Hypocholesterolemia in Barth Syndrome

by

Kristin Dawn Hauff

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

Doctor of Philosophy

Department of Pharmacology & Therapeutics

University of Manitoba

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THE UNIVERSITY OF MANITOBA

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Of

Doctor of Philosophy

Abstract

Barth Syndrome (BTHS) is a multifaceted disease, revealing no clear relationship between genotype and phenotype. In fact, over time, the same patient can exhibit a multitude of different symptoms. Of note, many patients relapse into a worsening disease state during the years often associated with the onset of puberty, a state heavily reliant on cholesterol for steroid production. For years hypocholesterolemia has been reported as a characteristic of patients with BTHS, without further inquiry. We have aimed to characterize the defect in cholesterol metabolism observed in BTHS, and to determine whether the same defect can be recreated by inhibiting CL remodeling, the phospholipid defective in BTHS.

Radiolabeling studies, in association with real time PCR and enzyme activity data suggests that cells deficient in mature CL, as demonstrated in BTHS, are able to maintain cholesterol levels, under normal cell culture conditions, at the expense of increased HMG-CoA reductase enzyme activity. This, however, results in the inability of these cells to further compensate when external cholesterol levels are low, as is the case with serum removal. These results were not due to any differences in cell cycle progression, as BTHS cells entered S-phase at the same rate as control cells. It is therefore possible that cholesterol synthesis in BTHS patients is limiting when demands are high.

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Finally, I would like to express my immense gratitude to my family, for passing along their genetic propensity for stubbornness, without which I wouldn't have made it this far, and constantly reminding me of what is important in life. I am forever grateful to know you will always be there for me no matter what direction my life takes me. In particular, my grandfather, who always made sure that I had the best out of life, including driving me an hour, daily, to every skating practice, every summer. My mother, who sacrificed everything she could to ensure that I had all that I needed, and drove me to plenty of skating practices herself, and Dan, who has been there for me every day. Especially Dan, who has learned acutely to manipulate my stubborn streak, and kept me from ever giving up. You have the power to make my frustrations fade, without which, I would have never made it through.

For you all, I am grateful.

"Let me tell you the secret that has led me to my goal. My strength lies solely in my tenacity."
- Louis Pasteur

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Abbreviations

3-MGA	-	3-methylglutaconic aciduria
7-AAD	-	7-aminoactinomycin D
AA	-	arachadonic acid
ABCA1	-	ATP-binding cassette protein A1
ACAT	-	acyl-CoA: cholesterol acyltransferase
ACC	-	acetyl-CoA carboxylase
ACS	-	ATP-dependent acyl-CoA synthetase
AD	-	acyl-CoA dehydrogenase
ADP	-	adenosine 5'-diphosphate
AGPAT	-	1-acylglycerol-3-phosphate O-acyltransferase
ALCAR	-	acetyl-L-carnitine
ALCAT1	-	acyl-CoA lysocardiolipin acyltransferase
ApoB	-	apolipoprotein B
ATP	-	adenosine 5'-triphosphate
BAD	-	Bcl-2-associated death promoter
BAK	-	Bcl-2 homologous antagonist/killer
Bax	-	Bcl-2–associated X protein
Bcl_2	-	B-cell lymphoma 2
Bcl-xL	-	basal cell lymphoma-extra large
Bid	-	BH3 interacting domain death agonist

BiP	-	immunoglobulin heavy chain binding protein
BrdU	-	5-bromo-2'-deoxyuridine
BSA	-	bovine serum albumin
BTHS	-	Barth Syndrome
C:AT	-	carnitine:acylcarnitine translocase
cAMP	-	cyclic adenosine monophosphate
CCPT	-	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CDP-DG	-	cytidine- 5'-diphosphate-1,2-diacyl-*sn*-glycerol
CDR1	-	Yeast CDP-DG synthase (phosphatidate cytidylyltransferase) 1/2
CDS1/2	-	CDP-DG synthase (phosphatidate cytidylyltransferase) 1/2
CE	-	cholesterol ester
CEPT	-	CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase
CH	-	cholesterol
CHO	-	Chinese Hamster Ovary cells
CK	-	choline kinase
CL	-	cardiolipin
CPTI/II	-	carnitine palmitoyltransferase-1
Crd1	-	yeast homologue for CDS1
CT	-	CTP:phosphocholine cytidylyltransferase
CTP	-	cytidine- 5'-triphosphate
CTP	-	cytidine 5'-triphosphate

