CTα-deficient macrophages were more susceptible to cholesterol-induced cell death than wildtype macrophages.

It is notable that PEMT activity was reduced after PH in comparison to sham in LCTα-*floxed* and LCTα$^{-/-}$ mice. The inverse relationship between the expression of PEMT and rapid cell proliferation has been shown previously in cell and animal models. This may indicate a yet uncharacterized role of PEMT in cell division [8, 26].

2.4.2 Why PEMT activity is not increased in LCTα$^{-/-}$ mice after sham operation

Despite an 85% decrease in hepatic CT activity, total PC biosynthesis was only decreased by 50% in LCTα$^{-/-}$ mice and total PC content was normal after sham operation. Previously, the up-regulation of PEMT was proposed to be responsible for maintaining hepatic PC levels in LCTα$^{-/-}$ mice [10]. In contrast, we did no observe an increase in PEMT activity in LCTα$^{-/-}$ mice after sham in comparison to wildtype control animals. The difference in data could be that we analyzed hepatic tissue from mice which continuously had access to food. In the contrasting study, the mice were fasted for 16 h before tissue collection. Corresponding to our analysis that the fed metabolic state may impair PEMT

activity, PEMT activity was increased by 53% in type I diabetic rats and administration of insulin was found to prevent the stimulation [27, 28].

2.4.3 Why hepatic *de novo* PC synthesis is not enhanced during liver regeneration in LCTα-*floxed* and LCTα$^{-/-}$ mice

From *in vitro* and *in vivo* assays, we determined hepatic *de novo* PC synthesis was not enhanced during liver regeneration under normal or CTα deficient conditions. Thus, we propose that PC synthesis is not up-regulated after PH. Quiescent hepatocytes appear to have enough capacity to maintain PC metabolism during liver regeneration. The liver can secrete up to 23 mg of PC a day into bile alone, an amount equal to the total PC present in the liver [29]. The liver is also able to obtain PC through the uptake of plasma lipoproteins (e.g. low density lipoproteins, high density lipoproteins) [30, 31]. Furthermore, reduced CT activity/PC synthesis impaired hepatocyte proliferation in cell culture but not *in vivo* may be due to the absence of circulating lipoproteins, which would have provided a source of extraneous PC for the cultured cells.

The increased accumulation of hepatic TG in LCTα$^{-/-}$ mice after PH also supports the hypothesis that LCTα$^{-/-}$ mice may be obtaining PC from circulating lipoproteins. Previous studies have shown that a fraction of the PC obtained from LDL and HDL is converted to TG through phospholipase C activity [30, 31]. Therefore, if LCTα$^{-/-}$ mice have increased uptake of PC associated LDL and/or HDL to compensate for decreased PC synthesis, LCTα$^{-/-}$ mice would also have a higher accumulation of hepatic TG. Alternatively, the remaining 25-30% CT activity provided by CTβ2 and PEMT activity in LCTα$^{-/-}$ mice could also explain why PC synthesis in LCTα$^{-/-}$ hepatocytes is not impaired during liver regeneration.

Previous studies in rats have suggested that the CDP-choline pathway was significantly upregulated after PH during S phase of the cell cycle [32]. However,

the authors, who measured the rate of PC synthesis *via* the incorporation of [^{3}H]choline to PC, failed to take into consideration that the resected livers have a higher rate of [^{3}H]choline uptake in comparison to sham operated livers [33]. Therefore, the increased PC synthesis reported by Houweling *et al* [32] may be a reflection of increased pools of [^{3}H]choline in the resected liver rather than increased PC synthesis from [^{3}H]choline.

2.4.4 Conclusion

In conclusion, hepatic CTα is not necessary for normal liver regeneration after PH. Basal PC synthesis appears to be sufficient to support liver regeneration after PH since we show that PC synthesis is not stimulated after PH in control mice. In fact, liver regeneration is still unimpaired even when total hepatic PC biosynthesis is reduced by 50% as in LCTα$^{-/-}$ mice. This suggests that PC can be obtained from other sources (e.g. lipoprotein uptake) or that the remaining PC biosynthetic enzymes are enough to maintain PC metabolism during liver regeneration. In addition, we show that attenuated hepatic PC content (before and after PH) does not impair regeneration of liver mass after PH.

Interestingly, decreased PC content in LCTα$^{-/-}$ mice as result of a HF diet increased mortality after PH. PC/PE and TG content were also altered in these mice and may play a factor. This will be investigated in the next chapter.

Figure 2.1 Diagram of a mouse liver

A mouse liver is comprised of the left lobe (35% w/w), the right and left median lobes (33%), the right lobe (26%), and the cauldate lobe (6%). In 70% partial hepatectomy, the resection of the left lobe, the right median lobe, and the left median lobe result in ~70% removal of total liver mass. Dashed lines represent the lines of surgical resection.

Figure 2.1

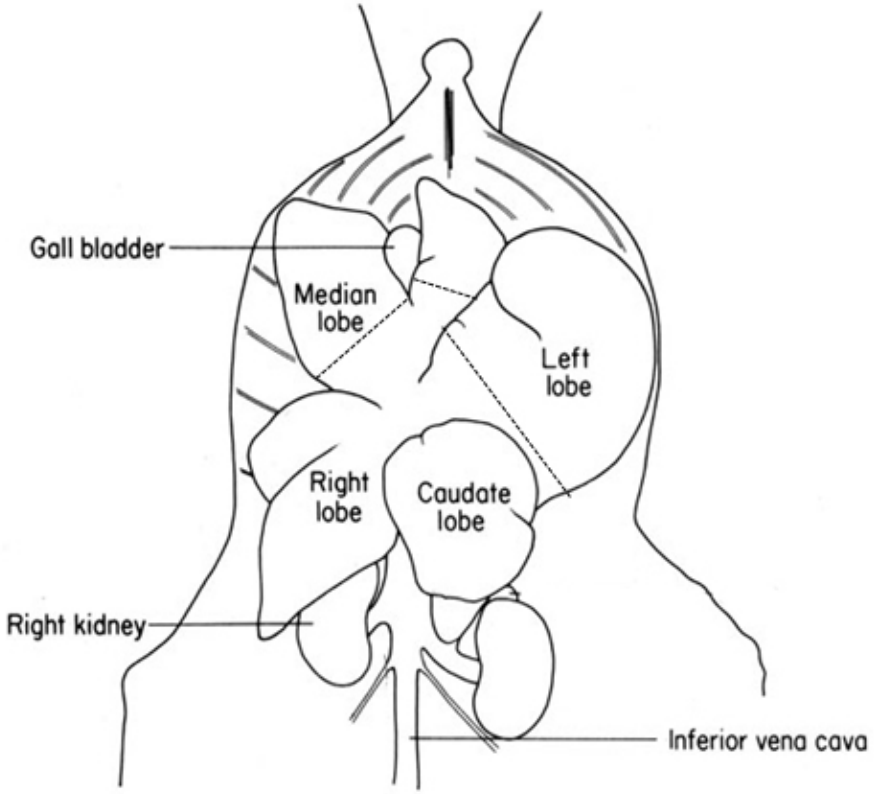

Gall bladder
Median lobe
Left lobe
Right lobe
Caudate lobe
Right kidney
Inferior vena cava

Figure 2.2 Timeline for liver regeneration after PH

LCTα-*floxed* mice were subjected to PH. Regeneration of liver mass was determined up to 10 days following surgery. Liver mass at each time point is expressed as percent of original liver weight. Original liver weight was measured in LCTα-*floxed* mice not subjected in any surgery. (n=2-4)

Figure 2.2

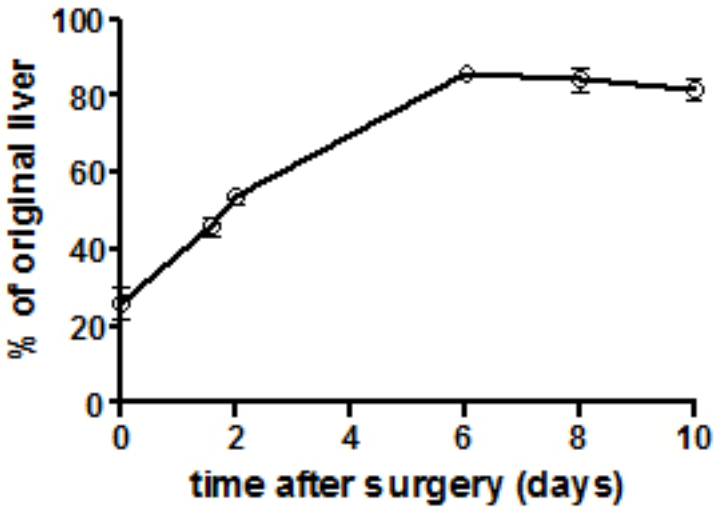

100
80
60
40
20
0
% of orlginal liver
0
2
4
6
8
10
time after surgery (days)

Figure 2.3 Liver regeneration after PH is not impaired in LCTα$^{-/-}$ mice.

(A) LCTα-*floxed* and LCTα$^{-/-}$ mice were subjected to either a sham operation or PH. Liver weight after hepatectomy was calculated as percent of sham liver weight and measured 38 and 192 h after surgery (n=5-13). Liver weight after sham operation is the pooled percent of original liver weight measured 38 and 192 h after surgery (n=10). **(B, C)** Rate of DNA synthesis was measured *via* the incorporation of BrdU into hepatocytes following sham, 38 h after PH, and 192 h after PH. (n=3-6) **(D)** Hepatic expression of PCNA was measured after sham and 38 h after PH (n=3). Values are mean ± S.E. # $p < 0.05$ *vs.* sham of the same genotype.

Figure. 2.3

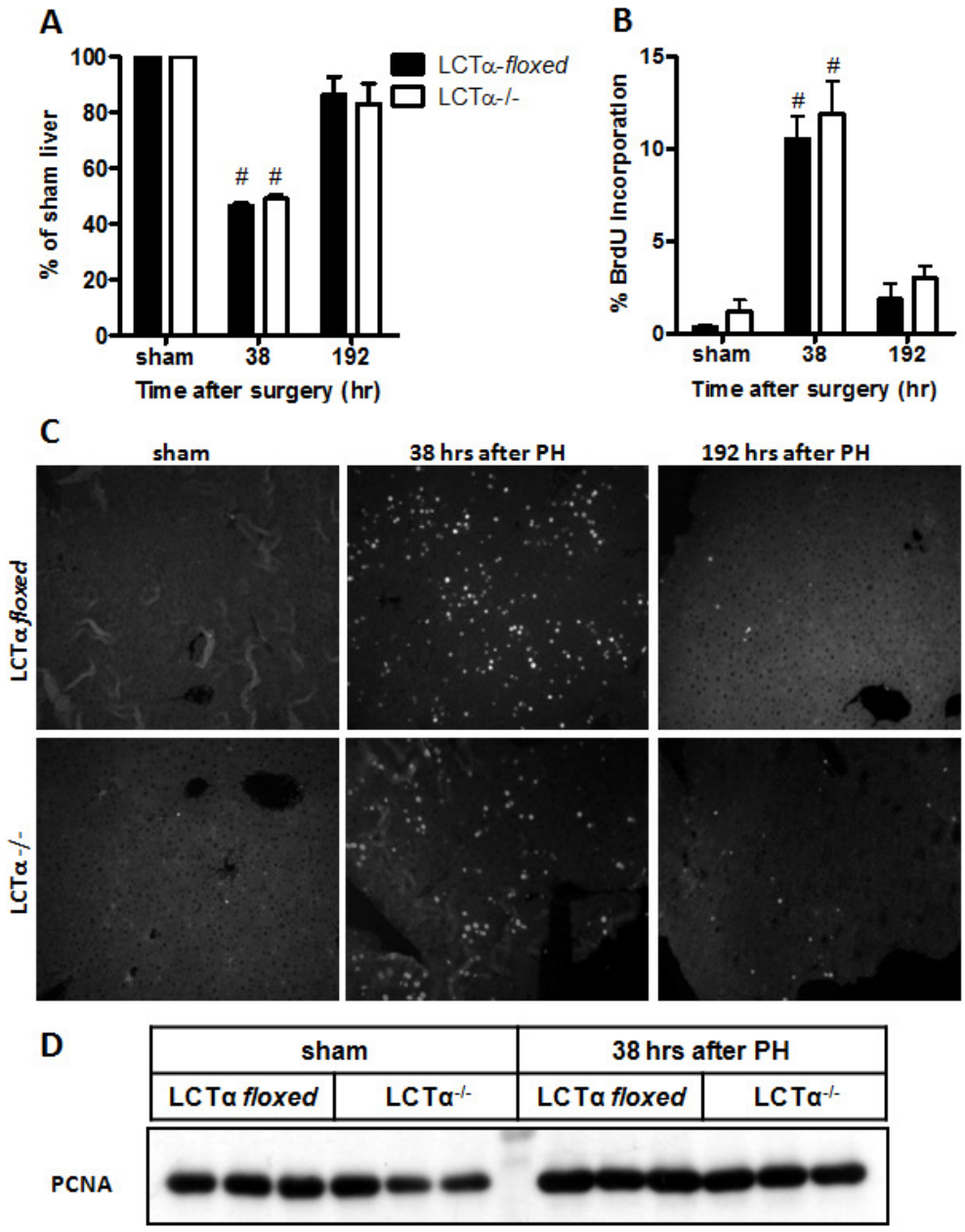

A
% of sham liver
100
80
60
40
20
0
LCTα-floxed
LCTα-/-
# #
sham
38
192
Time after surgery (hr)
B
% BrdU Incorporation
15
10
5
0
# #
sham
38
192
Time after surgery (hr)
C
sham
38 hrs after PH
192 hrs after PH
LCTα floxed
LCTα -/-
D
sham
38 hrs after PH
LCTα floxed
LCTα-/-
LCTα floxed
LCTα-/-
PCNA

Figure 2.4 Hepatic phospholipid content is maintained after 70% partial hepatectomy (PH) in LCTα$^{-/-}$ mice.

(A) Hepatic PC, **(B)** PE, and **(C)** PC/PE were measured in LCTα-*floxed* and LCTα$^{-/-}$ mice after sham, 38 h after PH, and 192 h after PH (n=3-8). Values are mean ± S.E. * p <0.05 *vs*. LCTα *floxed.* # p <0.05 *vs.* sham of the same genotype.

Figure 2.4

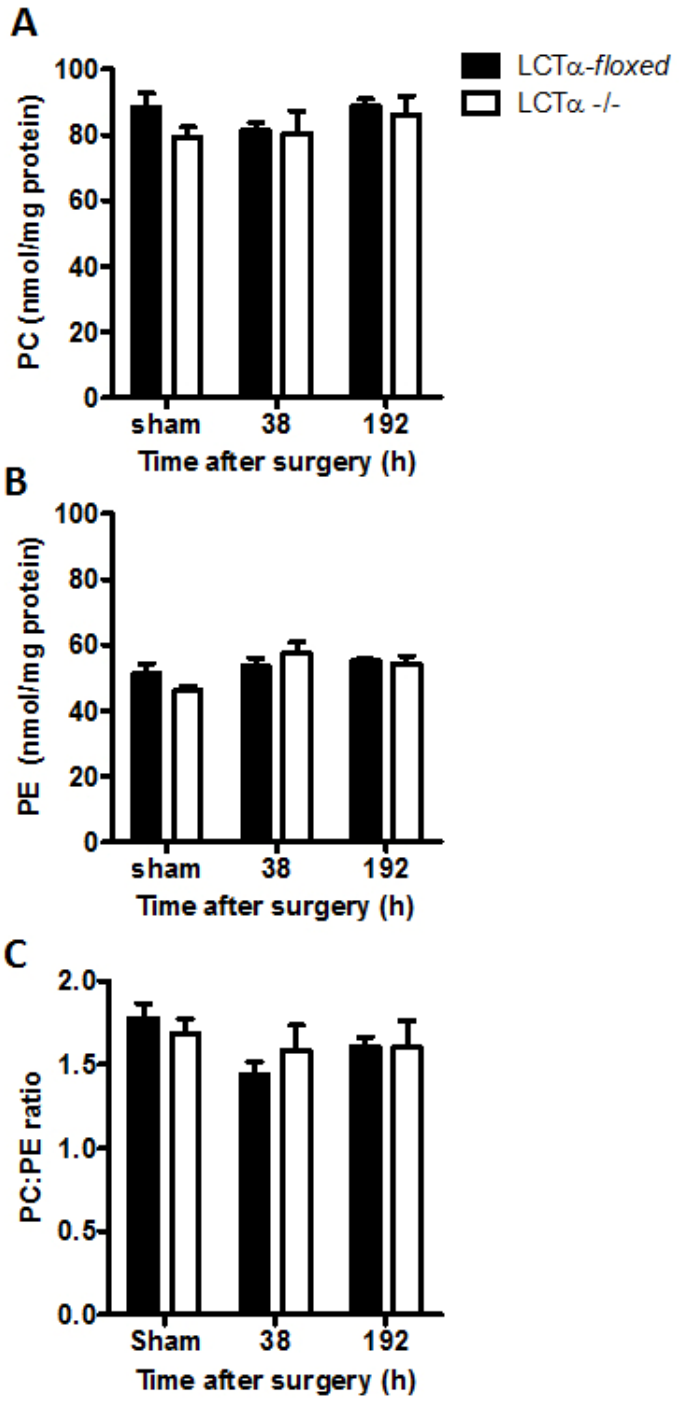

A
LCTα-floxed
LCTα -/-
PC (nmol/mg protein)
100
80
60
40
20
0
sham
38
192
Time after surgery (h)
B
PE (nmol/mg protein)
100
80
60
40
20
0
sham
38
192
Time after surgery (h)
C
PC:PE ratio
2.0
1.5
1.0
0.5
0.0
Sham
38
192
Time after surgery (h)

Figure 2.5 Histology of liver isolated from LCTα-*floxed* and LCTα$^{-/-}$ mice after sham or PH.

Liver sections isolated from LCTα-*floxed* and LCTα$^{-/-}$ mice after sham, 38 h after PH, and 192 h after PH were fixed in 10% buffered formalin and stained with H&E. Slides were visualized with a dissecting light microscope and images were captured with the Nikon DXM 1200 camera (University of Alberta Advanced Microscopy Facility).

Figure 2.5

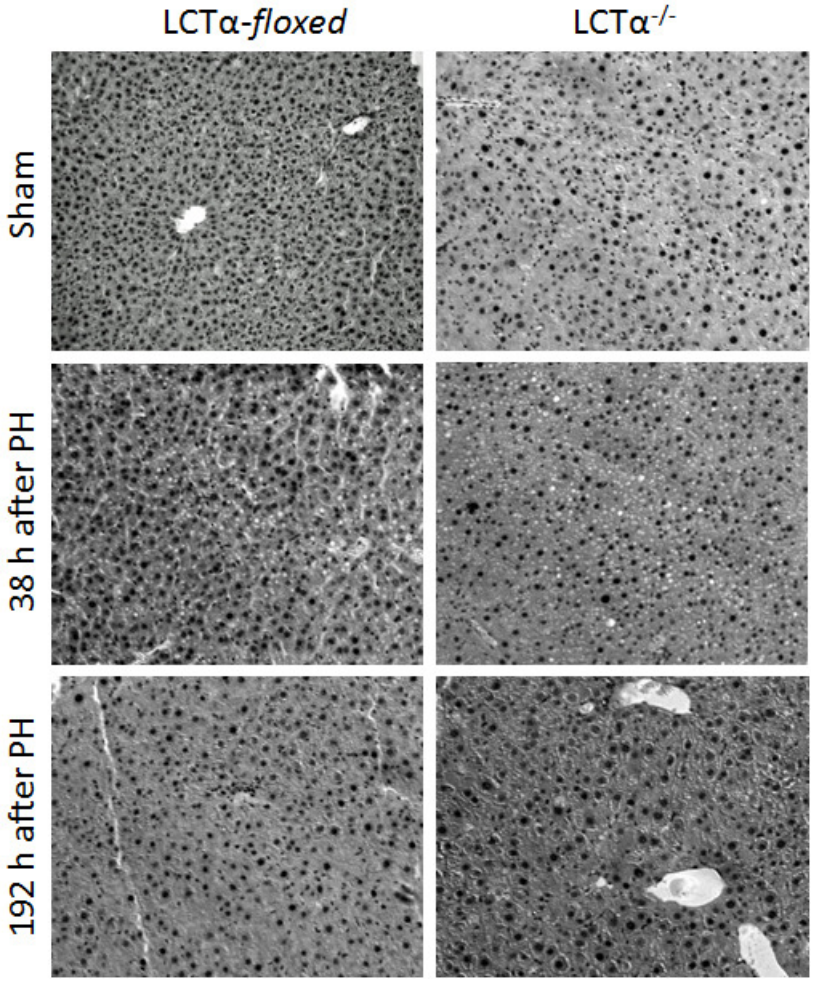


Figure 2.6 Hepatic neutral lipid content in LCTα-*floxed* and LCTα$^{-/-}$ mice after PH

(A) Hepatic TG, **(B)** cholesteryl ester, and **(C)** cholesterol were measured in LCTα-*floxed* and LCTα$^{-/-}$ mice after sham, 38 h or 192 h after PH (n=3-8). Values are mean ± S.E. * p <0.05 *vs*. LCTα *floxed.* # p <0.05 *vs.* sham of the same genotype.