Cyt c	-	cytochrome c
DG	-	diacylglycerol
DGAT	-	diacylglycerol acyltransferase
DHA	-	docosahexanoic acid
DHAP	-	dihydroxyacetone phosphate
DMEM	-	Dulbecco's modified eagle medium
DNA	-	deoxyribonucleic acid
DTT	-	dithiothreitol
EDTA	-	ethylenediamine-tetraacetic acid
EH	-	enoyl-CoA hydratase
eIF2α	-	eukaryotic translation initiation factor 2α
EK	-	ethanolamine kinase
ER	-	endoplasmic reticulum
ET	-	CTP:phosphoethanolamine cytidyldyltrasnferase
ETC	-	electron transport chain
FACS	-	fluorescent activated cell counting
FBS	-	fetal bovine serum
G-3-P	-	glycerol-3-phosphate
G-CSF	-	granulocyte colony stimulating factor
GLUT1	-	glucose transporter 1
GPAM	-	glycerol-3-phosphate acyltransferase, mitochondrial

GPAT	-	glycerol-3-phosphate-O-acyltransferase
GRP78	-	78kDa glucose regulated protein
hCLS1	-	human cardiolipin synthase1
HD	-	L-3-hydroxyacyl-CoA dehydrogenase
HDL	-	high density lipoprotein
HEPES	-	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HMG-CoA	-	3-hydroxy-3-methylglutaryl-CoA
HMGR	-	3-hydroxy-3-methylglutaryl-CoA reductase
HMGS	-	HMG-CoA synthase
HSP70	-	heat shock protein 70
HUVEC	-	human umbilical vein vascular endothelial cells
IFNγ	-	interferon gamma
Insig-1	-	insulin responsive gene
INTERHEART	-	International Heart Study
KT	-	3-ketoacyl-CoA thiolase
L_4-CL	-	tertralinoelyl-CL
LA	-	linoleic acid
LAD	-	long chain dehydrogenase
LDL	-	low density lipoprotein
LDLR	-	low density lipoprotein receptor
LPAAT	-	lysophosphatidic acid acyltransferase

LPS	-	lipopolysaccharide
LRC	-	Lipid Research Clinics trial
LXR	-	liver X receptor
MAD	-	medium chain dehydrogenase
MAPK	-	mitogen activated protein kinase
MLCL	-	monolysocardiolipin
MLCL AT	-	monolysocardiolipin acyltransferase
MRFIT	-	Multiple Risk Factor Intervention Trial
NADH	-	nicotinamide adenine dinucleotide, reduced
NAO	-	10-N-nonyl acridine orange
NCEP	-	National Cholesterol Education Program
NIH3T3	-	National Institutes of Health 3T3 cells
NTC	-	no template control
PA	-	phosphatidic acid
PAP	-	PA phosphatase
PBS	-	phosphate buffered saline
PC	-	phosphatidylcholine
PDC	-	pyruvate dehydrogenase complex
PDK2/4	-	pyruvate dehydrogenase kinase 2/4
PE	-	phosphatidylethanolamine
PEL1	-	yeast phosphatidylglycerol synthase

PEPCK	-	phospho-enol pyruvate carboxy kinase
PERK	-	PKR-like ER kinase
PG	-	phosphatidylglycerol
PGP	-	PG phosphate
PGS1	-	phosphatidylglycerol synthase
PGS-S	-	CHO cell line defective in PGS1
PI	-	phosphatidylinositol
PL	-	phospholipid
PLA_2	-	phospholipase A_2
PPAR	-	peroxisome proliferator activated receptor
PS	-	phosphatidylserine
qRT-PCR	-	quantitative, or real time reverse transcription-polymerase chain reaction
RNA	-	ribonucleic acid
RPMI	-	Roswell Park Memorial Institute
RT-PCR	-	reverse transcription-polymerase chain reaction
S1/2P	-	site 1/2 protease
SAC	-	short chain dehydrogenase
SCAP	-	SREBP cleavage activating protein
siRNA	-	small interfering ribonucleic acid
SLOS	-	Smith-Lemli-Opitz Syndrome
SM	-	sphingomyelin