Figure 2.6

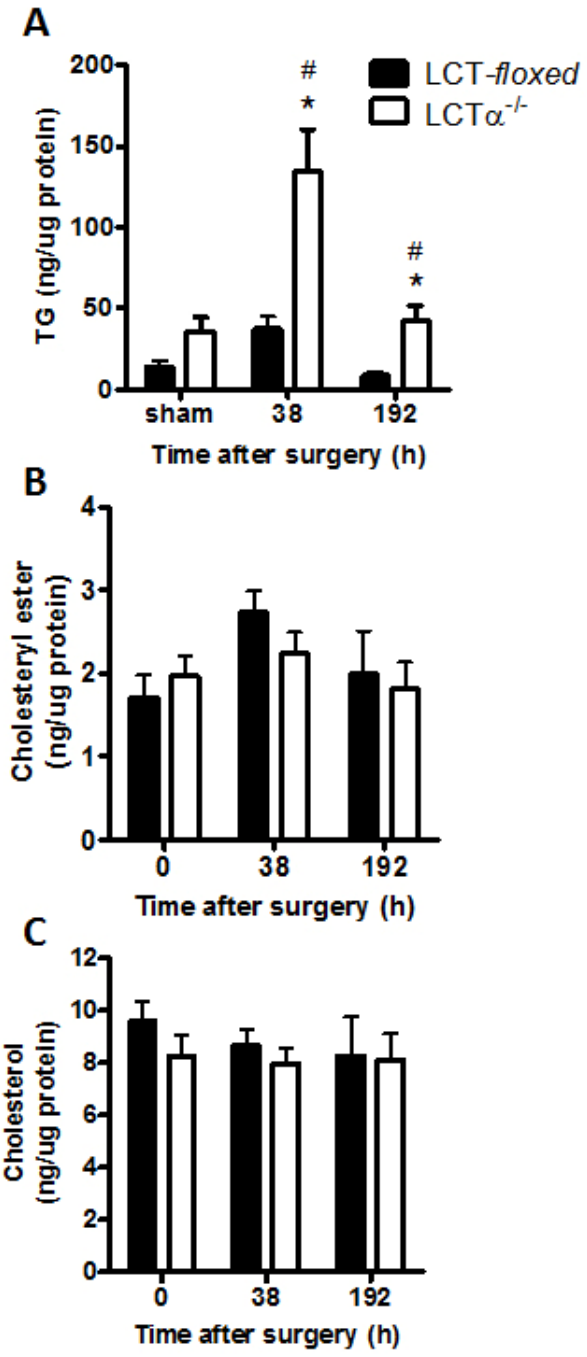

A
200
150
100
50
0
TG (ng/ug protein)
#
*
#
*
LCT-floxed
LCTα-/-
sham
38
192
Time after surgery (h)
B
4
3
2
1
0
Cholesteryl ester (ng/ug protein)
0
38
192
Time after surgery (h)
C
12
10
8
6
4
2
0
Cholesterol (ng/ug protein)
0
38
192
Time after surgery (h)

Figure 2.7 CT and PEMT activity in the liver of LCTα-*floxed* and LCTα$^{-/-}$ mice after sham or PH

(A) *In vitro* activities of CT and **(B)** PEMT were measured in total liver homogenate isolated from LCTα-*floxed* and LCTα$^{-/-}$ mice after sham, 38 h after PH, and 192 h after PH. (n=3-8). Values are mean ± S.E. * p <0.05 *vs*. LCTα *floxed.* # p <0.05 *vs.* sham of the same genotype.

Figure 2.7

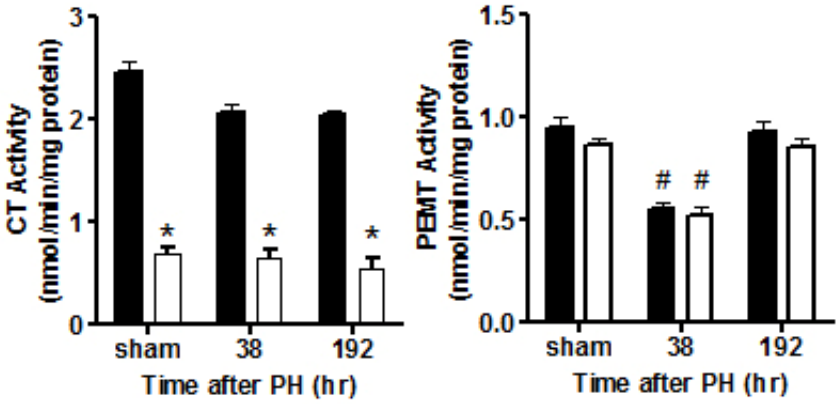

CT Activity
(nmol/min/mg protein)
3
2
1
0
*
*
*
sham
38
192
Time after PH (hr)
PEMT Activity
(nmol/min/mg protein)
1.5
1.0
0.5
0.0
# #
sham
38
192
Time after PH (hr)

Figure 2.8 *De novo* synthesis of hepatic lipids after sham or PH in LCTα-*floxed* and LCTα$^{-/-}$ mice.

(A) [^{3}H]glycerol (0.5 µCi/g body weight) was injected into animals 34 h after sham or PH. The animals were sacrificed 1 h after injection. Total radioactivity was counted in 10 µl of liver homogenate (n=5-6). Total incorporation of [^{3}H]glycerol into hepatic **(B)** PE, **(C)** (PC) and **(D)** TG was measured and expressed as % [^{3}H]PE, % [^{3}H]PC, and % [^{3}H]TG *vs.* total hepatic radioactivity (n=5-6). Values are mean ± S.E. * p <0.05 *vs*. LCTα *floxed.* # p <0.05 *vs.* sham of the same genotype.

Figure 2.8

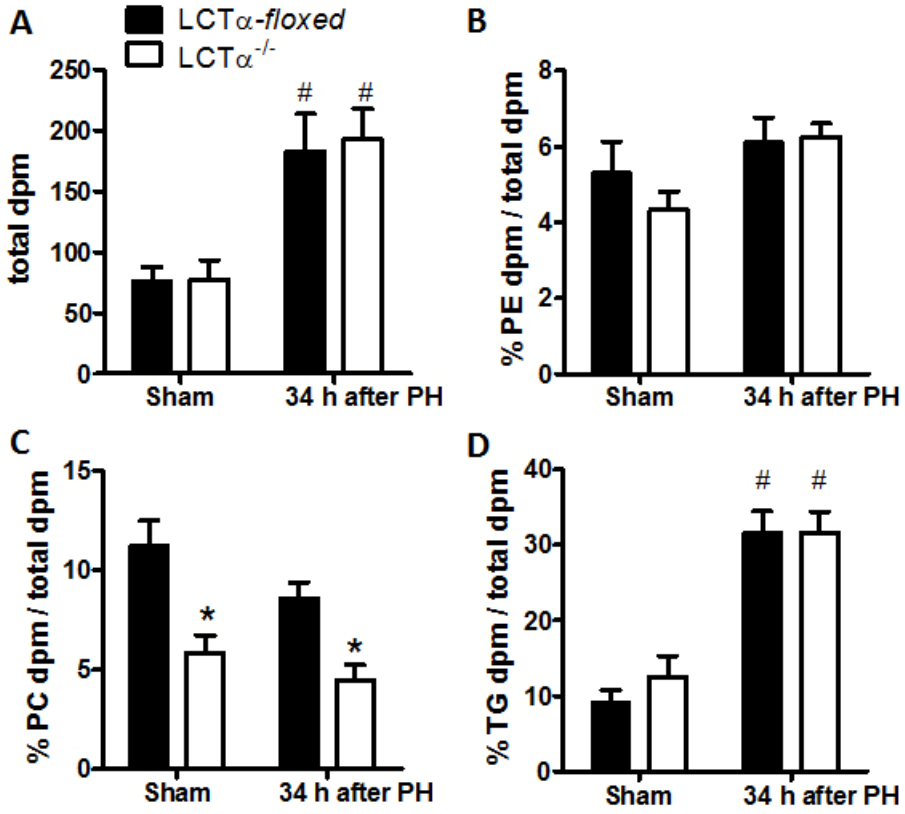

A
LCTα-floxed
LCTα-/-
total dpm
250
200
150
100
50
0
#
#
Sham
34 h after PH
B
% PE dpm / total dpm
8
6
4
2
0
Sham
34 h after PH
C
% PC dpm / total dpm
15
10
5
0
*
*
Sham
34 h after PH
D
% TG dpm / total dpm
40
30
20
10
0
#
#
Sham
34 h after PH

Figure 2.9 Hepatic lipid content after sham or PH in LCTα-*floxed* and LCTα$^{-/-}$ mice which have been fed a HF diet.

LCTα-*floxed* and LCTα$^{-/-}$ mice were fed a HF diet for 2 weeks before sham or PH was performed. **(A)** Hepatic PC, **(B)** PE, and **(C)** PC/PE ratio were measured after sham operation or 48 h after PH. Neutral lipids such as **(D)** TG, **(E)** CE, **(F)** were also measured by gas-liquid chromatography. * $p < 0.05$ *vs*. LCTα *floxed.* # $p < 0.05$ *vs.* sham of the same genotype.

Figure 2.9

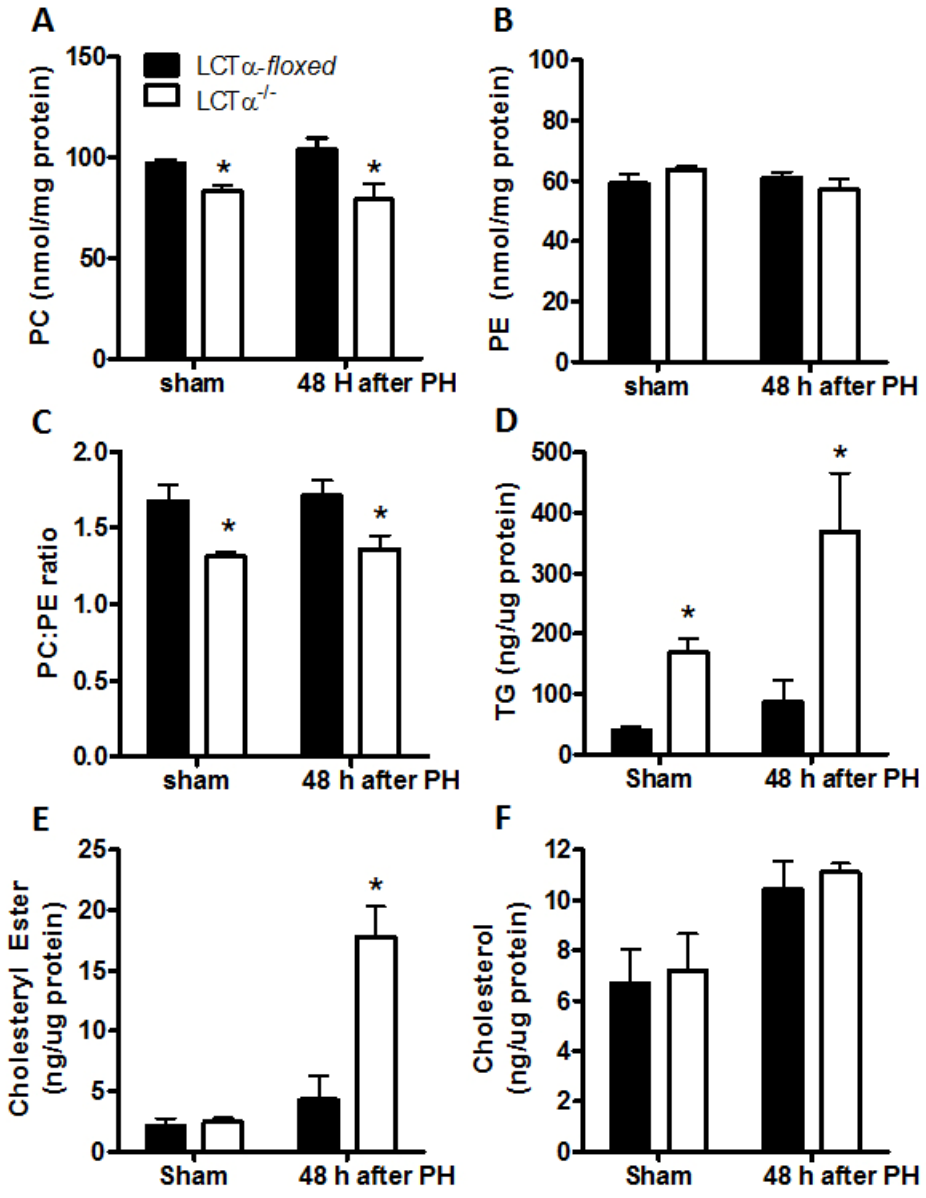
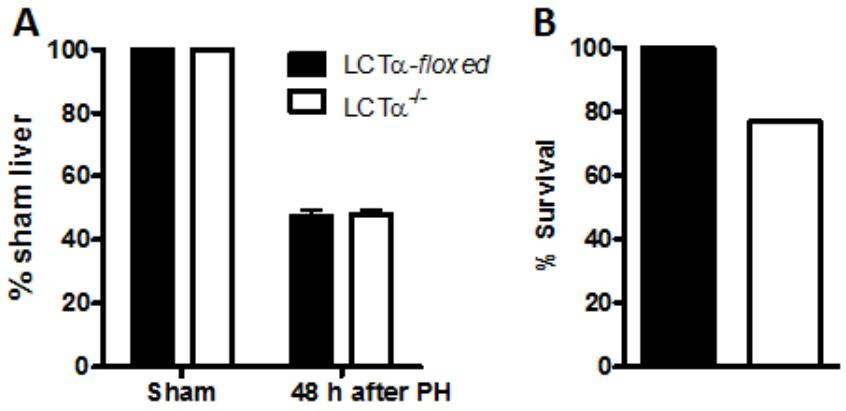

A
LCTα-floxed
LCTα-/-
PC (nmol/mg protein)
150
100
50
0
*
*
sham
48 H after PH
B
PE (nmol/mg protein)
100
80
60
40
20
0
sham
48 h after PH
C
PC:PE ratio
2.0
1.5
1.0
0.5
0.0
*
*
sham
48 h after PH
D
TG (ng/ug protein)
500
400
300
200
100
0
*
*
Sham
48 h after PH
E
Cholesteryl Ester (ng/ug protein)
25
20
15
10
5
0
*
Sham
48 h after PH
F
Cholesterol (ng/ug protein)
12
10
8
6
4
2
0
Sham
48 h after PH

Figure 2.10 Liver regeneration and survival after sham or PH in LCTα-*floxed* and LCTα$^{-/-}$ mice which have been fed a HF diet

(A) Regeneration of hepatic mass in LCTα-*floxed* and LCTα$^{-/-}$ mice which had been fed a HF diet prior to PH. Liver weight after sham and PH is calculated as percent of sham liver weight isolated 48 h after surgery. **(B)** Percent survival was measured 48 h after PH in LCTα *floxed* and LCTα$^{-/-}$ mice. (n=3-8) Values are mean ± S.E. *p<0.05 *vs*. LCTα-*floxed.* # p <0.05 *vs.* sham of the same genotype.

Figure 2.10


A
100
80
60
40
20
0
% sham liver
LCTα-floxed
LCTα-/-
Sham
48 h after PH
B
100
80
60
40
20
0
% Survival

2.5 References

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CHAPTER 3: Hepatic ratio of phosphatidylcholine to phosphatidylethanolamine predicts survival after partial hepatectomy in mice

3.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) consists of a wide spectrum of hepatic pathologies [1]. The disease can progress from steatosis (triacylglycerol (TG) accumulation) to non-alcoholic steatohepatitis (NASH; steatosis with inflammation), and eventually to liver failure. The pathology of NAFLD has been correlated with obesity and insulin resistance [2], which has risen exponentially in the last decade [3]. This is becoming increasingly important because NAFLD negatively influences patient outcome with regards to hepatic resection and liver transplantations [4-7]. Furthermore, steatohepatitis has been identified as a major cause of hepatocellular carcinomas [8]. Most often, the only recourse for patients diagnosed with hepatocarcinoma is liver resection or transplantation. Indeed, the percentage of patients who have undergone liver transplantation due to NASH has increased dramatically in the last decade (1.2% in 2001 to 9.7 in 2009) [9]. Furthermore, nearly half of potential donors for liver transplantation presented with some form of NAFLD [10].

Recent clinical studies have shown NAFLD increases the chance of post-operative complications after major hepatectomy [6] [11]. In genetic rodent models of obesity (*ob/ob* mice, zucker *fa/fa* rats) as well as nutritional models of NAFLD (methionine-choline deficient diet, HF diet), NAFLD is associated with delayed liver regeneration, increased liver damage, and mortality after 70% partial hepatectomy [12-14]. Such phenotypes have been attributed to decreased cell proliferation, impaired energy production, and increased oxidative damage, which consequently impair hepatocellular recovery after acute injury.

While the mechanisms dictating the negative effects of NAFLD on hepatic surgery have become increasingly clear, therapies to improve the post-surgical outcomes of patients with NAFLD are still not well developed. Recent studies have linked NAFLD to decreased PC and PC/PE in human and mouse models [15,

16]. In this study, we provide evidence that decreased hepatic PC/PE is a major contributor to the pathogenesis of NAFLD. We also identify hepatic PC/PE as a predictor of post-hepatectomy outcome. Finally, we provide evidence that choline supplementation is a viable therapy to improve survival after hepatectomy by increasing hepatic PC/PE, which decreases inflammation and improves energy utilization.

3.2 Methods & materials

3.2.1 Material

Primers were purchased from The Institute of Biomolecular Design. The Platinum® SYBER® Green qPCR SuperMix-UDG was purchased from Invitrogen(# 11733-038). All other chemicals reagents were from standard commercial sources.

3.2.2 Animal diets & surgery

All procedures were approved by the University of Alberta's Institutional Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care. C57Bl/6 *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice were backcrossed >7 generations. All animals were exposed to a 12 hour light-dark cycle, fed *ad libitum* a rodent chow diet (LabDiet, #5053), and had free access to water before and after PH. In some experiments, animals were switched to a HF diet (Bio Serv, #F3282; 1.3 g choline chloride/kg diet) or a HF diet supplemented with 2.7g choline chloride/kg diet for indicated lengths of time. The HF diet is 36% (w/w) fat, 36% (w/w) carbohydrate, and 21% (w/w) protein. The chow diet contains 4.5% (w/w) fat, 39% carbohydrate, and 20% protein.

All experiments were performed on male mice between the ages of 12-15 weeks. Mice were subjected to 70% partial hepatectomy using a modified method of Higgins and Anderson [17, 18]. All surgeries were performed between

9am-12pm under isofluorane anesthesia by Dr. Lin-Fu Zhu (University of Alberta). Animals were euthanized by cervical dislocation while under anesthesia. In all cases, mice were not fasted before euthanasia.

3.2.3 Histology and histopathological analysis of NAFLD

At time of sacrifice, a portion of the liver was fixed in 10% buffered formalin for at least 24 hours. Fixed livers were embedded in paraffin, sectioned, and stained with H&E by the University of Alberta Advanced Microscopy Facility. H&E stained liver sections were graded for portal inflammation, lobular inflammation, steatosis, cell ballooning, and fibrosis by Dr. Todd Chaba (University of Alberta), who was blinded to the samples [19] (Table 3.1). A modified NAFLD activity score was used to assess the progression of NAFLD. NAFLD activity score was calculated as the unweighted sum of steatosis, ballooning, portal inflammation, and lobular inflammation score [20, 21].

3.2.4 Quantification of hepatic lipid levels

Liver tissues were homogenized in 10 volumes of 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4. Protein concentrations were determined by Bradford assay (Bio-Rad) with BSA as standard. Total lipid were extracted from tissue homogenates (1 mg protein) using the Folch method [22] (see Section 2.2.8). Phospholipids and neutral lipids were separated by one dimensional 2 system TLC (see Section 2.2.10). Bands corresponding to PC, PE, and TG were scraped into glass tubes. PC and PE were quantified by a phosphorus assay [23] (see Section 2.2.8). TG was quantified by a chemical assay [24].

a) Quantification of TG by a chemical assay

Triolein (Sigma) was used to establish the standard curve (0 ηmol to 310 ηmol). Standards were dried under N_2 for at least 45 min before use in the

assay. 1 mL of alkaline hydroxyamine was added to the scraped silica (sample) or the dried standards. Tubes were briefly vortexed, incubated at 65°C for 2 min, and then cooled for 5 min at room temperature. 2.5 mL of ferric perchlorate was then added to the tubes and immediately vortexed and allowed to sit at room temperature for 25 min. Finally, the tubes were centrifuged at 1000 rpm for 5 min and absorbance at 530 ηm was read immediately. Alkaline hydroxyamine was made by mixing equal volumes of 4% ethanolic hydroxylamine solution (2% dissolved in 2.5 mL water, diluted to 50 mL with absolute ethanol) and of an 8% ethanolic NaOH (4 g dissolved in 2.5 mL water diluted to 50 mL with absolute ethanol). The NaCl was separated by centrifugation and the supernatant was decanted for use. Ferric perchlorate was made from combining 4 mL of stock ferric perchlorate (5 g ferric perchlorate dissolved in 10 mL 70% perchloric acid and 10 mL water, diluted to 100 mL with cold absolute ethanol), 3 mL of 70% perchloric acid, and diluted to 100 mL with cold absolute ethanol.

3.2.5 Real-time qPCR

Total RNA was isolated from snap frozen liver tissue using TRIzol® reagent (Invitrogen, #15596-018) according to manufacturer's protocol. Roughly, 0.1 mg of liver was homogenized in 3 mL of TRIzol® Reagent and RNA was isolated according to the manufacturer's protocol. The resulting RNA was suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and kept at -80°C. Quality of the RNA was determined by A_{260}/A_{280} ratio. 2 µg of RNA was treated with DNase I (Invitrogen, #18068-015) and reverse transcribed using Superscript II (Invitrogen, #18064-014) and oligo$(dT)_{12\text{-}18}$ primers (Invitrogen, #18418-012) according to manufacturer's instructions.

Real time qPCR was performed using a Rotor-Gene 3000 instrument (Montreal Biotech). A single reaction contains 0.625 µL of forward primer (10 µM), 0.625 µL of reverse primer (10 µM), 5 µL of cDNA, 12.5 µl of qPCR

SuperMix-UDG, and 6.25 µl of sterile deionized water. The primer pairs used are listed in Table 3.2. The program used to amplify the cDNA are as follows:

1) initial hold step: 50°C for 2 min.

2) denaturing step: 95°C for 4 min.

3) annealing step: temperature varies depending on the primer. Typically, the annealing temperature was set at 5°C below the melting temperature provided by the the primer manufacturer (the Institute of Biomolecular Design). Annealing time was 20 s.

4) extension step: the extension occurs at 72°C. The extension time depends on the size of the product. Typically, 1 min of extension time was used for every kilo basepair. Acquisition of the reporter probe signal is set for this step.

5) cycling: repeat step 1 to 4 for 35 cycles

6) melt curve: acquisition of the reporter probe signal while temperature is increased from 55-99°C by 1°C increments. There is a 1 min delay before starting the melt curve.

Data was analyzed using the Rotor-Gene 6.0.19 program (Montreal Biotech). Briefly, the relative mRNA concentration of the target gene and cyclophilin (housekeeping gene) in each sample was calculated by using the standard curve generated of the targent gene and cyclophilin. The normalized concentration of the target gene in one sample was calculated as the relative mRNA concentration of the target gene divided by the relative abundance of cyclophilin.

3.2.6 Quantification of hepatic ATP

To extract ATP from liver tissue, 0.01 g of powdered frozen liver tissue was homogenized in 1 mL of 0.4 M perchloric acid with a dounce homogenizer (30 strokes) on ice. The homogenates were neutralized with 0.28 mL of 2.5 M potassium hydroxide so the pH of the homogenate was roughly 7.8. The resulting precipitate was then centrifuged at 2000 x g for 2 minutes at 4°C. The supernatant was diluted 1:10 with ice cold PBS (0.1mM, pH 7.8) and assayed for ATP using the ATP assay kit (Sigma, #213-579-1) according to manufacturer's instructions.

3.2.7 Quantification of hepatic glycogen

Glycogen was extracted from 0.02 g of powered frozen liver tissue. Tissue was boiled in 30% KOH for 30 min and glycogen was precipitated with 3mL of 100% ethanol. Glycogen was then hydrolyzed to glucose by boiling in 3N HCl for 3 hrs. Once cooled to room temperature, glucose was assayed using a glucose assay kit (BioAssay Systems, #DIGL-200) according to manufacturer's instructions.

3.2.8 Blood glucose concentration

Blood was sampled from the mouse tail vein. Blood glucose levels were monitored using a glucometer (Accu-chek) right before surgery and at indicated times after surgery.

3.2.9 Statistical analysis

All data are represented as mean ± S.E. Correlation was determined by Pearson's correlation. Unpaired t-test was used to compare 2 groups. One or two way ANOVA was used in all other comparisons. In all tests, $p<0.05$ was considered significant.

3.3 Results

3.3.1 Decreased PC/PE is linked to the progression of NAFLD disease in LCTα$^{-/-}$ and *Pemt*$^{-/-}$ mice on HF diets

Both the *Pemt*$^{-/-}$ mice and the LCTα$^{-/-}$ mice develop hepatic steatosis when challenged with a HF diet for 10 weeks [25]. To study the progression of NAFLD in these animals, we placed LCT*α*$^{-/-}$ and *Pemt*$^{-/-}$ mice and their control littermates on a HF diet for 0 to 14 days to induce different degrees of NAFLD (Figure 3.1).

The histopathological analysis of the livers of LCT*α-floxed* mice showed no increase in steatosis (Figure 3.2A, B), inflammation (Figure 3.2C) or hepatocellular ballooning (Figure 3.2D) after 2 weeks on the HF diet. By contrast, LCT*α*$^{-/-}$ mice displayed increases in steatosis and inflammation scores, but there was no significant difference in hepatocellular ballooning. This suggests LCT*α*$^{-/-}$ mice but not LCT*α-floxed* mice developed NAFLD after 2 weeks of HF diet (Figure 3.2E).

Similarly, the histopathological analysis of the livers of *Pemt*$^{+/+}$ mice showed no development of NAFLD after 2 weeks on the HF diet. There was no increase in steatosis (Figure 3.4A,B), inflammation (Figure 3.4C) or hepatocellular ballooning (Figure 3.4D) in the *Pemt*$^{+/+}$ mice. By comparison, *Pemt*$^{-/-}$ mice exhibited increasing levels of steatosis with the HF challenge. This was confirmed with the quantification of hepatic TG content. Histopathological analysis also showed increasing hepatocellular ballooning and inflammation in *Pemt*$^{-/-}$ mice. However, no fibrosis was detected at any stage. Thus, *Pemt*$^{-/-}$ mice rapidly developed NAFLD over the course of the HF challenge (Figure 3.4E).

We next determined whether the progression of NAFLD in either of our mouse models was associated with a decline in hepatic PC/PE. LCTα$^{-/-}$ mice showed a decreased hepatic PC and consequently reduced PC/PE when placed

on a HF diet for 2 weeks in comparison to control mice (Figure 3.5A-C). Similarly, hepatic PC and PE content of the *Pemt*$^{-/-}$ mice were altered during HF feeding in comparison to *Pemt*$^{+/+}$ mice such that hepatic PC/PE decreased in *Pemt*$^{-/-}$ mice with time on the HF diet (Figure 3.6A-C).

Using correlation curves, we further corroborated a significant association between reduced hepatic PC/PE and the development of NAFLD using *Pemt*$^{-/-}$ mice, LCTα$^{-/-}$ mice, and their corresponding wildtype littermates (Figure 3.7A). Decreased hepatic PC/PE was also correlated with increasing severity of steatosis (Figure 3.7B) and inflammation (Figure 3.7C). However, the correlation between hepatic PC/PE and hepatocellular ballooning was not very strong (Figure 3.7D).

3.3.2 Decreased pre-operative hepatic PC/PE is negatively associated with survival rates following PH

NAFLD increases the risk of morbidity and mortality after liver surgery [26]. Since we have shown hepatic PC/PE to be associated with the intensity of NAFLD, we next determined whether hepatic PC/PE can be used to predict survival after partial hepatectomy. *Pemt*$^{-/-}$ or LCT$\alpha^{-/-}$ mice and their control littermates were fed the HF diet for 0 to 14 days. Each group was subjected to 70% partial hepatectomy (PH). Post-surgical outcome was assessed as percent survival 24 h after surgery. Using correlation curves, we demonstrated that increasing the severity of NAFLD prior to surgery results in decreased survival after hepatectomy (Figure 3.8A). Both the degree of hepatic steatosis (Figure 3.8B, C) and that of inflammation (Figure 3.8D) before PH are also negatively correlated with survival. Hepatocellular ballooning was very weakly correlative (Figure 3.8E). Interestingly, pre-operative hepatic PC and PE levels alone have no or weak correlation with survival respectively (Figure 3.9A,B). However,

decreased pre-operative hepatic PC/PE was strongly predictive of mortality after PH (Figure 3.9C)

3.3.3 Choline supplementation increases hepatic PC/PE and improves survival after PH in *Pemt*$^{-/-}$ mice

Next, we determined whether increasing hepatic PC/PE prior to surgery could improve survival after PH in mice with NAFLD. Because *Pemt*$^{-/-}$ mice exhibited a greater range of NAFLD in comparison to LCTα$^{-/-}$ mice over the 2 weeks of HF challenge, we used *Pemt*$^{-/-}$ and *Pemt*$^{+/+}$ mice for the following experiments. *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed the HF diet for 1 week (1.3 g choline/kg diet) to trigger NAFLD and then fed a HF diet supplemented with choline chloride (HF-CS: 4 g choline/kg diet) for an additional week. We expected the additional choline would stimulate hepatic PC synthesis *via* the CDP-choline pathway, thereby improving the hepatic PC/PE [27]. PH was performed and survival was monitored 24 h after surgery.

The HF-CS diet increased hepatic PC levels (Figure 3.10A) in *Pemt*$^{-/-}$ mice compared to *Pemt*$^{-/-}$ mice fed a non-supplemented HF diet for 2 weeks. Hepatic PE levels in *Pemt*$^{-/-}$ mice fed the HF-CS diet were also increased in comparison to *Pemt*$^{-/-}$ mice fed the HF diet (Figure 3.10B). As such, hepatic PC/PE was significantly increased in *Pemt*$^{-/-}$ mice fed the HF-CS diet when compared to *Pemt*$^{-/-}$ mice fed the HF diet (Figure 3.10C). Correspondingly, survival after PH increased by almost two-fold in *Pemt*$^{-/-}$ mice fed the HF-CS diet when compared to the HF fed *Pemt*$^{-/-}$ mice (57% *vs.* 32%) (Figure 3.10D).

The HF-CS diet had no effect on liver size (Figure 3.11A) and HF-CS diet did not prevent hepatic steatosis (Figure 3.11B, C) in *Pemt*$^{-/-}$ mice. Choline supplementation also did not affect cellular ballooning in *Pemt*$^{-/-}$ mice (Figure 3.11D). However, the livers of *Pemt*$^{-/-}$ mice on the HF-CS diet exhibited reduced

inflammation (*via* histopathological analysis) and NAFLD activity score in comparison to *Pemt*$^{-/-}$ mice fed the HF diet (Figure 3.11E, F). Likewise, *Pemt*$^{-/-}$ mice fed the HF-CS diet showed attenuated hepatic macrophage infiltration as measured by decreased expression of macrophage markers CD68 and F4/80 (Figure 3.12A). Decreased expression of pro-inflammatory cytokines (e.g. IL-6 and TNFα) was also observed in HF-CS fed *Pemt*$^{-/-}$ mice in comparison to HF diet. Furthermore, hepatic mRNA concentration of NADPH oxidase, an oxidative stress marker [28, 29], was also reduced in HF-CS fed *Pemt*$^{-/-}$ mice in comparison to HF diet.

Choline supplementation of the *Pemt*$^{-/-}$ mice on the HF diet prevented the depletion of hepatic ATP levels observed in *Pemt*$^{-/-}$ mice fed the HF diet (Figure 3.12B). Correspondingly, the HF diet fed *Pemt*$^{-/-}$ mice displayed increased hepatic expression of uncoupling protein-2 (UCP2), an inner mitochondrial membrane protein that decreases ATP production [30], which was reversed when the *Pemt*$^{-/-}$ mice fed the HF-CS diet (Figure 3.12C).

3.3.4 Choline supplementation prevented hypoglycemia in Pemt$^{-/-}$ mice resulting in decreased mortality after PH

After PH, the remaining liver undergoes regeneration while preserving tissue-specific functions necessary for survival, which includes the maintenance of euglycemia [31]. Both *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed the HF diet experienced drops in plasma glucose levels after PH (Figure 3.13A). However, the *Pemt*$^{-/-}$ mice had significantly lower glucose levels at 24 h. The *Pemt*$^{-/-}$ mice were also less ambulatory and displayed signs of hypothermia (e.g. shivering, cold to the touch). Coinciding with this, the survival rate of the *Pemt*$^{-/-}$ mice was low while all the *Pemt*$^{+/+}$ mice survived (Figure 3.10D). However, when the mice were fed the HF-CS diet, there were no significant differences in plasma glucose between the *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice 24 h after PH (Figure 3.13A). Thus, more of the

Pemt$^{-/-}$ mice fed the HF-CS diet recovered from the PH than the *Pemt*$^{-/-}$ mice fed the HF diet (Figure 3.10D).

We hypothesized the hypoglycemia experienced by the HF fed *Pemt*$^{-/-}$ mice after PH was the result of impaired gluconeogenesis and/or glycogenolysis [31]. We found glycogen levels in *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ on both diets to be significantly decreased after PH in comparison to the levels before surgery, suggesting that glycogenolysis occurs normally in *Pemt*$^{-/-}$ mice (Figure 3.13B). However, we found that *Pemt*$^{-/-}$ mice on 2 weeks HF diet had significantly lower glycogen levels before PH in comparison to *Pemt*$^{+/+}$ mice. No difference in pre-operative glycogen levels was observed in *Pemt*$^{-/-}$ mice on HF-CS in comparison to *Pemt*$^{+/+}$ mice. The expression of phosphoenolpyruvate carboxykinase (PEPCK), the rate limiting enzyme in hepatic gluconeogenesis, was much lower in *Pemt*$^{-/-}$ mice fed 2 weeks HF diet after PH in comparison to controls (Figure 3.13C). This difference in PEPCK expression was not observed in *Pemt*$^{-/-}$ mice on HF-CS diet in comparison to *Pemt*$^{+/+}$ mice.

3.4. Discussion

3.4.1 Decreased hepatic PC/PE promotes the progression of NAFLD and increases the risk of postoperative complications after PH

Pemt$^{-/-}$ mice developed NAFLD faster and to a larger degree than LCTα$^{-/-}$ mice when the animals were fed the HF diet for the same length of time. In line with this observation, PC/PE in *Pemt*$^{-/-}$ mice also dropped to a far greater degree than LCTα$^{-/-}$ mice. This was surprising because the CT mediated CDP-choline pathway is quantitatively the more important pathway for hepatic PC production (*vs.* the PEMT pathway) [32]. However, because PEMT catalyzes the conversion of PE to PC, PEMT may play a pivotal role in regulating the cellular balance between PC and PE. Indeed, PEMT activity *in vitro* has been shown to be up-

regulated as the consequence of elevated PE in the ER [33]. Chow fed *Pemt*$^{-/-}$ mice also have increased hepatic PE levels and decreased PC/PE in comparison to control mice [34]. Interestingly, PE level in *Pemt*$^{-/-}$ mice were normalized after 2 weeks of HF diet. This may reflect a compensatory decrease in CDP-ethanolamine pathway or increased PE degradation as result of elevated PE and/or decreased PC/PE [15, 35].

PC and PE are major components of the mammalian membrane [32]. Their distribution and composition are carefully regulated to maintain membrane integrity and to regulate the flow of substances across the plasma membrane and intracellular membranes [36, 37]. Decreased hepatic PC/PE can trigger inflammation and steatohepatitis as a result of an imperfectly packed plasma membrane that leads to the leaking of hepatocellular content into the extracellular space [15]. This can activate kupffer cells and promote the infiltration of neutrophils and other macrophages, which will increase cytokine-mediated injury of hepatocytes. In particular, TNFα is known to induce oxidative damage to the mitochondrial DNA and increase the permeability of the mitochondrial outer membrane, thus impairing the mitochondrial respiratory chain (MRC) [38, 39]. Dysfunction of the MRC increases the production of reactive oxygen species (ROS), which further contributes to hepatocellular injury and the pathogenesis of NASH [39]. Indeed, patients with NASH and rodent models of diet induced steatohepatitis often present with depleted hepatic ATP content [40, 41].

Pemt$^{-/-}$ mice on 2 weeks HF diet showed increased production of TNFα, elevated NADPH oxidase expression, and depletion of hepatic ATP content. We also observed increased UCP2 expression in 2 weeks HF fed *Pemt*$^{-/-}$ mice. UCP2 mediates the movement of protons across the inner mitochondrial membrane into the matrix, which decreases the formation of mitochondrial ROS in the MRC,

but at the cost of ATP synthesis [42]. In combination, this suggests *Pemt*$^{-/-}$ mice on 2 weeks HF diet have an impaired MRC and increased oxidative stress.

Choline supplementation increased hepatic PC/PE in *Pemt*$^{-/-}$ mice with NAFLD, prevented TNFα production, reduced UCP2 and NADPH oxidase expression, as well as normalized ATP content. This suggests that decreased hepatic PC/PE may facilitate the development of NASH by promoting inflammation and MRC dysfunction. Interestingly, *Pemt*$^{-/-}$ mice still accumulated hepatic TG when with choline supplementation, which increased hepatic PC/PE. Therefore, we hypothesize that the accumulation of hepatic TG may not be the major cause of the metabolic injuries that leads to the progression of NAFLD. Instead, decreased PC/PE appears to have a more significant role in contributing to the development of NAFLD.

The majority of patient mortality and morbidity after liver resection is caused by inadequate function of the remnant liver, leading to liver failure [43]. To support the demands of rapid cellular proliferation and essential liver function after PH, a sufficient supply of ATP is required [31]. Our data suggests that decreased hepatic PC/PE may lead to impaired MRC function. Therefore, a low ratio of hepatic PC/PE may contribute to a negative outcome after PH due to a failure of the remnant liver to meet energy requirements. We noted that the post-operative mortality experienced by *Pemt*$^{-/-}$ mice fed the HF diet for 2 weeks coincided with the onset of hypoglycemia. This can be attributed to an impaired induction of gluconeogenesis (a highly endergonic process) after PH, and depleted hepatic glycogen stores pre-surgery. Of note, IL-6 inhibits insulin-stimulated glycogen synthesis in rat hepatocytes [44, 45]. Therefore, the inflammatory response induced by a low hepatic PC/PE in *Pemt*$^{-/-}$ mice fed the HF diet for 2 weeks may also contribute to mortality after PH by impairing glycogen storage.

3.4.2 Decreased PC/PE as a clinical marker of postoperative risk after hepatectomy

Clinical and animal studies have shown NAFLD impairs recovery after liver resections [26]. In liver transplantations, steatotic grafts increase the chance of primary non-function and recipient morbidity and mortality [46]. The degree of NAFLD also predisposes the liver to injury due to ischemia and reperfusion (IR) during hepatectomy, further contributing to liver dysfunction and mortality after surgery [47]. Therefore, it is now common practice to take into account the presence of NAFLD before hepatic surgery.

Currently, NAFLD is diagnosed by the histopathological evaluation of liver biopsies stained with either H&E or lipid specific stains (e.g. Oil Red O, Sudan IV) [26]. However, staining and histological analysis of the liver samples are also highly operator-dependent, and often more than one sample from liver biopsies is required for accurate analysis [48]. This may increase the chance of internal bleeding and even mortality [49].

In our studies, we found hepatic PC/PE predicts survival after PH as well as the histological analysis of NAFLD. In practice, assaying hepatic PC/PE may be safer than histological analysis in determining risk of liver surgery since the measure of hepatic PC/PE would require one *vs.* multiply biopsies. Quantification of hepatic PC/PE would also avoid personal bias in the staining and the histopathological evaluation of the liver samples. Therefore, it may be worthwhile to further evaluate the correlation between hepatic PC/PE to post-operative risk in humans and other rodent models of NAFLD/obesity.

3.4.3 Choline supplementation as potential therapy for steatohepatitis and steatohepatitis related injury

Our results show increasing hepatic PC/PE by dietary choline supplementation improved the survival rate of PH in *Pemt*$^{-/-}$ mice with severe NAFLD by preventing inflammation and MRC dysfunction; thus avoiding the detrimental drop in blood glucose after surgery. Preventing inflammation and MRC dysfunction also protects hepatocytes from cytokine/ROS mediated necrosis during IR and liver regeneration [47]. Therefore, a high dietary intake of choline before PH would be a beneficial therapy to improve the post-operative outcome of hepatectomy or liver transplantation in individuals presenting with inflammation (e.g., patients with NASH, hepatocellular carcinoma, or hepatitis).

Reduced hepatic PC and PC/PE has been observed in patients with NASH [15, 16]. This suggests that a low hepatic PC/PE may be a common characteristic of inflammation of the liver, not just a negative effect of genetic inhibition of hepatic PC biosynthesis. As such, choline supplementation (to increase hepatic PC/PE) may also be used as a preventative therapy by individuals susceptible to NASH and/or NASH patients before substantial liver injury has occurred (e.g. cirrhosis, hepatic carcinomas).

Increasing the dietary intake of choline has been shown to prevent NAFLD in humans and animal models. For example, intravenous administration of choline was effective in resolving hepatic steatosis, high serum alanine aminotransferase levels, and low plasma choline levels in human patients receiving long term intravenous feeding [50-52]. Choline supplementation also prevented steatosis and fibrosis induced by a high sucrose diet or 15% ethanol in rats [53]. In addition, a high dietary intake of choline has been found to be beneficial in reducing plasma inflammatory biomarkers such as C-reactive protein and TNFα in the Greek population [54].

3.4.4 Conclusions

In conclusion, we provide evidence that hepatic PC/PE is inversely correlated with the degree of NAFLD. As such, quantification of hepatic PC/PE could be an excellent determinant of NAFLD and predictor of post-liver surgery outcome. Choline supplementation in mice with NAFLD prevented hepatic inflammation and NASH development by increasing hepatic PC/PE. Therefore, choline supplementation may be a viable therapy to prevent NASH development and to decrease the mortality rate post-liver surgery in patients with NAFLD.

Table 3.1 Histological evaluation of NAFLD

H&E stained liver sections were scored for steatosis, hepatocellular ballooning, and inflammation according to the guidelines stated above. All liver sections were analyzed by a single pathologist, who was blinded to the samples [19-21].

Table 3.1

Classification	Grade	Definition	Description
Steatosis	0	none	The percent of hepatocytes that present with cellular lipid accumulation when evaluated under low or moderate magnification
	1	<33%	
	2	33-66%	
	3	>66%	
Hepatocellular ballooning	0	none	The number of hepatocytes which are enlarged 1.5–2 times the normal hepatocyte diameter and contain rarefied cytoplasm.
	1	mild	
	2	moderate	
	3	marked	
Lobular inflammation	0	0 foci	The number of inflammation foci (clusters) observed under 200x field magnification. Inflammatory cells may include polymorphonuclear leukocytes, lymphocytes, eosinophils and microgranulomas.
	1	<2 foci	
	2	2-4 foci	
	3	>4 foci	
Portal inflammation	0	0 foci	The number of inflammation foci (clusters) observed under 200x field magnification around the portal tract. Inflammatory cells may include lymphocytes, eosinophils, and plasma cells.
	1	<2 foci	
	2	2-4 foci	
	3	>4 foci	

Table 3.2 Primer sequences for real-time qPCR analysis

The genes analyzed are: *Cyp*, cyclophilin; *Cd68*, cluster of differentiation 68; *F4/80*, F4/80; *Il6*, interleukin 6; *Cybb*, NADPH oxidase; *Pck1*, phosphoenolpyruvate carboxykinase 1; *Tnf*, tumor necrosis factor α.

Table 3.2

Gene	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
Cyp	TCC AAA GAC AGC AGA AAA CTT TCG	TCT TCT TGC TGG TCT TGC CAT TCC
Cd68	GCG GCT CCC TGT GTG TCT GAT	GGG CCT GTG GCT GGT CGT AG
F4/80	CCC TCG GGC TGT GAG ATT GTG	TGG CCA AGG CAA GAC ATA CCA G
Il6	CAA AGC CAG AGT CCT TCA GAG	TTG GAT GGT CTT GGT CCT TAG
Cybb	GAC TGG ACG GAG GGG CTA T	ACT TGA GAA TGG AGG CAA AGG
Pck1	GAA CTG ACA GAC TCG CCC TAT	TTC CCA CCA TAT CCG CTT C
Tnf	GTC TAC TGA ACT TCG GGG TGA	CAC CAC TTG GTG GTT TGC TAC GAC

Figure 3.1 Accumulation of hepatic lipids in LCTα$^{-/-}$ mice after a high fat (HF) diet

LCTα-*floxed* and LCTα$^{-/-}$ mice were fed a HF diet for 0 (chow) or 14 days. Representative livers from chow fed (left) and HF fed (right) mice were fixed in 10% buffered formalin and stained with hematoxylin and eosin (n = 3-7/group).