SRE	-	sterol response element
SREBP-2	-	sterol response element binding protein-2
STAR	-	steroid acute response
TAZ	-	tafazzin
tBid	-	truncated Bid
TCA cycle	-	tricarboxylic acid (Krebs) cycle
TG	-	triacylglycerol
TLC	-	thin layer chromatography
T_m	-	transition temperature
TNFα	-	tumor necrosis factor-alpha
U937	-	human monoblastic cells
UPR	-	unfolded protein response
VAD	-	very long chain acyl-CoA dehydrogenase
VLDL	-	very low density lipoprotein

Chapter 1: Cardiolipin Metabolism and Barth Syndrome

Chapter 1: Cardiolipin Metabolism and Barth Syndrome

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Abstract

Many advances have occurred in the field of Barth Syndrome biology in the 26 years since it was first described as an X-linked cardiomyopathy. Barth Syndrome is the first human disease recognized in which the primary causative factor is an alteration in cardiolipin remodeling. Cardiolipin is required for the optimal function of many proteins within the mitochondria, particularly in the respiratory chain and is involved in the mitochondrial-mediated apoptotic process. The appropriate content of cardiolipin appears to be critical for these functions. Cardiolipin is synthesized *de novo* in mitochondria and is rapidly remodeled to produce cardiolipin enriched in linoleic acid. The Barth Syndrome gene TAZ has been identified and expression of the gene yields proteins known as tafazzins. Mutations in TAZ result in a decrease in tetra-linoleoyl species of cardiolipin and an accumulation of monolysocardiolipin within cells from Barth Syndrome patients. Although the protein product of the TAZ gene displays sequence homology to the glycerolipid acyltransferase family of enzymes, its precise biochemical function remains to be elucidated. In this review we highlight some of the recent literature on cardiolipin metabolism and Barth Syndrome.

Keywords: Cardiolipin; Barth Syndrome; Mitochondria; Cardiomyopathy; neutropenia; X-linked genetic disease; tafazzin; phospholipid remodeling; acyltransferase

Abbreviations: BTHS, Barth Syndrome; CL, cardiolipin; TAZ, tafazzin; PA, phosphatidic acid; CDP-DG, cytidine-5'-diphosphate-1,2-diacyl-*sn*-glycerol; CDS, CDP-DG synthetase; PG, phosphatidylglycerol; PGP, PG phosphate; PLA_2, phospholipase A_2; MLCL, monolysocardiolipin; MLCL AT, monolysocardiolipin acyltransferase; PE,phosphatidylethanolamine; PC, phosphatidylcholine; L_4-CL, tetralinoleoyl-CL; ALCAT1, acyl-CoA:lysocardiolipin acyltransferase-1; 3-MGA, 3-methylglutaconic aciduria.

1.1. Introduction

The first description of an X-linked cardiomyopathic disease was made in the 1970s, where the triad of cardiomyopathy, neutropenia and 3-methylglutaconic aciduria (3-MGA) caused death in infancy when left untreated (Neustein *et al.*, 1979). Barth Syndrome (MIM302060, BTHS) was first characterized by Barth *et al* in 1983 (Barth *et al.*, 1983) and further characterized by Kelley *et al* in 1991(Kelley *et al.*, 1991b). Since then, BTHS boys have been recognized earlier and are receiving the medical attention they require. Early recognition appeared to be the key to living longer, even into adulthood, as the average life expectancy of a male diagnosed with BTHS was increased from the high mortality that occurred in infants previously (Barth *et al.*, 1983; Ades *et al.*, 1993; Christodoulou *et al.*, 1994; Gedeon *et al.*, 1995). Although the incidence has been estimated to be as high as 1 in 100 000, BTHS is still a poorly recognized disease.

1.1. The BTHS Defect

1.1.1. Genotype

BTHS occurs due to mutation in the Tafazzin (TAZ) gene known previously as G4.5. The 10 966 base pair long TAZ gene was localized, by linkage analysis, to the Xq28.12 region of the human genome (Bolhuis *et al.*, 1991; Ades *et al.*, 1993; Bione *et al.*, 1996; D'Adamo *et al.*, 1997). Since then, over 90 different pathogenic mutations have been described (Gonzalez, 2004; Gonzalez, 2005), including frameshifts, non-sense, splice-site, and missense mutations (as reviewed in (Barth *et al.*, 2004)). A publicly maintained

log of mutations in TAZ has been initiated in association with the BTHS Foundation (Gonzalez, 2004).