Figure 3.1

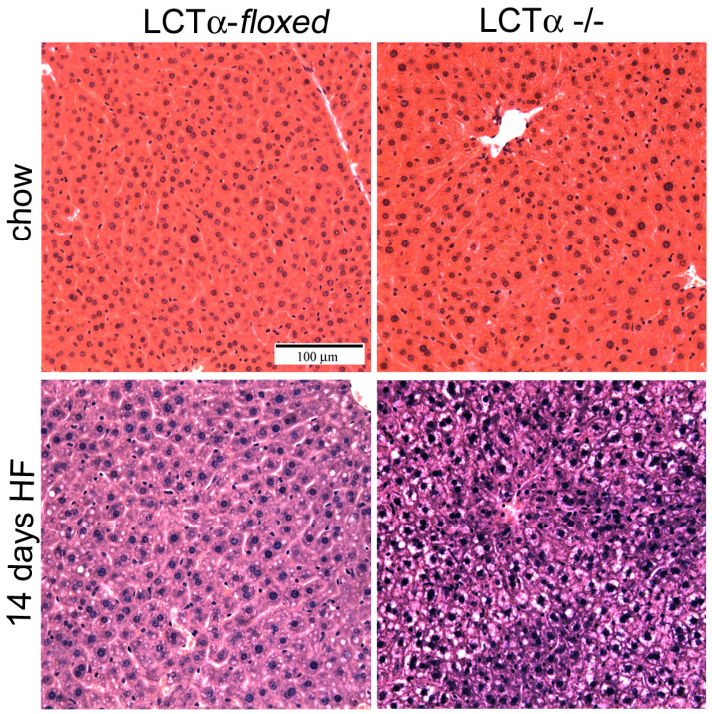


Figure 3.2 Progression of NAFLD in LCTα-*floxed* and LCTα$^{-/-}$ mice after a high fat (HF) diet

Livers from LCTα-*floxed* and LCTα$^{-/-}$ mice fed the HF diet for 0 and 14 days were fixed and stained with hematoxylin and eoxin. The liver sections were histologically analyzed for NAFLD: each was graded for **(A)** steatosis, **(C)** inflammation, **(D)** cell ballooning, and **(E)** NAFLD activity score. **(B)** The livers were also analyzed from triacylglycerol (TG) content. Values are means ± S.E.M (n = 3-4/group) * p <0.05 *vs.* LCTα-*floxed* fed the same diet. NAFLD, non-alcoholic fatty liver disease; N.D., not detected.

Figure 3.2

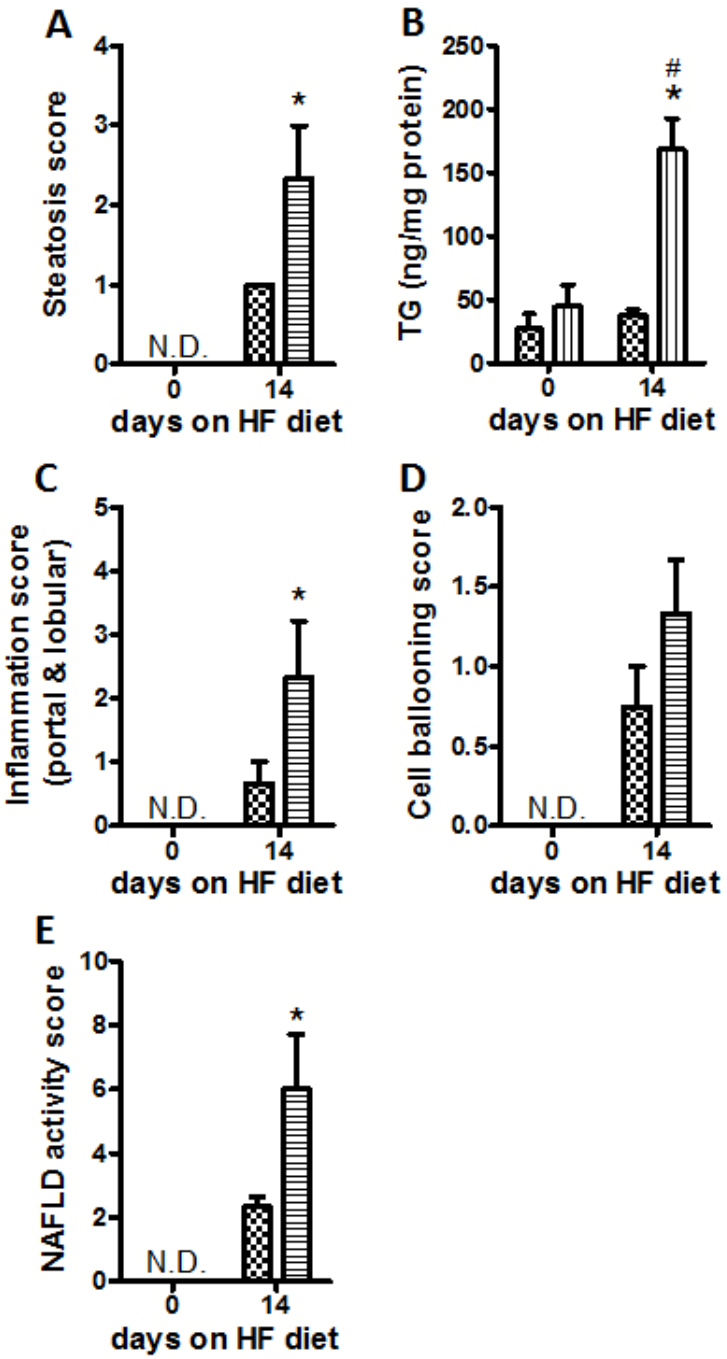

A
Steatosis score
4
3
2
1
0
N.D.
*
0
14
days on HF diet
B
TG (ng/mg protein)
250
200
150
100
50
0
#
*
0
14
days on HF diet
C
Inflammation score
(portal & lobular)
5
4
3
2
1
0
N.D.
*
0
14
days on HF diet
D
Cell ballooning score
2.0
1.5
1.0
0.5
0.0
N.D.
0
14
days on HF diet
E
NAFLD activity score
10
8
6
4
2
0
N.D.
*
0
14
days on HF diet

Figure 3.3 Hepatic lipid accumulation in *Pemt*$^{-/-}$ mice fed a high fat (HF) diet.

Pemt $^{+/+}$ and *Pemt*$^{-/-}$ mice were fed either the HF diet for 0-14 days or fed the HF diet for 1 week and the HF diet supplemented with choline for an additional week (HF-CS). Livers from mice fed either diet were fixed in 10% buffered formalin and stained with hematoxylin and eosin. A representative micrograph for each group of animals is displayed (n = 3-7/group).

Figure 3.3

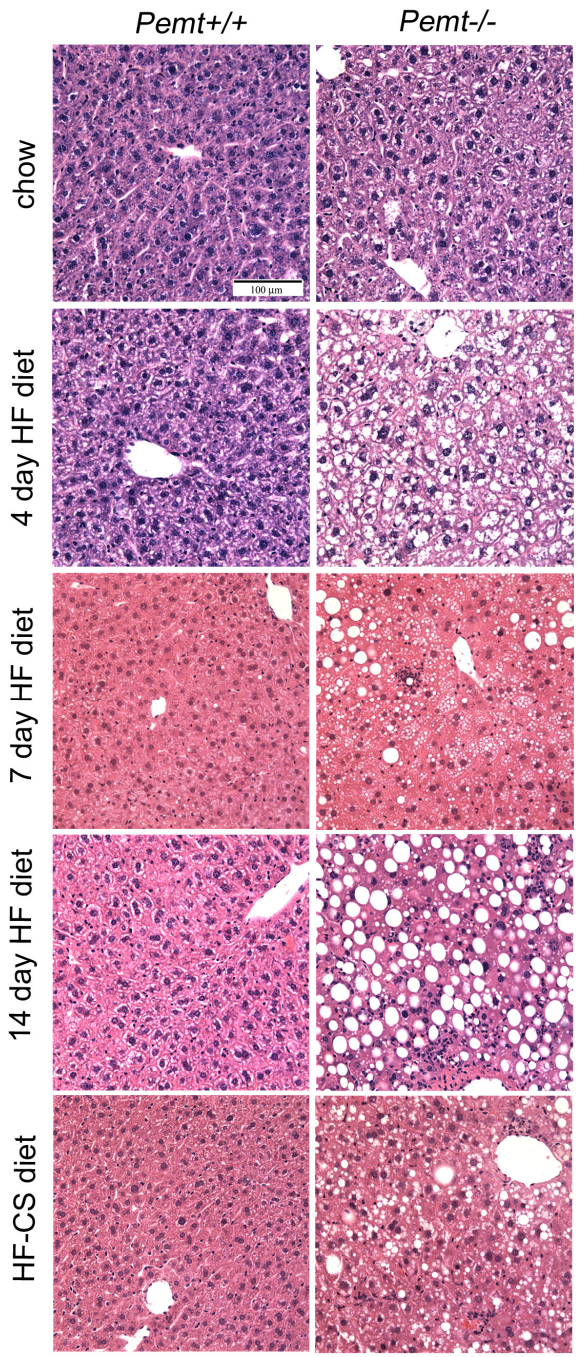

Pemt+/+
Pemt-/-
chow
4 day HF diet
7 day HF diet
14 day HF diet
HF-CS diet
100 µm

Figure 3.4 Progression of NAFLD in *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed a high fat (HF) diet.

Pemt$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed a HF diet for 0-14 days. Livers sections were stained with hematoxylin and eosin and graded by histology for **(A)** steatosis, **(C)** inflammation, **(D)** cell ballooning and NAFLD activity score. **(B)** The livers were also analyzed from TG content. (n = 3-7/group). Values are means ± S.E.M * $p<0.05$ *vs*. *Pemt*$^{+/+}$; # $p<0.05$ *vs.* t=0 of the same genotype. NAFLD, non-alcoholic fatty liver disease.

Figure 3.4

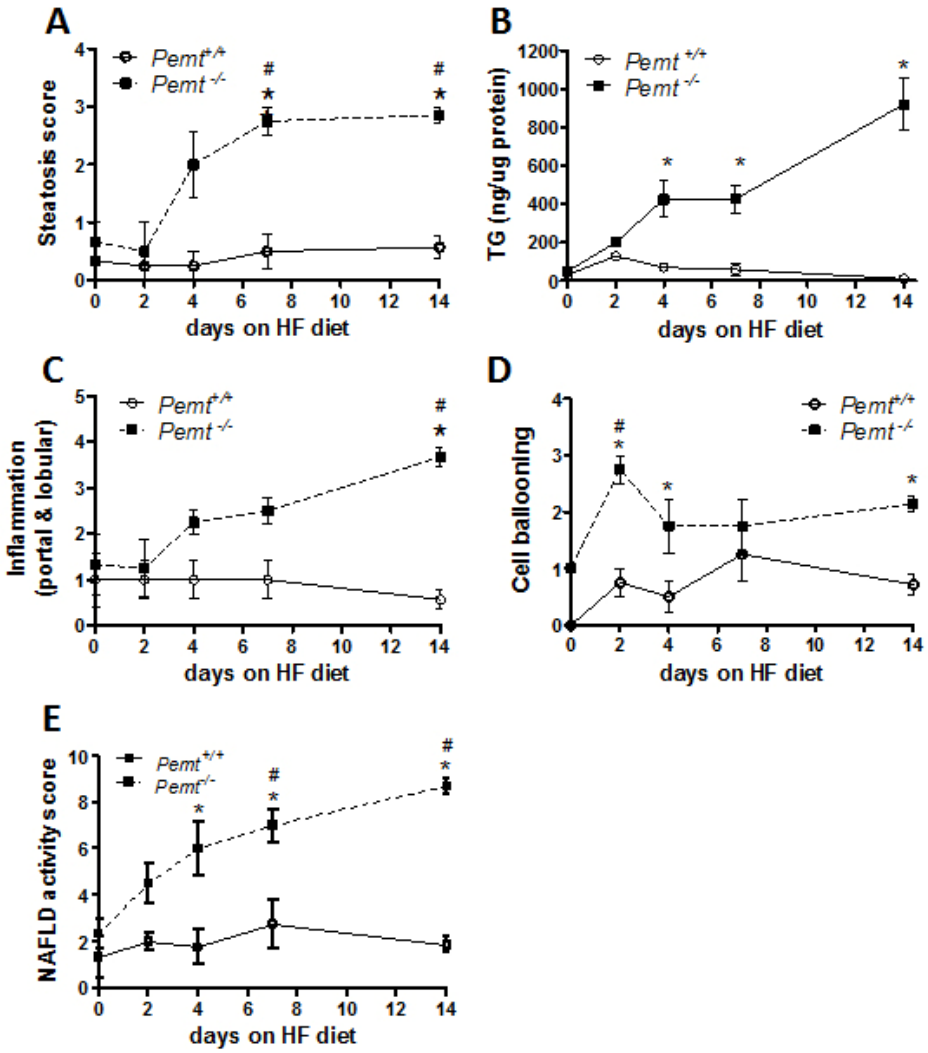

A
Steatosis score
Pemt+/+
Pemt-/-
days on HF diet
B
TG (ng/ug protein)
days on HF diet
C
Inflammation (portal & lobular)
days on HF diet
D
Cell ballooning
days on HF diet
E
NAFLD activity score
days on HF diet

Figure 3.5 Hepatic PC/PE is decreased in LCTα$^{-/-}$ mice when fed high fat (HF) feeding

(A) Hepatic phosphatidylcholine (PC) and **(B)** phosphatidylethanolamine (PE) content, as well as **(C)** PC/PE ratio was measured in LCTα-*floxed* and LCTα$^{-/-}$ mice after 0 and 14 days of the HF diet. Values are means ± S.E.M (n=5-12/group) * p <0.05 *vs.* LCTα-*floxed* mice fed the same diet.

Figure 3.5

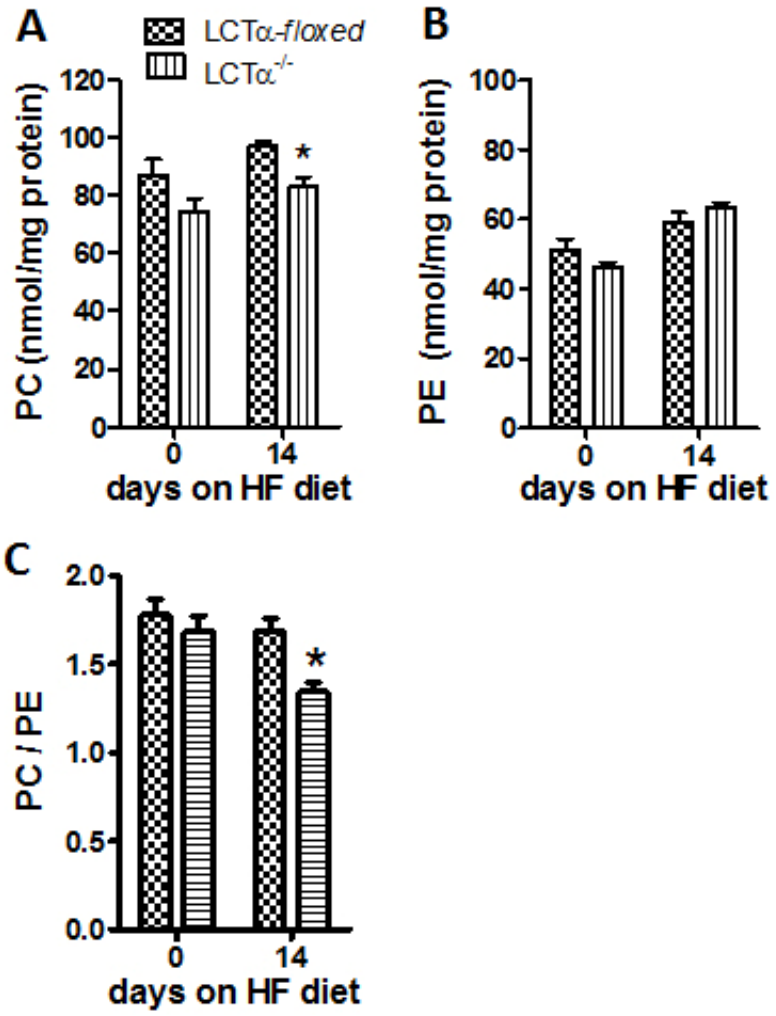

A
LCTα-floxed
LCTα-/-
PC (nmol/mg protein)
120
100
80
60
40
20
0
*
0
14
days on HF diet
B
PE (nmol/mg protein)
100
80
60
40
20
0
0
14
days on HF diet
C
PC / PE
2.0
1.5
1.0
0.5
0.0
*
0
14
days on HF diet

Figure 3.6 Hepatic PC/PE is decreased in *Pemt*$^{-/-}$ mice when fed high fat (HF) feeding

(A) Hepatic PC, **(B)** PE, and **(C)** PC/PE were measured after *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed the HF diet for 0-14 days. (n=4-11/group). Values are means ± S.E.M * $p<0.05$ *vs*. *Pemt*$^{+/+}$; # $p<0.05$ *vs.* t=0 of the same genotype.

Figure 3.6

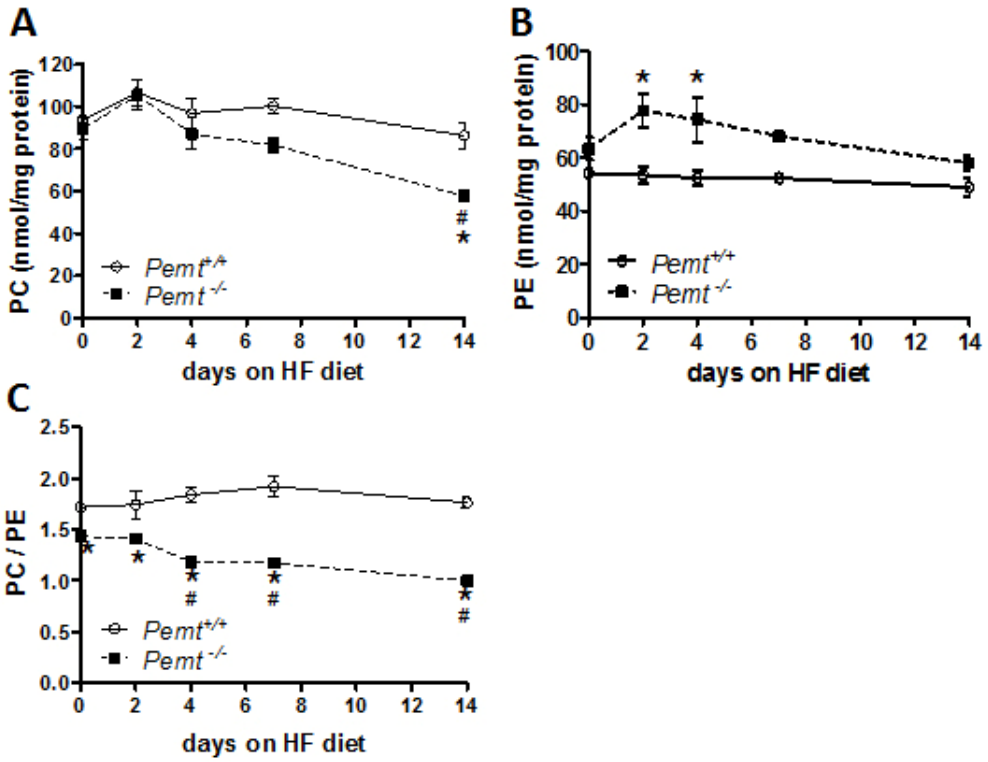

A
PC (nmol/mg protein)
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14
days on HF diet
Pemt+/+
Pemt -/-
B
PE (nmol/mg protein)
100
80
60
40
20
0
0 2 4 6 8 10 12 14
days on HF diet
Pemt+/+
Pemt -/-
C
PC / PE
2.5
2.0
1.5
1.0
0.5
0.0
0 2 4 6 8 10 12 14
days on HF diet
Pemt+/+
Pemt -/-

Figure 3.7 Correlation of hepatic PC/PE with the progression of NAFLD in *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice

Correlation graph shows the association between hepatic phosphatidylcholine to phosphatidylethanolamine ratio (PC/PE) to **(A)** steatosis, **(B)** inflammation, **(C)** cell ballooning, and **(D)** NAFLD activity score (n = 42). Linear regression, coefficient of determination (r^2) and p-value are as shown on each graph.

Figure 3.7

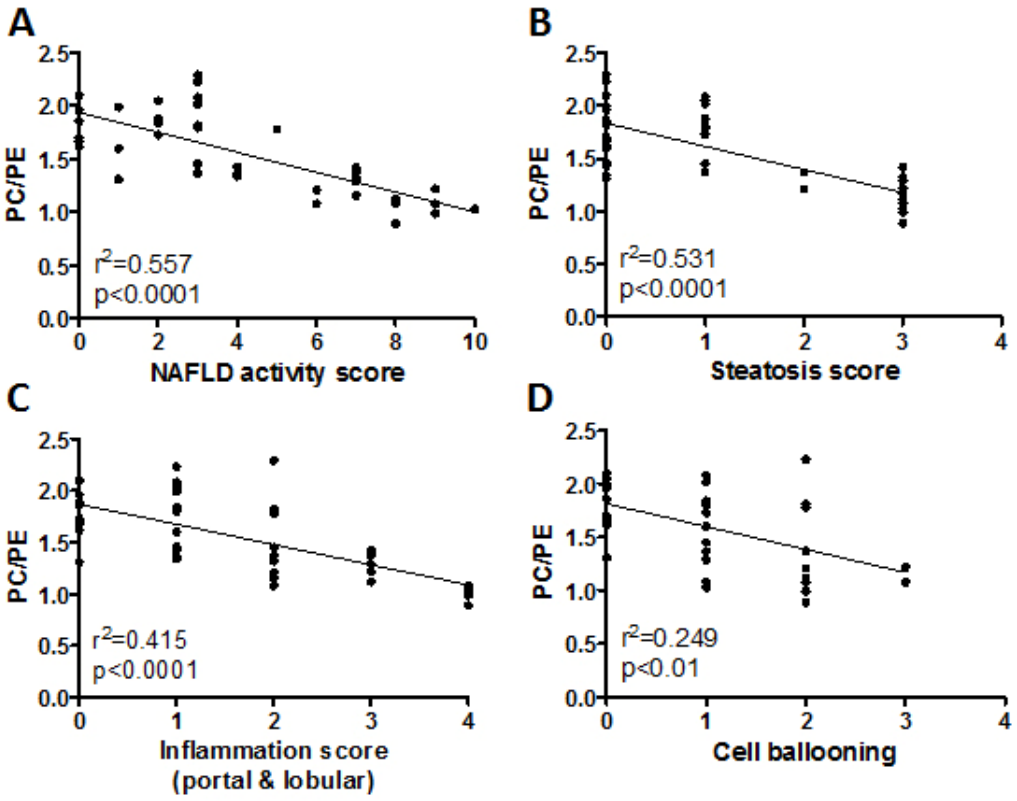

A
PC/PE
NAFLD activity score
r²=0.557
p<0.0001
B
PC/PE
Steatosis score
r²=0.531
p<0.0001
C
PC/PE
Inflammation score
(portal & lobular)
r²=0.415
p<0.0001
D
PC/PE
Cell ballooning
r²=0.249
p<0.01

Figure 3.8 Correlation of survival rate after 70% partial hepatectomy with pre-operative histological analysis of NAFLD.

Pemt$^{-/-}$, LCTα$^{-/-}$, and control littermates were fed the high fat diet for 0-14 days and then subjected to liver resection (n = 3-7/group). Survival rate 24 h after surgery was associated with pre-operative **(A)** NAFLD activity score, **(B)** steatosis, **(C)** inflammation, and **(D)** cell ballooning. Linear regression, coefficient of determination (r^2) and p-value are as shown on each graph. NAFLD, non-alcoholic fatty liver disease.

Figure 3.8

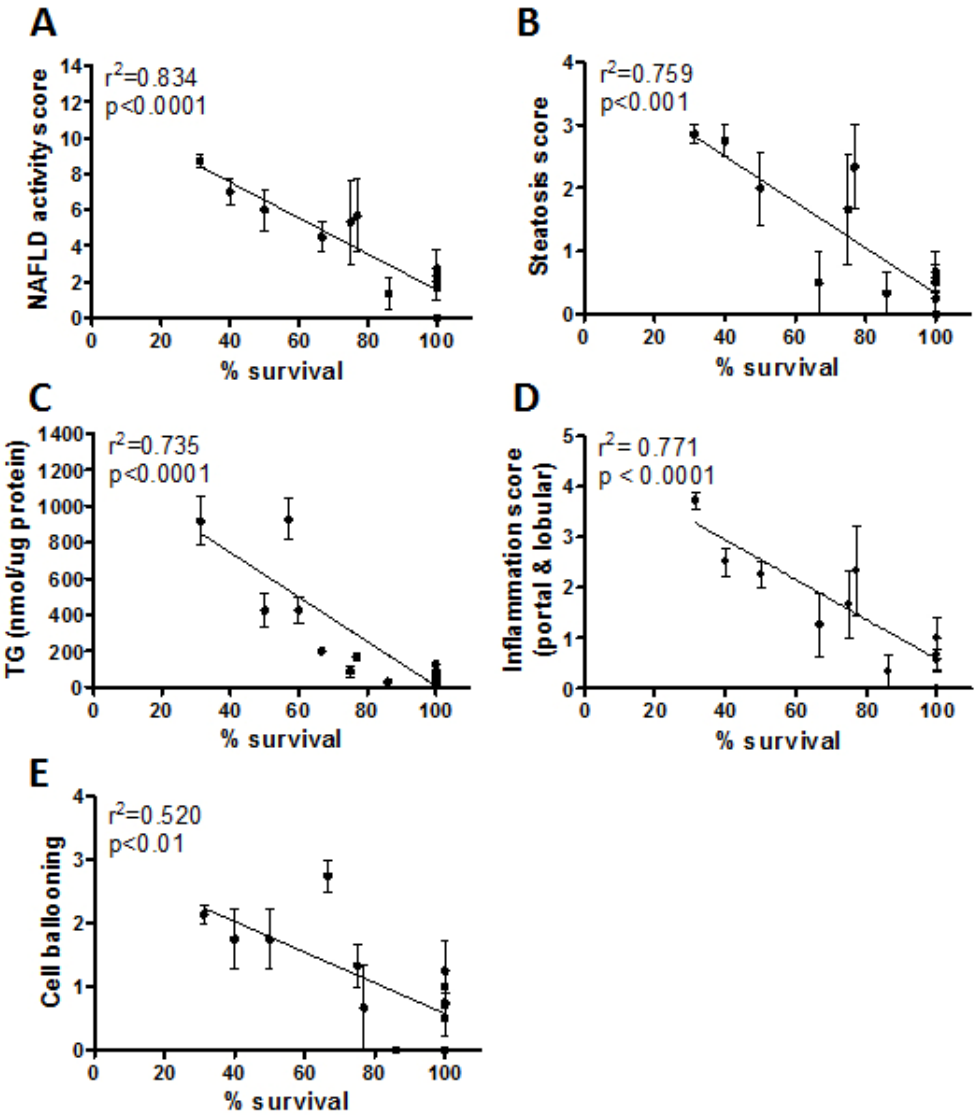

A
NAFLD activity score
r²=0.834
p<0.0001
% survival
B
Steatosis score
r²=0.759
p<0.001
% survival
C
TG (nmol/ug protein)
r²=0.735
p<0.0001
% survival
D
Inflammation score (portal & lobular)
r²= 0.771
p < 0.0001
% survival
E
Cell ballooning
r²=0.520
p<0.01
% survival

Figure 3.9 Correlation of survival rate after 70% partial hepatectomy with pre-operative hepatic phospholipid content

Pemt$^{-/-}$, LCTα$^{-/-}$, and control littermates were fed the high fat diet for 0-14 days and then subjected to liver resection (n = 3-7/group). Survival rate 24 h after surgery was associated with pre-operative hepatic **(A)** phosphatidylcholine (PC), **(B)** phosphatidylethanolamine (PE), and **(C)** PC to PE ratio (PC/PE). Linear regression, coefficient of determination (r^2) and p-value are as shown on each graph. NAFLD, non-alcoholic fatty liver disease.

Figure 3.9

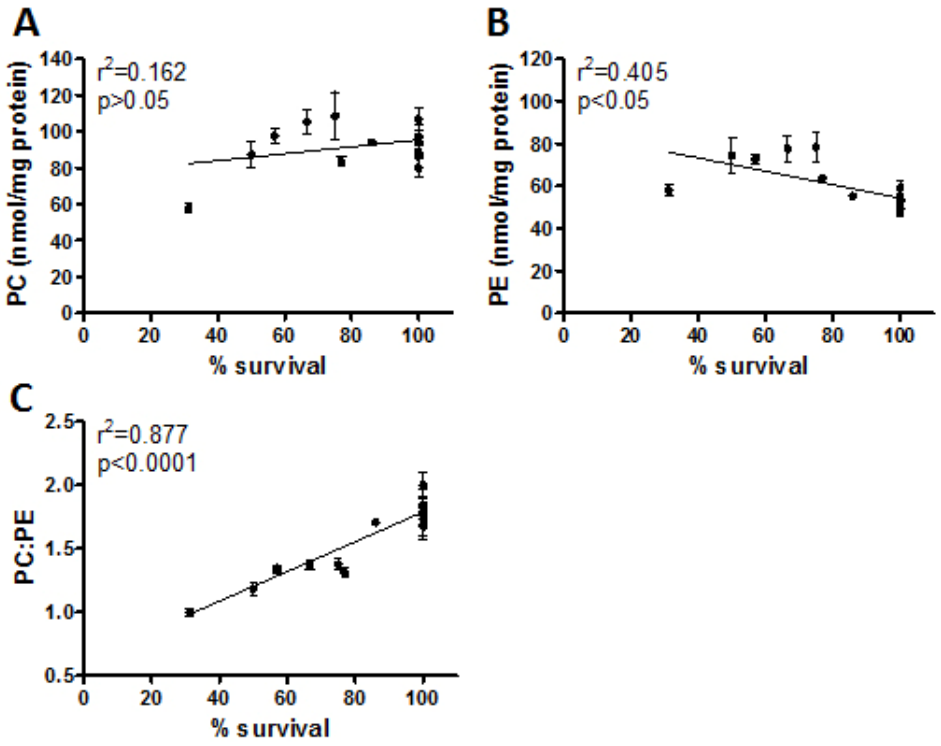

A
r^2=0.162
p>0.05
PC (nmol/mg protein)
% survival
B
r^2=0.405
p<0.05
PE (nmol/mg protein)
% survival
C
r^2=0.877
p<0.0001
PC:PE
% survival

Figure 3.10 Choline supplementation to the high fat (HF) diet improves hepatic PC/PE and survival after 70% partial hepatectomy (PH)

Pemt$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed for 2 weeks the HF diet or for 1 week the HF diet plus 1 week of the HF diet supplemented with choline (HF-CS). **(A)** Pre-operative hepatic phosphatidylcholine (PC) level, **(B)** phosphatidylethanolamine (PE) level, and **(C)** PC to PE ratio (PC/PE) were measured (n = 4-7/group). **(D)** Survival rate was determined 24 h after surgery (n = 6-16/group). *p<0.05 *vs*. *Pemt*$^{+/+}$; # p<0.05 *vs.* 2 wks HF.

Figure 3.10

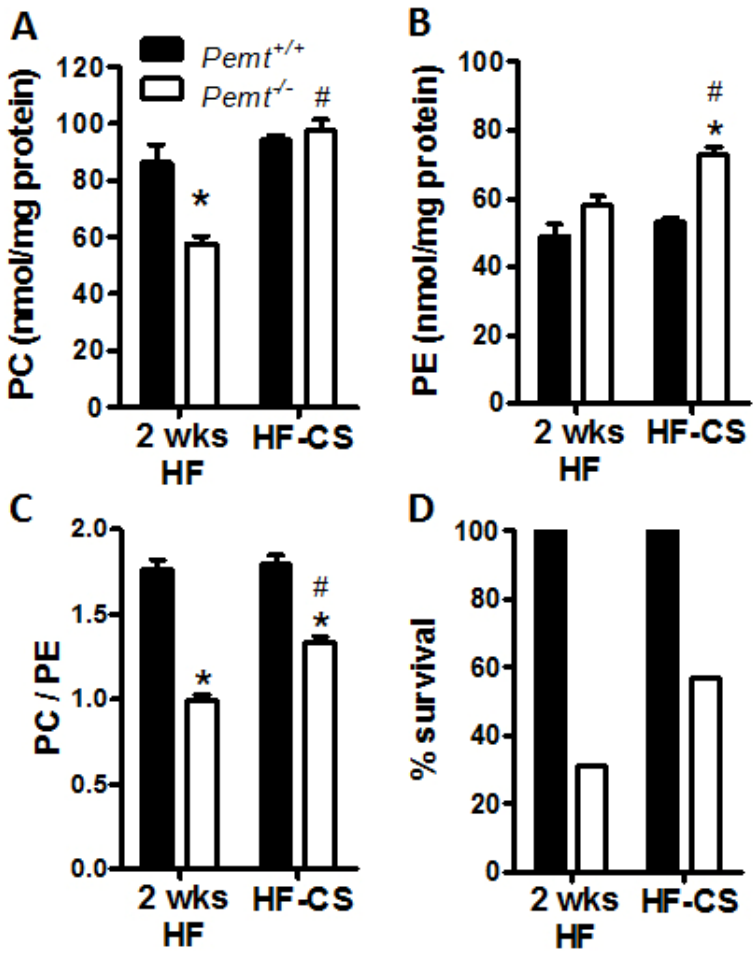

A
Pemt+/+
Pemt-/-
PC (nmol/mg protein)
120
100
80
60
40
20
0
*
#
2 wks HF
HF-CS
B
PE (nmol/mg protein)
100
80
60
40
20
0
#
*
2 wks HF
HF-CS
C
PC / PE
2.0
1.5
1.0
0.5
0.0
*
#
*
2 wks HF
HF-CS
D
% survival
100
80
60
40
20
0
2 wks HF
HF-CS

Figure 3.11 Choline supplementation to the high fat (HF) diet attenuates NAFLD in *Pemt*$^{-/-}$ mice

Pemt$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed for 2 weeks the HF diet or for 1 week the HF diet plus 1 week the HF diet supplemented with choline (HF-CS). **(A)** Liver size was calculated as % liver *vs.* body weight. Livers were scored for **(B)** steatosis, and **(D)** inflammation, **(E)** cell ballooning, and **(F)** NAFLD activity score (n = 4-7/group). **(C)** Hepatic triacylgycerol (TG) content was also quantified. * $p<0.05$ *vs*. *Pemt*$^{+/+}$; # $p<0.05$ *vs.* 2wks HF. NAFLD, non-alcoholic fatty liver disease.

Figure 3.11

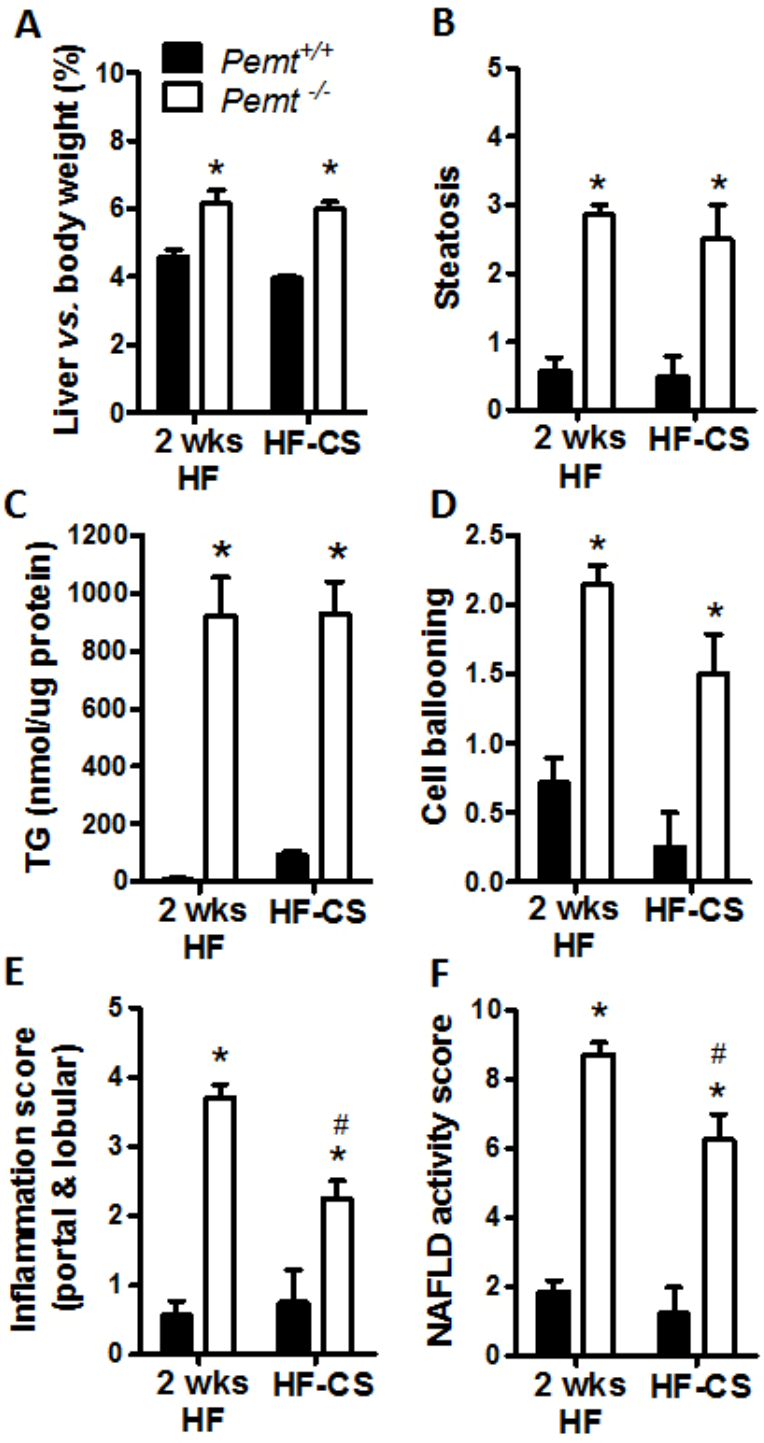

A
Pemt+/+
Pemt -/-
Liver vs. body weight (%)
10
8
6
4
2
0
*
*
2 wks HF
HF-CS
B
Steatosis
5
4
3
2
1
0
*
*
2 wks HF
HF-CS
C
TG (nmol/ug protein)
1200
1000
800
600
400
200
0
*
*
2 wks HF
HF-CS
D
Cell ballooning
2.5
2.0
1.5
1.0
0.5
0.0
*
*
2 wks HF
HF-CS
E
Inflammation score (portal & lobular)
5
4
3
2
1
0
*
#
*
2 wks HF
HF-CS
F
NAFLD activity score
10
8
6
4
2
0
*
#
*
2 wks HF
HF-CS

Figure 3.12 Choline supplementation to the high fat (HF) diet reduces inflammation and increases ATP level in *Pemt*$^{-/-}$ mice

Pemt$^{+/+}$ and *Pemt*$^{-/-}$ mice were fed the HF diet for 2 weeks or the HF diet for 1 week plus 1 week of the HF fat diet supplemented with choline (HF-CS). **(A)** mRNA expressions of CD68, F4/80, TNFα, IL-6, and NADPHox (n = 3-8/group). **(B)** Hepatic ATP content (n = 5/group). **(C)** mRNA expression of uncoupling protein 2 (UCP2) (n = 3-4/group). * $p<0.05$ *vs*. *Pemt*$^{+/+}$; # $p<0.05$ *vs.* 2 wks HF. CD68, Cluster of differentiation 68; TNFα, Tumor necrosis factor α; IL-6, Interleukin 6; ATP, adenosine triphosphate; NADPHox, NADPH oxidase.

Figure 3.12

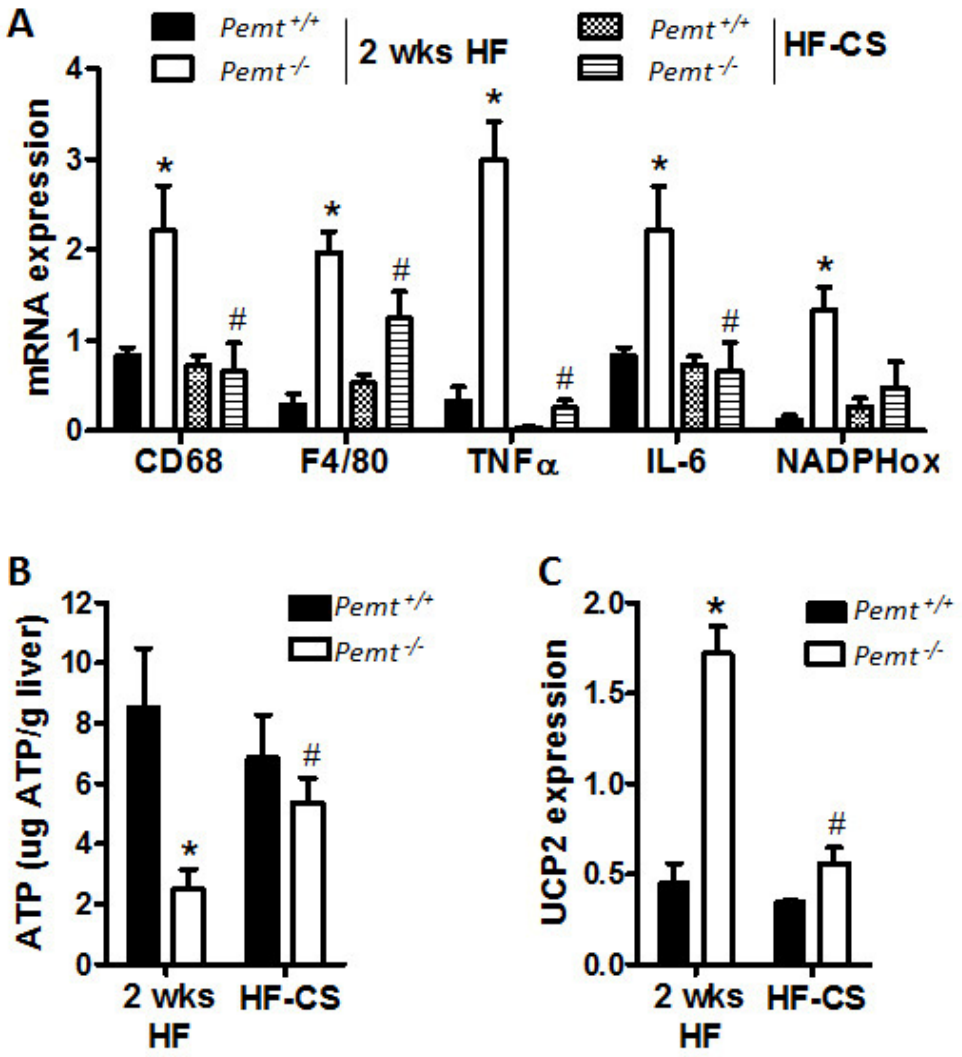
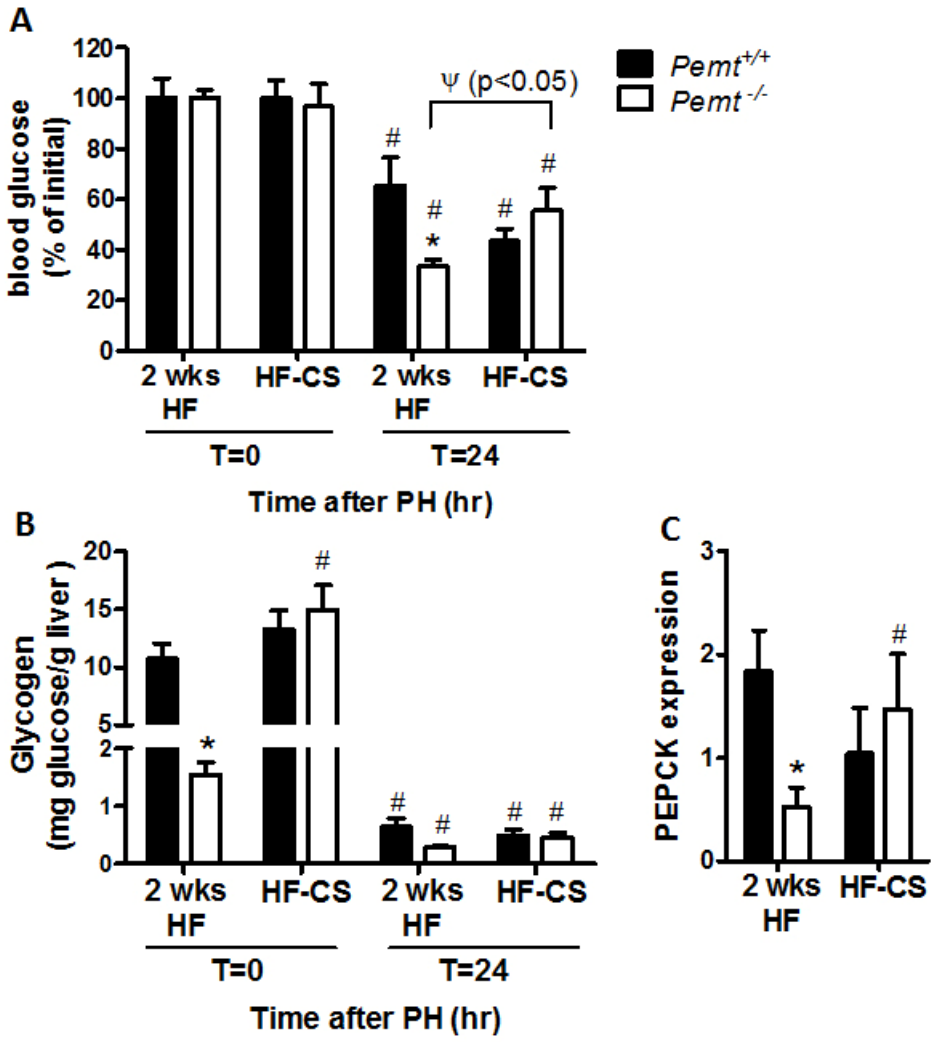