A clinical disease state has not been reported in female carriers due to the X chromosomal location. A certain proportion of cells should randomly inactivate the X chromosome with mutation while others inactivate the wild type gene, resulting in a milder BTHS phenotype (Lyon, 1961). However, it seems that more severe diseases cause a skewed X-inactivation, leading to a larger than predicted population of wild-type cells in the carrier-state (Orstavik et al., 1998). The exact mechanism of this skewed inactivation is unknown, but may be a result of elimination of mutant cells due to selective pressures.

The TAZ gene product, tafazzin, displayed a high sequence homology to a family of glycerolipid acyltransferases (Neuwald, 1997), and thus, its function has been proposed as an acyltransferase. The 1.8 kb transcript contains 11 exons in humans, leading to a 292 amino acid protein (**Figure 1-1**) (Bione et al., 1996). A theoretical potential for 16 alternatively spliced products existed based on the combination of exons-5-7 that could be excluded during transcription and alternative splicing revealing two putative translational initiation sequences (Bione et al., 1996). The existence of separate initiation codons in the transcripts has led to the belief that the TAZ gene product may exist in a membrane bound form, utilizing the ATG sequence in exon-1, producing an N-terminal hydrophobic region, and a soluble form by utilizing the initiation site just upstream of exon-4. However, none of the human TAZ splice variants missing the N-teminal hydrophobic region were able to reconstitute function in a yeast *taz1Δ* mutant (Vaz *et al.*, 2003). This observation was corroborated in BTHS patients with mutations in exons-1 or

-2 (Johnston et al., 1997). These patients did not exhibit milder symptoms than their counterparts displaying mutations downstream of the second initiation site. The function of this second site of initiation, as well as the presence of other potential sites of initiation, remains to be determined.

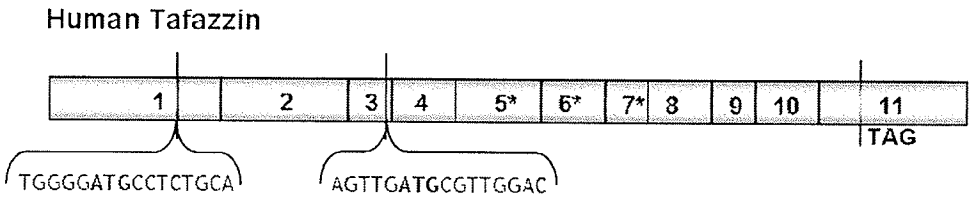

Figure 1-1: Schematic Diagram of the Human Tafazzin cDNA, illustrating the exon alignment and two putative translational initiation sites.

Starred (*) exons indicate potential for alternative splicing. Adapted from Bione et al. 1996 (Bione et al., 1996).

A *Saccharomyces cerevisiae* model of BTHS was developed and has been utilized to screen human TAZ splice variants for functional reconstitution of the *taz1Δ* defect (Vaz *et al.*, 2003). From these studies the authors determined two variants that were able to recover the temperature sensitive growth defect of the *taz1Δ* mutant. The variant lacking exon-5 was able to fully recover the slow growth defect. Cells transformed with the full-length cDNA failed to restore growth of *taz1* null strains to wild type levels. The authors attributed this to the fact that the full-length cDNA transformant may not actually represent a physiologically relevant mRNA, as exon-5 exhibited no sequence homology to other organisms. The presence of multiple mammalian TAZ splice variants in murine tissues, human monoblastic (U937) cells and human umbilical vein vascular endothelial cells (HUVECs) and a species specific difference in these variants between mice and humans has been demonstrated (Lu *et al.*, 2004). In addition to the full length tafazzin, three variants (lacking exon-5, or exon-7, or exon-5 + 7) were observed in the human U937 cells and HUVECs. In contrast, in murine tissues only TAZ splice variants lacking exon-5 and exon-9 were observed. *In vitro* translation of the variants lacking exon-5 resulted in only two protein products, corresponding to the use of alternative sites of initiation. Moreover, in that study five motifs were identified in human tafazzin that have been established as critical for function of the acylglycerolphosphate acyltransferase (AGPAT) superfamily of enzymes further implicating TAZ as an acyltransferase. Mutations occurring within these five regions in some BTHS patients have been established.