A
Pemt +/+
Pemt -/-
2 wks HF
Pemt +/+
Pemt -/-
HF-CS
mRNA expression
CD68
F4/80
TNFα
IL-6
NADPHox
B
ATP (ug ATP/g liver)
Pemt +/+
Pemt -/-
2 wks HF
HF-CS
C
UCP2 expression
Pemt +/+
Pemt -/-
2 wks HF
HF-CS

Figure 3.13 Choline supplementation to the high fat (HF) diet improves energy metabolism after 70% partial hepatectomy (PH) in *Pemt*$^{-/-}$ mice

(A) Blood glucose levels in *Pemt*$^{+/+}$ and *Pemt*$^{-/-}$ mice fed either the 2 weeks HF diet or the 1 week HF diet and then 1 week HF diet with choline supplementation (HF-CS) (n = 6-7/group). Blood glucose levels were measured before and after PH. **(B)** Hepatic glycogen content before and after PH (n = 7-9/group). **(C)** mRNA expression of phosphoenoylpyruvate carboxykinase (PEPCK) 24 h after PH (n = 3-6/group). * $p<0.05$ *vs*. *Pemt*$^{+/+}$; # $p<0.05$ *vs.* t=0 of the same genotype.

Figure 3.13


A
blood glucose (% of initial)
120
100
80
60
40
20
0
Ψ (p<0.05)
Pemt+/+
Pemt -/-
#
*
2 wks HF
HF-CS
2 wks HF
HF-CS
T=0
T=24
Time after PH (hr)
B
Glycogen (mg glucose/g liver)
20
15
10
5
2
1
0
2 wks HF
HF-CS
2 wks HF
HF-CS
T=0
T=24
Time after PH (hr)
C
PEPCK expression
3
2
1
0
2 wks HF
HF-CS

3.5 References

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CHAPTER 4: Characterization of lipid and lipoprotein metabolism in primary human hepatocytes

4.1 Introduction

The use of primary human hepatocytes is becoming very common with the increased availability of cryopreserved human hepatocytes and better isolation techniques from human liver tissue [1-3]. The use of primary human hepatocytes has played a critical role in the study of hepatitis C virus infection which affects 175 million people worldwide and is a major cause of chronic liver diseases (e.g. liver failure, cirrhosis, liver cancer) [4]. Unlike rodent hepatocytes and some human hepatoma cells, primary human hepatocytes are sensitive to hepatitis C virus infection and support viral replication [5-7]. Thus, primary human hepatocytes provide a valuable tool in the understanding of hepatitis C virus infection and replication, as well as for the evaluation of antiviral molecules. In addition, the development of the chimeric mouse in which the mouse liver is repopulated with primary human hepatocytes has also provided a robust and sustainable animal model to study hepatitis viruses [8, 9].

The most common use of primary human hepatocytes is in study of drug metabolism. Multiple studies have shown primary human hepatocytes express drug transporters and cytochrome P450 enzymes at a significantly higher level than human hepatoma cell lines (e.g. HepG2) [10-12]. As such, primary human hepatocytes provide a relevant human model for studying potential drug-drug interactions, active drug transport mechanisms, and the effects of drug metabolites formed during the metabolic transformation [13, 14].

Aside from drug metabolism, the liver is also a major site of lipid and lipoprotein metabolism. However, there are only a handful of publications in the primary literature on the study of lipid and lipoprotein metabolism in primary human hepatocytes. In 1987, Bouma *et al* showed primary human hepatocytes secrete 3 lipoprotein fractions that corresponded to the density of VLDL, LDL, and HDL [15]. The secretion rates of the lipoproteins were comparable to that

found in cultures of rat hepatocytes. Kuipers and colleagues demonstrated the size of VLDLs secreted by primary human hepatocytes (40 ηm) is within the range of VLDL found in human plasma (30 ηm-80 ηm) [16]. The relative amounts of TG, cholesterol, and phospholipid in VLDL secreted by primary human hepatocytes were also comparable to those reported for human serum VLDL. However, the relative amount of CE was markedly lower [16].

Consistent with studies on primary rodent hepatocytes and human-derived hepatoma cell lines (e.g. HepG2), insulin inhibits the secretion of apoB containing lipoprotein in primary human hepatocytes are are insulin [17]. This suggests lipid metabolism in freshly isolated human hepatocytes responds to insulin signalling. The selective uptake of HDL associated CE, which has been characterized in several cell models, was also shown to occur in primary human hepatocytes [18]. Interestingly, Havekes *et al* showed LDL receptor activity in primary human hepatocytes is only weakly down-regulated by LDL, whereas LDL receptor activity was completely inhibited by HDL [19]. The authors suggest this could be the reason why human liver maintains LDL receptor activity even under physiological concentration of plasma LDL. In addition, there is evidence to suggest that primary human hepatocytes respond differently to activation of peroxisome proliferator-activated receptor (PPAR) α in comparison to primary rodent hepatocytes [20]. Fenofibric acid, a known PPARα agonist, increased fatty acyl-CoA oxidase mRNA expression and activity in rat hepatocytes but not in human hepatocytes. Moreover, the expression of other known peroxisome proliferator-activated receptor α regulated genes (e.g. carnitine palmitoyl transferase-1) was also not regulated by fenofibric acid in primary human hepatocytes [20]. Thus, studies in rodent hepatocytes are not always recapitulated in human hepatocytes.

Currently, mouse models of lipid metabolism are widely used to elucidate potential drug targets for metabolic diseases such as obesity and atherosclerosis. However, if therapies are to be developed, it is essential that our knowledge of the mouse metabolism is translatable to that of the human. In this study, we characterized some of the fundamental lipid and lipoprotein metabolism in primary human hepatocytes. We also evaluate the synthesis of phospholipids in primary human hepatocytes, which has never been reported, and its relation to lipoprotein metabolism. Lastly, we determined the effect of nicotinic acid (niacin, NA) on lipid and lipoprotein metabolism in primary human hepatocytes.

4.2 Material and method

4.2.1 Materials

Hoechst stain was purchased from Invitrogen (#H1398). The anti-albumin (rabbit) and anti-TG hydrolase (rabbit) antibody was a gift from Dr. Richard Lehner. The CTα antibody (rabbit) was a gift from Dr. Mallampalli. The PEMT antibody (rabbit) was produced in the lab of Dr. Dennis Vance. The goat anti-apoB antibody was purchased from Chemicon (#AB742). The goat anti-PDI antibody was purchased from Cell Signalling (#2446S). The rabbit anti-apoA1 (#K23001R) and goat anti-apoE (#K74190G) antibody was purchased from Biodesign. The [^{3}H]-glycerol of purchased from MP Biomedicals (#27059H).

4.2.2 Isolation and culture of primary human hepatocytes

The human liver tissues used for hepatocyte isolation were obtained from patients undergoing operations for therapeutic purposes at the Service of Digestive Tract Surgery, University of Alberta. Liver samples were obtained from patients with colorectal metastasis, intrahepatic hemangioma, or benign liver tumor. Ethical approval was obtained from the University of Alberta's Faculty of Medicine Research Ethics Board and all patients consented to participate in the study.

Primary human hepatocytes were prepared by the lab of Dr. Norman Kneteman (University of Alberta) and were isolated and purified by collagenase-based perfusion of liver fragments (~20g) obtained from resection specimens far away from the tumor margin [8]. Briefly, the liver was perfused with 0.33 mg/ml Liberase (Roche #1666720) in digestion buffer (1.95 g NaCl, 0.25 KCl, 0.35 $CaCl_2$, 12 g HEPES buffer, 500 mL sterile water) for 20-25 min. After the digestion was completed, the liver was roughly dissected and the isolated cells were filtered sequentially through 2 mesh screens (course then fine). Next 3 volumes

of cold HBSS (GIBCO #14065-065) and 10% FBS was added to the cells to prevent further digestion. The resulting cell preparation was centrifuged at 500 rpm for 4 min at 4°C. The cell pellet was then resuspended in 5 mL of cold HBSS and layered on top of 7.5 mL of 1.04 g/ml of the Percol solution (Sigma # P-1664). The cells were then centrifuged at 1500 rpm for 4 min at 4°C. The pellet, which contains only viable hepatocytes were collected and resuspended in DMEM media (Sigma D5796) containing 1 ηM insulin and 1 ηM dexamethasone.

Cell count (hemocytometer) and viability (trypan blue exclusion) were measured before use; viability was routinely >80%. Isolated primary human hepatocytes were plated in 60 mm collagen-coated dishes (BD BioCoat™ #356401) at a density of 1.5 x 10^6 cells per dish and kept at 37°C in humidified air containing 5% CO_2. The cells were cultured in modified Roswell Park Memorial Institute (RPMI)-1640 culture medium (GIBCO #11875-093) containing 10% fetal bovine serum (FBS) for 3 h to allow the cells to attach to the dish, after which the old media was replaced with fresh media.

4.2.3 Cell viability analysis

Hoechst staining of fixed cells were used to determine cell viability. Briefly, media were aspirated from dishes containing hepatocytes 24, 48, and 72 h after attachment and the cells were fixed in 10% buffered formalin at room temperature for 15 min. Plates were quickly rinsed with PBS (0.1 M, pH7.4) 3 times and 0.2% TritonX-100 was added to permeablize the cells (5 min). Again the plates were rinsed with PBS. Finally, the cells were stained with Hoechst 33258 (0.5 μg/mL PBS) for 15 min in the dark and examined under a Leica DM IRE2 fluorescence microscope (Leica Microsystems) at the excitation wavelength of 351 ηm. Images were captured using OpenLab 3.1.4 (PerkinElmer) and the number of apoptotic and viable cells were counted.

Cell viability was also determined in primary human hepatocytes by direct staining of plated cells with Trypan blue (GIBCO #15250-061). At indicated times after plating, 1.5 mL of Trypan blue was added to each dish already containing 1.5 mL of medium and allowed to incubate at room temperature for 5 min. Dishes were rinsed 3 times with PBS and examined under a Leica DM IRE2 microscope with white light. Images were captured using OpenLab 3.1.4 (PerkinElmer) and number of dead and viable cells were counted.

4.2.4 Immunoblot analysis

Cells were collected in buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) and homogenized by sonication (15 s). Protein concentration was determined by the Bradford Assay (Bio-Rad) using BSA as standard. Cell lysates (20 μg of protein) were heated for 10 min at 95°-100°C in western blot buffer (50mM Tris, 10% glycerol, 5% SDS, 1% 2-mercaptoethanol, 0.02% bromophenol blue, pH 6.8) and the samples were electrophoresed on a 12% gel. Proteins were transferred to a polyvinylidene fluoride membrane and the membrane was incubated in 5% skim milk in T-TBS (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) for 1 h at room temperature. The membrane was immunoblotted overnight at 4°C with antibodies against TGH (1:50,000 dilution in T-TBS), PEMT (1:300 dilution in T-TBS) or CTα (1:2000 dilution in 25mM Tris, 150 mM NaCl, 0.25% Tween 20, 1% gelatine, pH7.4) [21]. The membrane was then washed 4 times with T-TBS for 15 min each and then incubated with the secondary antibody. The membrane was washed again 4 times with T-TBS for 15 min each and then visualized with the enhanced chemiluminescence system (Amersham Biosciences) according to the manufacturer's instructions.

For analysis of albumin and lipoprotein secretion, primary human hepatocytes were washed and incubated in RPMI medium 16 h before collection.

Media were then collected, briefly centrifuged to remove cellular debris, and 1 mL of media was incubated with 5 mg Cab-O-Sil for 1 h at 4°C on a rotator. The Cab-O-Sil pellet was washed twice with ice cold water, resuspended in 40 µl of western blot buffer, and heated at 95°- 100°C for 10 min. 20 µl of each sample was electrophoresed on a 5% or 10% SDS-PAGE gel and immunoblotted with antibodies raised against ApoB (1:7500 dilution), ApoA1 (1:5,000 dilution), ApoE (1:600), and albumin (1:1000). The membrane was then washed 4 times with T-TBS for 15 min each and then incubated with the secondary antibody. The membrane was washed again 4 times with T-TBS for 15 min each and then visualized with the enhanced chemiluminescence system (Amersham Biosciences) according to the manufacturer's instructions.

4.2.5 Enzymatic assays

Total CT activity was measured in total cell lysate (50 µg of protein) by monitoring the conversion of [^{3}H]phosphocholine into CDP-choline [22] (see Section 2.2.7a). PEMT activity was measured in primary hepatocytes by following the production of [^{3}H]PC from PMME and S-adenosyl-[methyl-^{3}H]methionine in total cell lysates (50 µg of protein) [23] (see Section 2.2.7b).

4.2.6 Lipid Synthesis and secretion in primary human hepatocytes

To measure lipid secretion, primary hepatocytes were incubated with 2 µCi [^{3}H]glycerol in RPMI plus 10 % FBS for 4 h. After incubation, dishes were washed twice with RPMI media and re-incubated with RPMI medium for an additional 4 h. Both cells and media were collected and analyzed for secretion of radiolabelled lipids. Lipids were extracted by the Folch method [24] (see Section 2.2.8) and phospholipids and neutral lipids were resolved by TLC using the 1 dimentional 2 phase system (see Section 2.2.10). Individual lipids were visualized by I_2 vapour and the bands corresponding to PC and TG were scraped and radioactivity was measured by a scintillation counter. The percentage of PC

secreted was calculated as percent of [^{3}H]PC (dpm) in the medium *versus* total [^{3}H]PC (dpm) in medium and cells.

To measure lipid synthesis, hepatocytes were incubated with 2 µCi [^{3}H]glycerol and 0.4 mM oleic acid/0.5% fatty acid-free BSA for 4 h. After 4 h, cells were collected and [^{3}H]PC and [^{3}H]TG were analyzes by using the 1 dimentional 2 phase system (see Section 2.2.10).

4.2.7 Quantification of lipids in primary human hepatocytes

Lipids from whole cell lysates (0.5 mg of protein) were extracted by the method of Folch *et al* [24] (see Section 2.2.8). Neutral lipids were quantified in liver homogenates by gas-liquid chromatography [25] (see Section 2.2.9). Tridecanoin (2 µg) was used as the internal standard.

4.2.8 Fast protein liquid chromatography analysis

Size exclusion fast protein liquid chromatography (FPLC) was used to separate lipoprotein particles secreted by cultured primary human hepatocytes into media. Plated hepatocytes were washed and incubated in RPMI medium 16 h before collection. Media was collected and concentrated using centrifugal filter unit (Amicon Ultra-15). Concentrated media (100 µl) was subjected to FPLC by the Lipid Analysis Core (University of Alberta). The lipoprotein classes was resolved from each other on a Superose 6 column attached to a Beckman systems Gold or Nouveau Gold apparatus. Quantification of cholesterol or TG after separation was achieved by in-line assays using a post-column T-connector/solvent delivery module (Beckman Coulter, Mississauga) and passed through a CH-30 column heater (Eppendorf) set at 37°C. Total cholesterol and TG were measured by the Infinity Cholesterol Reagent (Sigma) and Triglyceride GPO Trinder kit (Sigma), respectively. Reaction products were monitored at 500 ηm in real-time using a programmable Detector Module (Beckamn Coulter)

4.2.9 PEMT adenovirus infection

The PEMT recombinant adenovirus was constructed by subcloning the cDNA encoding human PEMT into pAdTrack-CMV shuttle vector. The vector was then linearized with PmeI and inserted into the adenovirus genome by using the pAdEasy-1 system for homologous recombination in *Escherichia coli*. The pAdTrack-CMV shuttle vector contains a gene encoding green fluorescent protein (GFP). Therefore, the adenovirus used to express PEMT also expressed GFP, which served as a marker of a successful viral infection. The GFP recombinant adenovirus was constructed by subcloning an empty pAdTrack-CMV shuttle vector into the adenovirus. The concentration of each adenovirus preparation was estimated by fluorescent titering. Attached primary human hepatocytes were infected with adenovirus expressing human PEMT (advPEMT) or GFP (advGFP) (MOI = 5). Hepatocytes were maintained in RPMI media with 10 % FBS and cells were infected with virus for 24 h or 48 h.

4.2.10 Nicotinic acid treatment

Attached primary human hepatocytes were treated with 1) RPMI media containing 10% FBS, 2) RPMI media with 10% FBS and 3mM NA (Sigma #N0761), or 3) RPMI media with 10% FBS and 3mM nicotinamide (Sigma #N0636). Hepatocytes were incubated in treatment media 20 h before and during experiment.

4.2.11 Statistical analysis

All data are represented as mean ± S.E. Unpaired t-test was used to compare 2 groups. One or two way ANOVA was used in all other comparisons. In all tests $p<0.05$ was considered significant.

4.3 Results

4.3.1 Cell viability and lipoprotein secretion

Viability of primary human hepatocytes in our cell culture system was analyzed over 3 days by Hoechst staining (Figure 4.1). Primary human hepatocytes were found to have 90% cell viability after 1 day in culture and dropped to cell viability of 65% after 3 days in culture. The significant drop in hepatocyte viability after 3 days in culture was also confirmed with direct staining of plated cells with Trypan blue (Figure 4.2).

Lipoprotein secretion is an essential liver function. We assessed the ability of primary human hepatocytes to secrete lipoproteins over 3 days in culture by measuring the accumulation of apolipoproteins in media (Figure 4.3A). We show that primary human hepatocytes are able secrete apoB, apoA1, apoE, and albumin 3 days after initial plating. However, amount of lipoprotein and albumin secreted decreases after the first 24 h. This finding was recapitulated with radiolabelling experiments. Efflux of cellular [^{3}H]TG by primary human hepatocytes is decreased by 50% after 1 day in culture (Figure 4.3B). Secretion of cellular [^{3}H]PC, another major component of VLDLs, was not impaired (Figure 4.3C).

FPLC was used to assess the size and composition of the lipoprotein secreted by primary human hepatocytes (Figure 4.4A, B). Three distinct lipoprotein fractions were isolated from the media. The size of the lipoprotein fractions corresponded to VLDL, LDL, and HDL particles. The majority of the TG secreted was packaged into VLDL-sized particles; some TG was also detected in LDL-like particles. We found cholesterol to be packaged into VLDL, LDL, and HDL –like fractions. Interestingly, a major fraction of cholesterol was found in VLDL-sized particles.

4.3.2 TG synthesis in primary human hepatocytes

We found an accumulation of TG (Figure 4.5A) and cholesterol (Figure 4.5B) mass during the 3 days in culture but there was no increase of cholesterol ester (Figure 4.5C) or phospholipid content (Figure 4.5D). This coincides with an increase in cytosolic lipid droplets that could be observed in microscopic pictures taken of plated hepatocytes after 2 days in culture (Figure 4.2). Furthermore, when we assessed the incorporation of [^{3}H]glycerol into TG, there was an increase in [^{3}H]TG after 2 days in culture in the presence or absence of exogenous fatty acid (Figure 4.5E).

4.3.3 Phospholipid synthesis and lipoprotein secretion

There are 2 distinct pathways for PC biosynthesis in the liver: the PEMT pathway and the CDP-choline pathway [26]. The CDP-choline pathway is the major pathway for PC biosynthesis, producing 70% of total hepatic PC, while the PEMT pathway contributes the remaining 30% [27]. The protein expression of CTα, which is the major hepatic isoform of CT [26], and total CT activity were not altered during the 3 days the primary human hepatocytes are in culture (Figure 4.5) (Figure 4.6 B, D). However, PEMT protein levels were decreased after 24 h in culture and *in vitro* activity was down by 50% after 3 days in culture (Figure. 4.6A, C). We also measured total PC biosynthesis *via* the incorporation of [^{3}H]glycerol in cellular PC (Figure 4.6E). The amount of radiolabel incorporated into [^{3}H]PC in primary human hepatocytes remained constant over the 3 days in culture.