A recent study indicated the ability of human cultured lymphoblasts to utilize the full length protein as well as that lacking exon-5 (Gonzalez, 2005). Utilizing RT-PCR, the

expression pattern in BTHS patients, normal controls and primates were characterized. In that study, evidence was presented that supported a non-functional secondary initiation site and indicated that the putative "short" soluble TAZ mRNA did not exist. Although none of the eleven lymphoblast samples from BTHS and control subjects produced an alternatively spliced "short" RNA product, the second initiation site upstream of exon-4 might still be utilized as a translational initiation site. The results of this study supported our finding of four transcripts produced by U937 cells and HUVECs (full-length, exon-5Δ, exon-7Δ, exon-5 + 7Δ) (Lu *et al.*, 2004). The author resolved the controversy of exon-5 by an evolutionary analysis, wherein TAZ gene conservation was compared (Gonzalez, 2005). It was concluded that non-primates are devoid of exon-5 and that the sequence did not occur until somewhere in the monkey lineage. Furthermore, the Old World Monkeys did not have an exon-5 splice acceptor sequence in their TAZ. It is therefore likely that exon-5 became functional only after the AG splice sequence occurred in New World Monkeys. The conservation of sequence in TAZ exon-5 appeared strong when hominoid primates were compared, suggesting that a functional incentive for this conservation existed. It was further concluded that in humans there is probably a certain degree of function for both the full length and exon-5 deleted tafazzins, but that this would not necessarily be discovered in non-primate models. In fact, the exon-5 containing, full length product was transcribed but no functional outcome was determined. The reason that humans contain two tafazzin gene products whereas most other species have a highly conserved version that lacks exon-5 is unknown. No obvious sequence homology to other functional protein domains can be found to aid in the explanation for the existence of exon-5. However, the discovery of BTHS subjects

with mutations in exon-5 support a role for exon-5 in humans and promote the need to further understand the intricacies of TAZ and the function of the full length protein in humans.

1.1.2. Phenotype

The severity of BTHS has not been demonstrated to correlate between genotype and phenotype to date, suggesting that there may be other unknown factors that modulate disease severity (Johnston *et al.*, 1997). A characteristic feature of BTHS was the presentation of cardiomyopathy leading to weakness and fatigue. In a few cases, sudden, fatal ventricular tachycardia was demonstrated to occur even during a period of seemingly good health (Barth *et al.*, 1983). The skeletal and cardiomyocyte fatigue of BTHS lead to delayed motor development, and may even be a presenting factor. Myocyte fatigue has been attributed to mitochondrial deficiency and several studies have described deficiencies in the respiratory chain leading to a decrease in oxidative phosphorylation (Barth *et al.*, 1983; Figarella-Branger *et al.*, 1992; Ades *et al.*, 1993; Christodoulou *et al.*, 1994). One earlier study reported structural changes in cardiac mitochondrial appearance (Neustein *et al.*, 1979). Other signs commonly associated with BTHS were, neutropenia, 3-MGA, short stature, failure to thrive, and decreased plasma cholesterol (CH) (Kelley *et al.*, 1991b). Recently, the potential of a cognitive defect was proposed (Mazzocco *et al.*, 2001). However, these deficits could be effects of living with a chronic illness, especially one that prevents children from participating in regular school activities. More recently, a larger cohort was followed which reached the same conclusion, BTHS is associated with a mild cognitive phenotype (Mazzocco *et al.*, 2007).

Neutropenia has been described as cyclic, indicating that it may or may not be measurable at any given time, but is a major cause for concern, as chronic bacterial infections are a major complication in the treatment of BTHS (Kelley *et al.*, 1991b). Many neutropenic disorders have been attributed to an increase in apoptosis of the neutrophillic precursors, providing the rationale for therapy utilizing granulocyte-colony stimulating factor (G-CSF), a cytokine that suppresses myeloid cell apoptosis (Stein *et al.*, 2003). The deficiency has been described as resulting from a maturational stop at the premyelocyte stage in neutrophil development (Barth *et al.*, 1983). However, a more recent study by this same group has refuted the theory of increased neutrophillic apoptosis or reduced development in BTHS (Kuijpers *et al.*, 2004).