Mice with a disruption in the *Pemt* gene exhibit impaired VLDL secretion and hepatic TG accumulation [28, 29]. We wondered if the decrease in PEMT expression would contribute to impaired lipoprotein secretion in primary human hepatocytes over time. We infected primary human hepatocytes with adenovirus containing the human *Pemt* cDNA and monitored apolipoprotein secretion after infection. *In vitro* PEMT activity and PEMT protein expression was

significantly increased 2 days after infection in comparison to day 1 of infection and no infection control (Figure 4.7B). However, secretion of apoE, apoA1, and $apoB_{100}$ was not affected with increased PEMT activity (Figure 4.7B).

TG hydrolase (TGH), termed Ces3 in mice and Ces1 in humans, has also been suggested to play a role in VLDL assembly and secretion [30, 31]. Inhibition of TGH activity by a lipase inhibitor impaired mobilization of cellular TG stores and decreased VLDL secretion [32]. In addition, over expression of TGH specifically in the liver of transgenic mice increased fasting plasma TG and apoB levels [33]. We measured the protein expression of TGH in human hepatocytes and found that the reduction in apolipoprotein and lipid secretion over time in culture corresponded to a decrease in TGH protein level (Figure 4.8).

4.3.4 Effect of nicotinic acid on lipid and lipoprotein metabolism in primary human hepatocytes

Nicotinic acid (NA) is a lipid modifying drug used clinically to treat dyslipidemia and to reduce the occurrences of coronary heart disease [34]. Taken at high doses (grams), NA has been shown to increase plasma HDL-cholesterol and decrease plasma LDL-cholesterol, VLDL, and TG levels [34]. To determine whether NA could directly impair hepatic VLDL formation and secretion, we treated primary human hepatocytes with NA and measured lipid synthesis and lipoprotein secretion. Concurrently, hepatocytes were treated with nicotinamide (NAM), which is an amide analogue of NA but does not exert the same pharmacological effects as NA [35, 36].

To measure lipid synthesis, we determined the incorporation of [^{3}H]glycerol into cellular TG and PC. NA treatment did not affect the level of [^{3}H]TG or [^{3}H]-PC in comparison to control and NAM treated cells (Figure 4.9A,B), which suggests NA does not alter hepatic TG or PC synthesis. NA also did not

affect the cellular concentration of TG, cholesterol, and CE in comparison to control (Figure 4.9C-E). Correspondingly, we shown NA does not directly impair apoB, TG, and total cholesterol secretion in comparison to control treated cells (Figure 4.9F-H).

It is currently unclear how NA increases plasma HDL-cholesterol levels. We hypothesized that NA may raise HDL through increased formation of nascent HDL. We measured the secretion of apoA1 in primary human hepatocytes after NA treatment. However, we did not observe a difference in apoA1 secretion by primary human hepatocytes treated with NA compared to controls (Figure 4.10).

4.4 Discussion

4.4.1 Lipoprotein secretion by primary human hepatocytes

In this study we show that primary human hepatocytes plated on collagen coated dishes are able to maintain viability and liver specific function up to 3 days in culture. As such, human hepatocytes are better able to retain their ability to secret lipoproteins in comparison to primary rodent hepatocytes. Hepatocytes isolated from rats lose their ability to secret VLDL after 24 h in culture [37]. This is most likely due to de-differentiation as it is well established that the difference in hepatocytes gene expression with that of whole liver increases with time after isolation of hepatocytes [38-40].

PEMT expression has been shown to decrease with de-differentiation of primary rodent hepatocytes in culture. PEMT mRNA was undetectable in primary rat hepatocytes after 50 h in cell culture [41]. In addition, microarray analysis suggests that PEMT transcripts are decreased 12 h of isolation [40]. These results are consistent with the decrease in PEMT activity and protein expression we observed in human primary hepatocytes. This may suggest that *Pemt* is one of many genes that are associated with de-differentiation.

Furthermore, PC derived from the PEMT pathway is important for VLDL secretion in rodent hepatocytes [28, 29]. *Pemt*$^{-/-}$ mice secreted 50% less VLDL and accumulate more hepatic TG than wild-type controls when fed a high fat/high cholesterol diet [29]. In addition, over-expression of PEMT in McArdle RH 7777 (rat hepatoma) cells, that do not express PEMT, increased apoB and TG secretion by 3 fold [28]. We tested to see if decreased *PEMT* expression might be responsible for the reduced secretion of VLDL particles from primary human hepatocytes over time. However, when we increased PEMT activity in primary human hepatocytes after 2 days in culture, we did not observe a difference in lipoprotein secretion in comparison to control cells. Thus, decreased PEMT does not appear to have an important role in the decrease of lipoprotein secretion in primary human hepatocytes in prolonged culture. Alternatively, this could also mean that, unlike rodent hepatocytes, PEMT does not have a rate limiting role in VLDL secretion in primary human hepatocytes. Interestingly, the expression of TGH, another key protein in VLDL assembly and secretion, is also decreased. This could suggest that the attenuated expression of TGH or other proteins involved in VLDL assembly (e.g. microsomal TG transfer protein) could be responsible for the impaired VLDL secretion in primary hepatocytes over prolonged culture [42].

Variations in culture formats, cell density, and media supplements have been shown to vary morphology and cellular response to xenobiotics in primary human hepatocytes [43]. In addition, cultivation of primary rat hepatocytes in media supplemented with pyruvate, lactate, dexamethasone, and oleate stimulate a high rate of TG secretion for up to 3 days in culture [44]. Therefore, more research could be done to prevent de-differentiation and maintain liver-specific functions of primary human hepatocytes during long-term cultures.

4.4.2 Characterization of lipoprotein secreted by primary human hepatocytes

After incubation of primary human hepatocytes with serum free media, we isolated 3 distinct fractions of lipoproteins that corresponded to VLDLs, LDL, and HDLs by FPLC. This is consistent with a previous study in which 3 lipoprotein fractions were isolated from primary human hepatocytes cultures by density centrifugation [15]. It should be noted that the secretion of VLDL sized particles from the primary human hepatocytes were not stimulated with fatty acid incubation. In contrast, incubation with fatty acids is necessary to stimulate VLDL secretion in human derived hepatoma cell lines [45-47]. In the absence of fatty acid priming, HepG2 cells secrete little to no lipoproteins in the size and density of VLDLs [48]. Instead, most $apoB_{100}$ was associated with a fraction of lipoprotein similar in size and shape to plasma LDL [48]. This would result if hepatoma cells had attenuated expression of enzymes that play key roles in VLDL assembly and secretion (e.g. PEMT, TGH).

Consistent with human physiology [49], $ApoB_{100}$ was the only form of apoB found associated with the lipoproteins secreted by primary human hepatocytes. In contrast, rodent hepatocytes secrete $apoB_{48}$ as well as $apoB_{100}$ in association with VLDL particles [50]. We also show that VLDLs secreted by primary human hepatocytes contain a high CE content as well as TG. This differs from VLDL secreted by primary rodent hepatocytes which carries the majority of TG in VLDL and CE in HDL particles [51-53]. In human plasma, CE is associated with VLDL and HDL [51-53]. The major difference in lipoprotein-CE distribution between humans and rats or mice is due the activity of CE transfer protein (CETP) which catalyzes the transfer of CE from HDL to TG-rich lipoproteins (e.g. LDL, VLDL) in exchange for TG [54]. CETP is secreted by human hepatocytes but not rodent hepatocytes [54-56]. Therefore, CE could be transferred from HDL to VLDL by CETP in the media after secretion. Alternatively, CE could be packaged and secreted along with TG into VLDL in primary human hepatocytes. CE synthesis, specifically acyl-coenzyme A: cholesterol acyltransferase activity, has

been implicated in the regulation of nascent VLDL secretion [57]. However, acyl-coenzyme A: cholesterol acyltransferase is expressed in both mouse and human livers [58].

4.4.3 Effect of NA on lipid and lipoprotein metabolism in primary human hepatocytes

The prevailing hypothesis of VLDL (and LDL) lowering effects of NA has been attributed the inhibition TG lipolysis in adipocytes when NA binds to the G protein-coupled receptor GPR109A [59-61]. The antilipolytic action of NA is believed to decrease circulating non-esterified fatty acid levels, thereby impairing hepatic TG synthesis and VLDL secretion. This would in turn lead to a reduced formation of LDL particles, which are atherogenic. However, the lowering of circulating non-esterified fatty acid levels upon NA administration is followed by a marked rise in fatty acid levels within hours [62, 63]. Furthermore, circulating free fatty acid levels are continuously elevated during long-term NA treatment, whereas the lipid-lowering effects still persist [64]. NA has also been observed to impair diacylglycerol acyltrasferase 2 –dependent triglyceride synthesis, as well as apoB and VLDL secretion in human hepatoma cells [65, 66]. We show that NA does not alter TG synthesis or VLDL secretion in primary human hepatocytes. Furthermore, NA does not increase HDL levels *via* increased secretion of apoA1 from human hepatocytes. These data suggests that NA's mode of pharmacological action (e.g. reduced VLDL secretion, increased HDL) is not the result of NA's effect on the liver.

In conclusion, we show that primary human hepatocytes are less prone to de-differentiation than rodent primary hepatocytes after isolation. Primary human hepatocytes also express the enzymes involved in lipoprotein secretion. In addition, primary human hepatocytes secrete a lipoprotein profile similar to that of human plasma in comparison to human derived hepatoma cell line or

rodent primary hepatocytes. Therefore, primary human hepatocytes are a more relevant model to study lipid-related human disorders than hepatoma or rodent primary hepatocytes models. When we used primary human hepatocytes to study the effect of NA on hepatic lipid and lipoprotein metabolism, we determined the pharmacological action of NA is not the result of its effect on the liver.

Figure 4.1 Hoechst staining of primary human hepatocytes

Primary human hepatocytes were fixed in 10% buffered formalin and stained with Hoechst stain 24, 48, and 72 h after visible attachment of hepatocytes to dish. The number of apoptotic nuclei from >200 cells in each preparation was measured as a percentage of total number of hepatocytes. Data are means ± S.E. from 3 different preparations of primary human hepatocytes, each analyzed in duplicate. * $p<0.05$ (1-way ANOVA) in comparison to day 1.

Figure 4.1

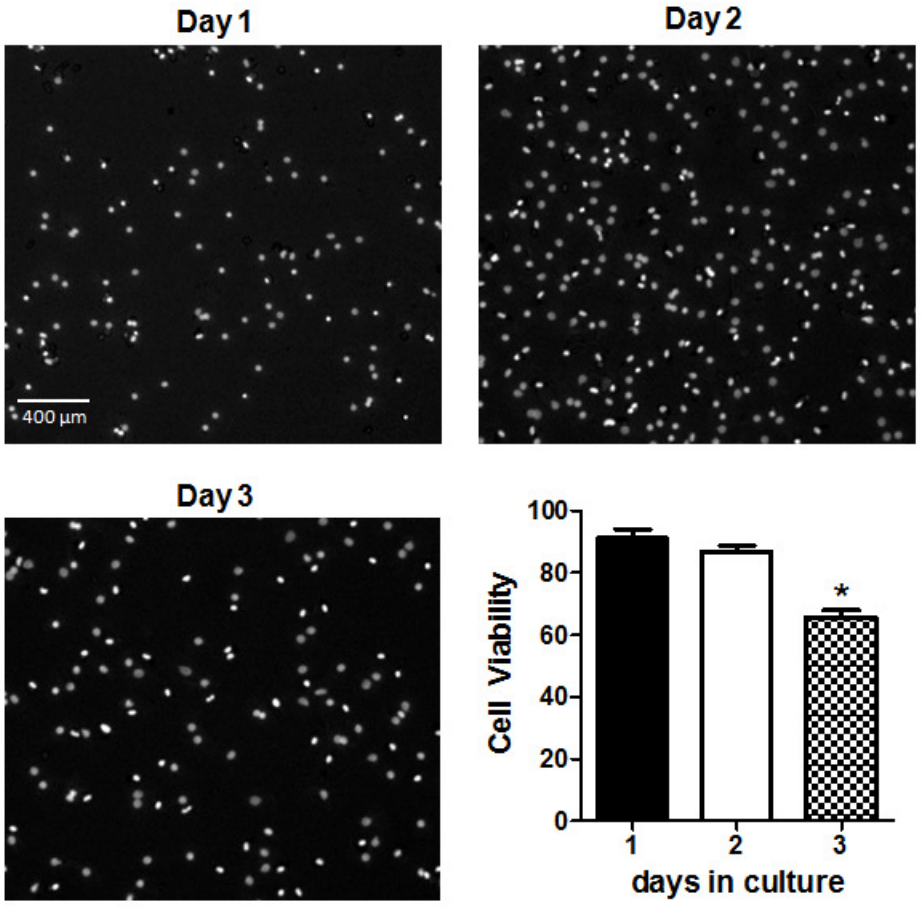

Day 1
Day 2
Day 3
400 µm
Cell Viability
100
80
60
40
20
0
1
2
3
*
days in culture

Figure 4.2 Trypan blue staining of primary human hepatocytes in culture.

Primary human hepatocytes in dishes were directly stained with Trypan blue. The number of dead cells from >100 cells was calculated as a percentage of total number of hepatocytes. Data are means ± S.E. from 3 different preparations of primary human hepatocytes and each were analyzed in duplicate. * $p<0.05$ (1-way ANOVA) in comparison to day 1.

Figure 4.2

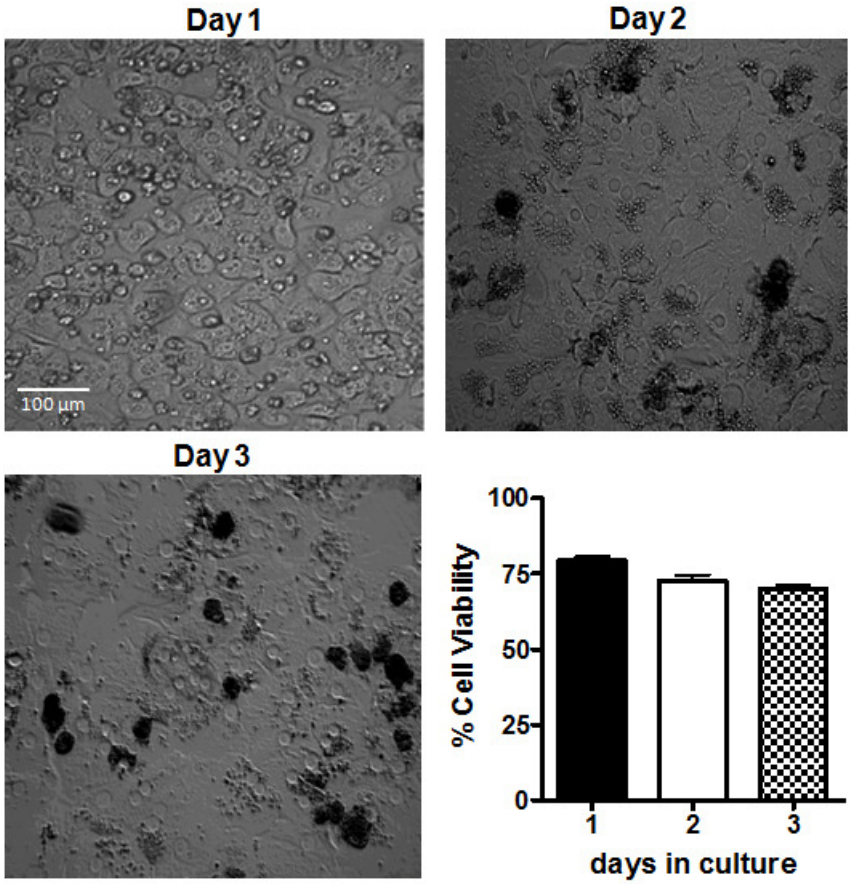

Day 1
Day 2
100 µm
Day 3
100
75
50
25
0
% Cell Viability
1
2
3
days in culture

Figure 4.3 Lipid and apolipoprotein secretion.

(A) The amounts apolipoprotein B_{100} ($apoB_{100}$), apolipoproteinA1 (apoA1), apolipoprotein E (apoE), and albumin were measured in the media of primary human hepatocytes cultured in serum free RPMI media. **(B, C)** The amounts of [^{3}H]triacylglycerol (TG) and [^{3}H]phosphatidylcholine (PC) secreted from primary hepatocytes over 4 h were measured as a percentage of total radiolabelled TG or PC in cell and media. Data are means ± S.E. from 3 different preparations of primary human hepatocytes and each were analyzed in duplicate. * $p<0.05$ (1-way ANOVA) in comparison to day 1.

Figure 4.3

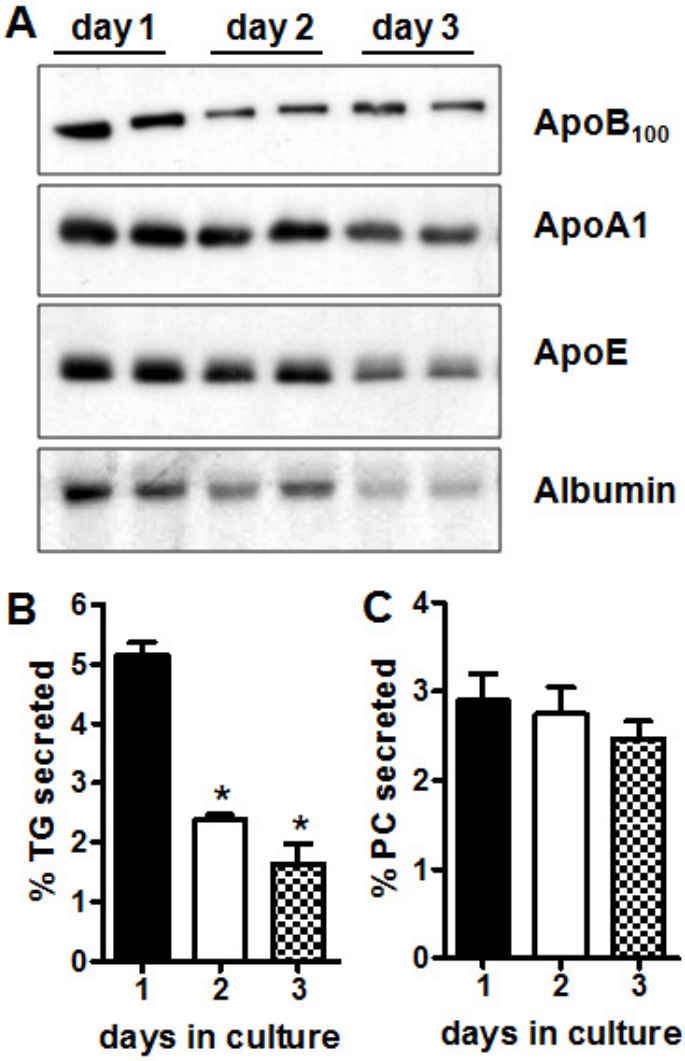

A
day 1
day 2
day 3
ApoB100
ApoA1
ApoE
Albumin
B
% TG secreted
6
5
4
3
2
1
0
*
*
1
2
3
days in culture
C
% PC secreted
4
3
2
1
0
1
2
3
days in culture

Figure 4.4 Lipoprotein profile of primary human hepatocytes.

Media from 3 dishes of primary human hepatocytes maintained in serum free RPMI were pooled, concentrated and analyzed for **(A)** TG and **(B)** cholesterol content in lipoprotein fractions by fast-protein liquid chromatography. Graph is representative of 3 independent preparations.

Figure 4.4

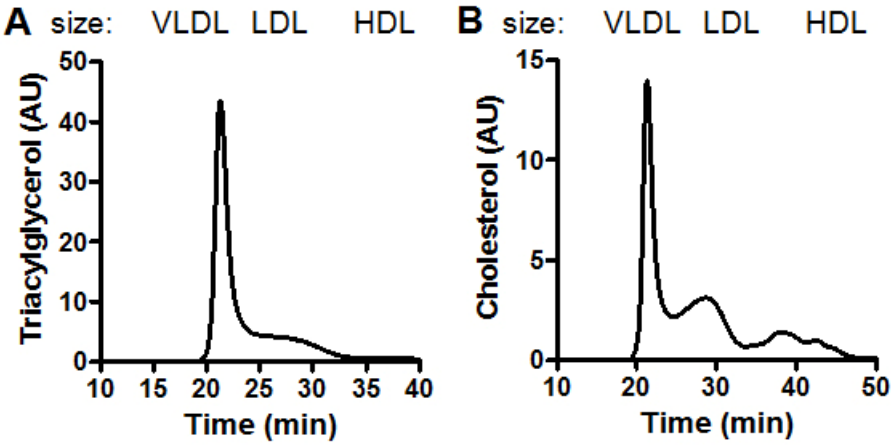

A size: VLDL LDL HDL
Triacylglycerol (AU)
50
40
30
20
10
0
10 15 20 25 30 35 40
Time (min)
B size: VLDL LDL HDL
Cholesterol (AU)
15
10
5
0
10 20 30 40 50
Time (min)

Figure 4.5 Lipid accumulation in primary human hepatocytes in culture

Cellular concentration of **(A)** TG, **(B)** cholesterol, **(C)** cholesteryl ester (CE), and **(D)** total phospholipids (PL) were measured in primary human hepatocytes. **(E)** Incorporation of [^{3}H]glycerol into cellular TG was measured after 4 h in the presence or absence of oleic acid (OA). Data are means ± S.E from 3 different preparations of primary human hepatocytes, with each analyzed in duplicate. * $p>0.05$ (1-way ANOVA) in comparison to day 1, # $p<0.05$ (2-way ANOVA) in comparison to –OA.

Figure 4.5

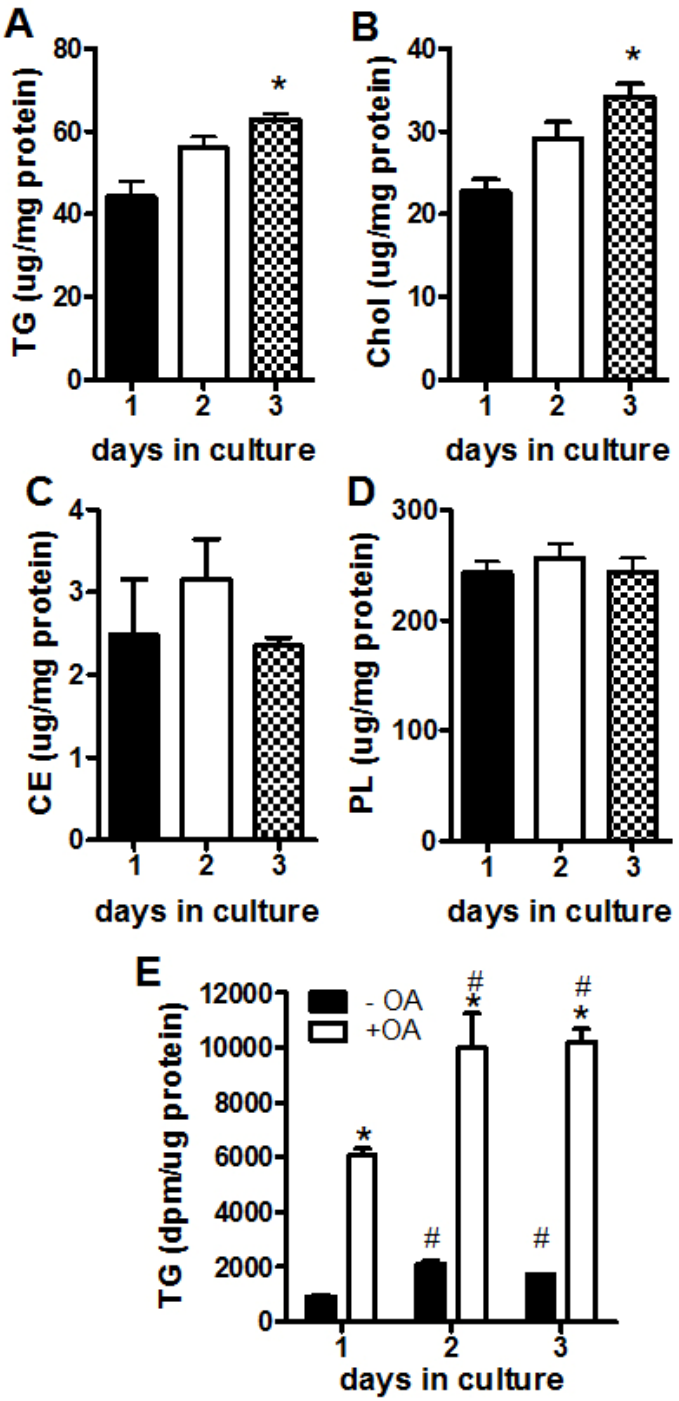

A
TG (ug/mg protein)
80
60
40
20
0
1
2
3
days in culture
*
B
Chol (ug/mg protein)
40
30
20
10
0
1
2
3
days in culture
*
C
CE (ug/mg protein)
4
3
2
1
0
1
2
3
days in culture
D
PL (ug/mg protein)
300
200
100
0
1
2
3
days in culture
E
TG (dpm/ug protein)
12000
10000
8000
6000
4000
2000
0
- OA
+OA
*
#
1
2
3
days in culture

Figure 4.6 Phosphatidylcholine biosynthesis in primary human hepatocytes in culture.

(A) Protein expression of phosphatidylethanolamine *N*-methyltransferase (PEMT) and **(B)** CTP:phosphocholine cytidylyltransferase (CT)α in primary human hepatocytes was measured by immunoblotting. *In vitro* activity of **(C)** PEMT and **(D)** CT was also measured. **(E)** Total phosphatidylcholine (PC) synthesis *via* the incorporation of [^{3}H]glycerol into PC was measured after 4 h in primary human hepatocytes. Data are means ± S.E from 2-3 different preparations of primary human hepatocytes with each analyzed in duplicate. * $p<0.05$ (1-way or 2-way ANOVA)

Figure 4.6

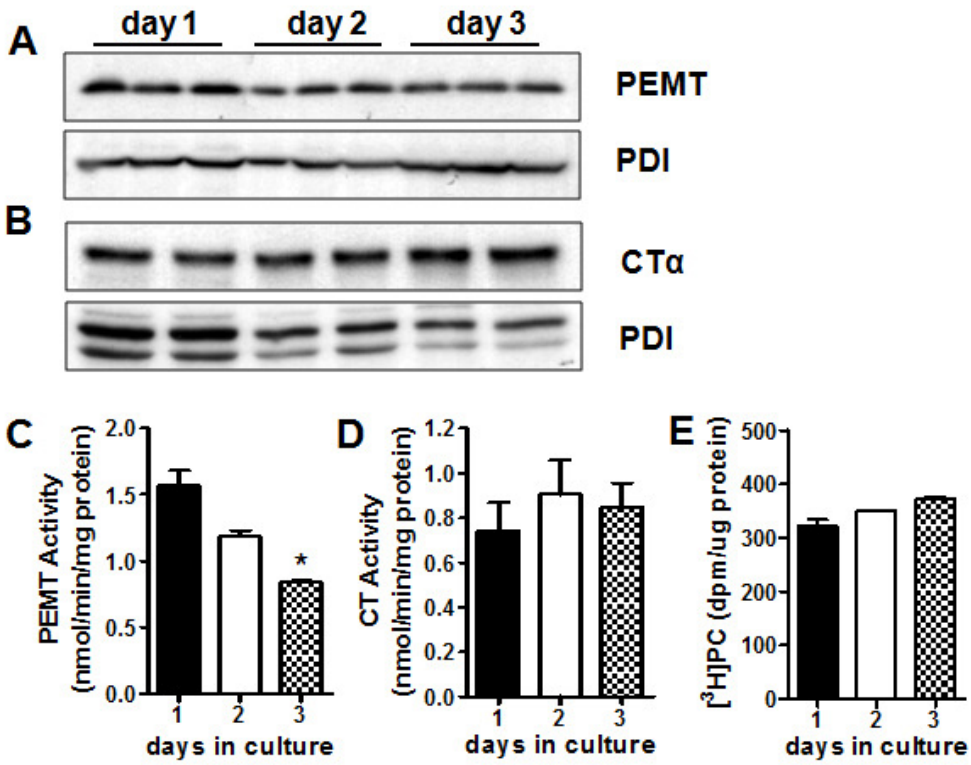

A
day 1
day 2
day 3
PEMT
PDI
B
CTα
PDI
C
PEMT Activity
(nmol/min/mg protein)
2.0
1.5
1.0
0.5
0.0
*
1
2
3
days in culture
D
CT Activity
(nmol/min/mg protein)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
days in culture
E
[3H]PC (dpm/ug protein)
500
400
300
200
100
0
1
2
3
days in culture

Figure 4.7 Apolipoprotein secretion in primary human hepatocytes overexpressing PEMT

Primary human hepatocytes were infected with no adenovirus, adenovirus containing green fluorescence protein (advGFP), or human phosphatidylethanolamine *N*-methyltransferase (advPEMT). **(A)** *In vitro* PEMT activity was measured after 1 and 2 days after infection. **(B)** Cellular PEMT protein level and protein levels of apolipoprotein (apo) B_{100}, apoA1, and apoE were also determined by immunoblotting. Graph is representative of 2 different preparations of primary human hepatocytes.

Figure 4.7

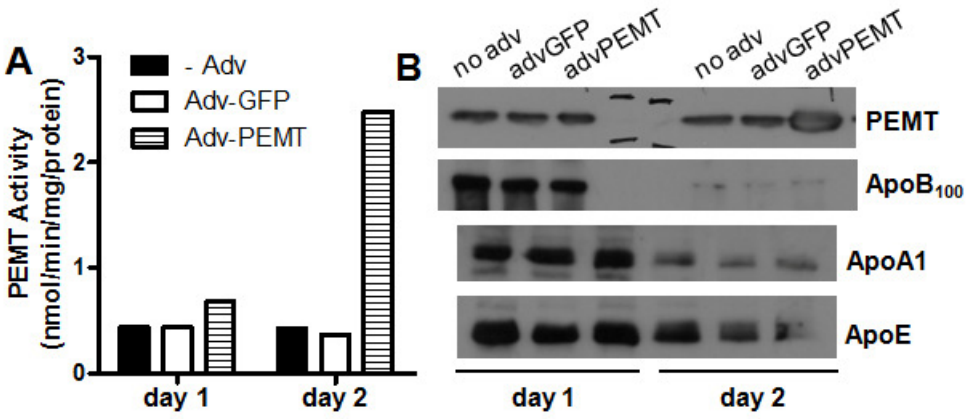

A
PEMT Activity
(nmol/min/mg/protein)
3
2
1
0
- Adv
Adv-GFP
Adv-PEMT
day 1
day 2
B
no adv
advGFP
advPEMT
no adv
advGFP
advPEMT
PEMT
ApoB100
ApoA1
ApoE
day 1
day 2

Figure 4.8 Expression of triacylglycerol hydrolase in primary human hepatocytes

Expression of triacylglycerol hydrolase in primary human hepatocytes. Protein level of triacylglcyerol hydrolase (TGH) was determined in primary human hepatocytes by immunoblotting (upper panel). Commassie blue staining of the membrane was used as loading control (lower panel). Graph is representative of 2 different preparations of primary human hepatocytes.

Figure 4.8

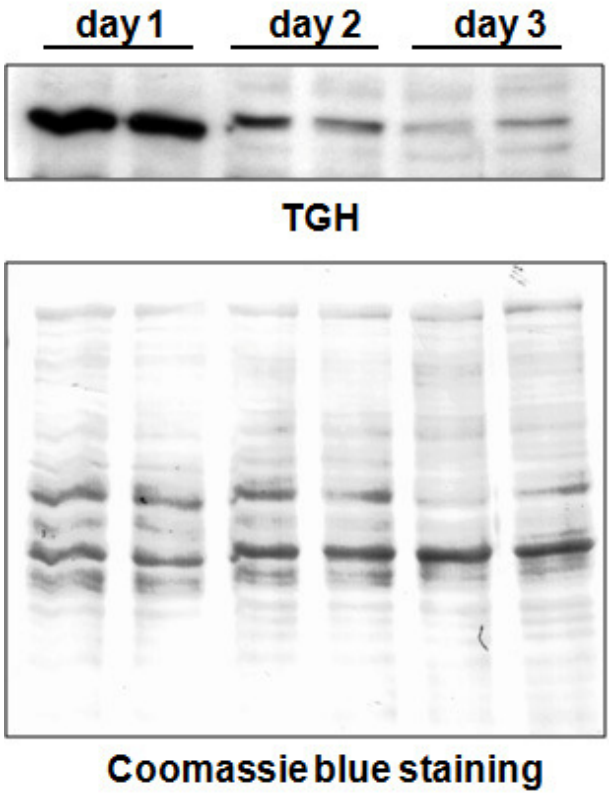


Figure 4.9. Lipid synthesis and VLDL secretion in nicotinic acid treated primary human hepatocytes

Primary human hepatocytes were pre-treated with media (-), 3mM nicotinic acid (NA), or 3mM nicotinamide (NAM). Incorporation of [^{3}H]glycerol into cellular **(A)** triacylglycerol (TG) and **(B)** phosphatidylethanolamine (PC) was measured after 4 h in the presence of oleic acid. **(C-E)** Cellular concentration of TG, cholesterol (chol), and cholesteryl ester (CE) were also measured in primary human hepatocytes in the presence or absence of NA. **(F)** The amount apolipoprotein B_{100} (apoB_{100}) was measured in the media of primary human hepatocytes cultured in serum free RPMI media containing only media, 3mM NA, or 3mM NAM. The concentration of **(G)** TG and **(H)** total cholesterol (chol and CE) in the media was analyzed in the presence and absence of NA. Data are means ± S.E from 3-9 different preparations of primary human hepatocytes, with each analyzed in duplicate or triplicate. * $p<0.05$ (1-way ANOVA) in comparison to no treatment (-). The apoB_{100} blot is representative of 3 independent preparations

Figure 4.9

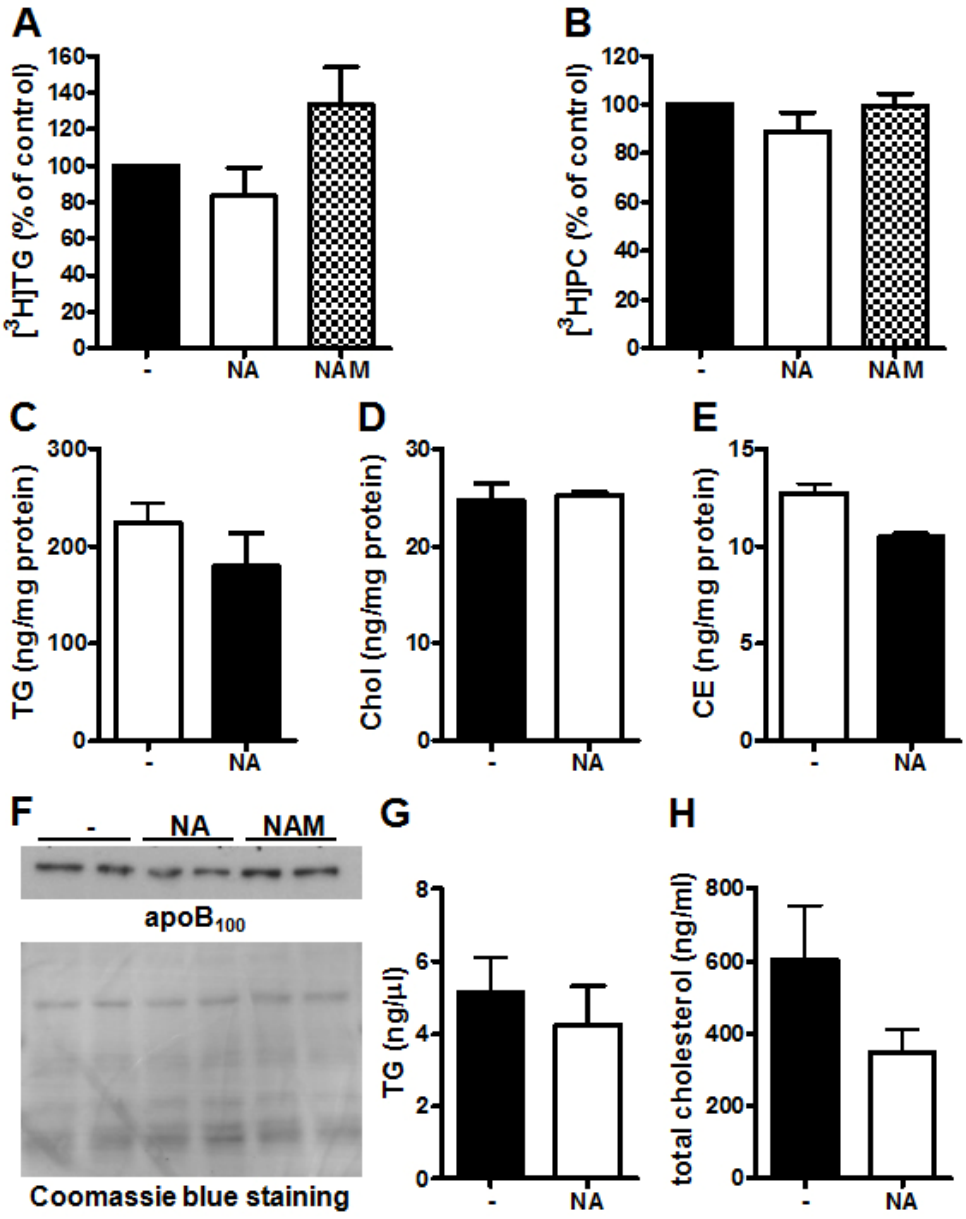
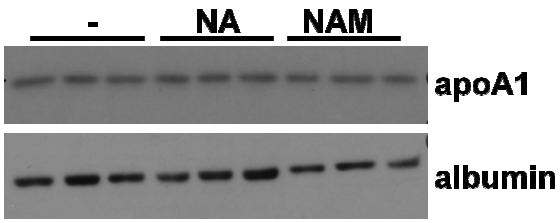

A
[3H]TG (% of control)
160
140
120
100
80
60
40
20
0
-
NA
NAM
B
[3H]PC (% of control)
120
100
80
60
40
20
0
-
NA
NAM
C
TG (ng/mg protein)
300
200
100
0
-
NA
D
Chol (ng/mg protein)
30
20
10
0
-
NA
E
CE (ng/mg protein)
15
10
5
0
-
NA
F
-
NA
NAM
apoB100
Coomassie blue staining
G
TG (ng/µl)
8
6
4
2
0
-
NA
H
total cholesterol (ng/ml)
800
600
400
200
0
-
NA

Figure 4.10 Apolipoprotein A1 secretion in nicotinic acid treated primary human hepatocytes

The amounts of apolipoprotein A1 (apoA1) and albumin were measured in the media of primary human hepatocytes cultured in serum free media containing only media (-), 3mM nicotinic acid (NA), or 3mM nicotinamide (NAM). Graph is representative of 3 independent preparations.

Figure 4.10



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CHAPTER 5: Conclusions and future directions

5.1 Conclusions

The physiological importance of PC biosynthesis in various cellular processes such as VLDL secretion [1-3] and cell proliferation [4] has been shown in various landmark studies. The experiments outlined in this thesis represent an attempt to identify the importance of PC metabolism to survival and hepatocyte proliferation after 70% partial hepatectomy in mice. We have also characterized some fundamental aspects of lipid and lipoprotein metabolism in primary human hepatocytes. In particular, we evaluated the synthesis of phospholipids in relation to lipoprotein secretion during the first 3 days of the primary human hepatocytes in culture.

In our main study, we dissected the importance of PC biosynthesis during liver regeneration after major liver resection. In contrast to previous *in vitro* work, we showed that the CDP-choline dependent pathway of PC biosynthesis is not required for normal hepatocyte proliferation and regeneration of liver mass after PH. Surprisingly, liver regeneration proceeded normally and PC levels were maintained in LCTα$^{-/-}$ mice despite a 50% reduction in total PC biosynthesis. We hypothesized that under physiological condition, CTα-deficient hepatocytes may obtain PC from other sources to sustain membrane synthesis during rapid cell division. As such, future studies should focus on identifying the mechanisms by which LCTα$^{-/-}$ hepatocytes sustain PC levels after PH. Possible avenues include decreased VLDL secretion and/or increased uptake of circulating lipoproteins (e.g. HDL, chylomicrons, LDL). In addition, CTα$^{-/-}$ deficient hepatocytes after hepatectomy may also be a good model for determining whether cells differentially respond to limiting PC under conditions of proliferation. An example is the changes in activation of gluconeogenesis, and SREBP.

When LCTα$^{-/-}$ mice were stressed with a HF diet, hepatic PC levels were reduced and hepatocytes accumulated TG. After PH, HF-fed LCTα$^{-/-}$ mice

displayed normal liver regeneration but the survival rate was decreased. Additional studies have demonstrated that decreased hepatic PC/PE, not PC content, impaired survival after PH. By using LCTα- deficient and PEMT-deficient mouse models, we also, for the first time, correlated PC/PE with hepatic TG accumulation and the progression of NAFLD pathology. We demonstrated that significantly reduced hepatic PC/PE increased cellular inflammation and oxidative stress, which are also associated with the development of NASH.

Decreased PC content and PC/PE have previously been observed in patients with NASH [5, 6]. Recently, altered PC/PE in leptin-deficient mice, a rodent model of obesity, has been linked to ER stress [7]. Therefore, hepatic PC/PE may be a useful measure of NAFLD. However, it would be necessary to assess the correlation between PC/PE and NAFLD development in other models of obesity and/or NASH such as leptin receptor-deficient (*db/db*) mice or SREBP-1c transgenic mice. In addition, plasma PC/PE as well as PC/PE in VLDLs were reduced in *Pemt*$^{-/-}$/*Ldlr*$^{-/-}$ mice fed a high fat/high cholesterol diet [8]. It would be interesting to determine whether decreased plasma or VLDL PC/PE also correlate with NAFLD progression. If it does, it would provide a non-invasive marker for NAFLD.

Our data suggests that increased dietary intake of choline may be a viable therapy to prevent the development of the more severe forms of NAFLD and to improve post-operative complications in NAFLD patients undergoing liver surgery. Supplementation of choline to NAFLD-*Pemt*$^{-/-}$ mice prevented inflammation, improved energy metabolism, and increased survival after PH. While the positive effects of choline can be attributed to increased PC and PC/PE, the contribution of choline to methionine-homocysteine pathway cannot be ignored. It is possible that methylation of gene promoters can regulate expression of genes involved in inflammation [9]. Since, betaine (a product of

choline), can act as a methyl group donor in the homocysteine-methionine methylation pathway, it would be interesting to see if betaine supplementation could also improve survival of NAFLD mice after PH.

Interestingly, choline supplementation of HF-fed *Pemt*$^{-/-}$ mice did not significantly reduce hepatic TG accumulation. Inhibition of MDR2 in *Pemt*$^{-/-}$ mice fed a choline deficient diet corrected the loss of hepatic PC but also did not completely prevent TG accumulation [6, 10]. This may suggest the lack of PEMT activity may play a role in hepatic TG accumulation independent of choline deficiency. Recent studies have implicated a role of PEMT and PC/PE in lipid droplet stability in adipocytes and drosophila, respectively [11-13]. Therefore, future studies should address the role of PEMT in hepatic lipid droplet metabolism, particularly in relation to VLDL assembly and secretion.

In the last study, we characterized the viability of primary human hepatocytes in culture by assessing hepatocyte specific functions such as lipoprotein metabolism. We found that primary human hepatocytes were less prone to de-differentiation than primary rodent hepatocytes and were able to continually secret lipoproteins up to 3 days in culture, without initial incubation with fatty acids. In addition, the primary human hepatocytes secreted a lipoprotein profile similar to that of human plasma. In this respect, primary human hepatocytes are a more relevant model to study lipid-related human disorders than hepatoma cell lines or rodent primary hepatocyte models. Future studies should further characterize the regulation of lipid synthesis and lipid secretion in the primary human hepatocytes. Initial studies should involve the regulation of SREBP, liver X receptor, and peroxisome proliferator-activated receptors in response to different nutrient conditions.

In summary, this body of work adds to the understanding of how PC metabolism is physiologically important after hepatectomy. While we

demonstrated that *de novo* hepatic PC synthesis was not essential for normal liver regeneration, we showed that PC synthesis and the maintenance of hepatic PC/PE was important for hepatic energy metabolism and survival after PH. We also began the preliminary analysis of lipid and lipoprotein metabolism in primary human hepatocytes. This will begin to highlight the differences and similarities between human and rodent hepatic lipid metabolism and allow better translatability of potential drug targets identified in rodent models.

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