A recent case reported a BTHS boy without clinically detectable levels of 3-MGA indicates that the presence of urinary 3-MGA may not be consistent, but a frequent clinical finding in BTHS (Schmidt *et al.*, 2004). Increased urinary 3-MGA excretion is a feature common to mitochondrial diseases [reviewed in (Barth *et al.*, 2004)]. The occurrence of 3-MGA was hypothesized to be the result of the breakdown of leucine possibly derived from catabolism of dietary proteins. The functional cause of 3-MGA in BTHS is unknown, and although it was considered to be most likely due to the presence of branched chain organic acids from dietary protein, fasting did not change the levels of 3-MGA detected (Christodoulou *et al.*, 1994). In addition, patient loading with leucine did not increase 3-MGA levels (Kelley *et al.*, 1991b; Christodoulou *et al.*, 1994) even when combined with fasting (Barth *et al.*, 1999). No enzymatic block in leucine breakdown in BTHS has been identified to date (Gibson *et al.*, 1991).

Although a relationship between the genetic dysfunction and the clinical presentation has intrigued researchers for some time, a correlation has not been elucidated to date. The recognition of certain BTHS-like syndromes that do not have a detectable TAZ mutation has further complicated the issue of understanding BTHS.

1.1.3. Therapy

Mutations in TAZ result in reduced levels of CL [reviewed in (Barth *et al.*, 2004)]. This feature is characteristic of BTHS cells, although current practice is to screen thrombocytes or fibroblasts for TAZ gene mutations. Current therapy for BTHS remains treating the symptoms and attempting to prevent serious episodes of infection and cardiac dysfunction, the leading causes of death in BTHS patients. A few therapies based on attempting to correct the metabolic disorder have been proposed, including supplementation with L-carnitine or pantothenic acid as an acyl chain co-factor (Ino *et al.*, 1988; Ostman-Smith *et al.*, 1994). Although initial studies seemed promising (Ostman-Smith *et al.*, 1994), a recent study did not observe a functionally significant outcome in long term treatment of three BTHS patients treated with pantothenate, a Coenzyme-A precursor (Rugolotto *et al.*, 2003). The ability of dietary linoleic acid to supplement the decreased species of tetralinoleoyl-CL (L_4-CL), based on its ability to restore levels in BTHS fibroblasts, has been suggested, but no conclusive trials have been reported to date (Valianpour *et al.*, 2003; Barth *et al.*, 2004).

1.2. Cardiolipin

CL is present in all mammalian cells containing mitochondria, but it is most abundant in cells with large numbers of mitochondria such as cardiac and skeletal myocytes [reviewed in (Hatch, 2004)]. For example, CL was shown to comprise 15-20% of the entire phospholipid (PL) phosphorous content in the mammalian heart. CL was shown to be localized almost exclusively to the inner mitochondrial membrane and was associated with membranes functional in myocardial electrical conductivity (Reig *et al.*, 1993; Hatch, 2004). Although the predominant molecular species of CL in mammalian cells was the L_4-CL and trilinoleoyl-oleoyl-CL species, there was a difference in the specific ratios of each in different cell types within an individual (Schlame *et al.*, 1999). The difference in this ratio between the cell types corresponded to the condensation of the mitochondrial cristae structure implicating the importance of the specificity in the acylation of CL.

In 2000, the possible involvement of CL in BTHS was first postulated (Vreken *et al.*, 2000). This represented the first time that a disorder had been linked to the aberrant metabolism of CL. These authors demonstrated that the rate of phosphatidylglycerol (PG) and CL synthesis was normal in cultured skin fibroblasts from BTHS patients, compared to controls. However, the total pool of CL and linoleic acid incorporation into CL were reduced. The reduction in total CL was initially attributed to an increase in the degradation of CL in BTHS.

1.2.1. Cardiolipin metabolism

1.2.1.1. De novo biosynthesis of cardiolipin

Biosynthesis of CL results in a unique di-phosphatidylglycerol structure [reviewed in (Schlame *et al.*, 2000; Hatch, 2004)]. Cardiolipin was shown to be synthesized *de novo* in mitochondria in mammalian cells (**Figure** 1-2). Phosphatidic acid (PA) is condensed with cytidine-5'-triphosphate (CTP) to form cytidine-diphosphate-1,2-diacyl-*sn*-glycerol (CDP-DG) with the release of pyrophosphate catalyzed by CDP-DG Synthetase (CDS-1 or -2), also known as PA:CTP cytidylyltransferase. We previously demonstrated that this is a major rate limiting step of CL biosynthesis in myoblastic heart cells (Hatch *et al.*, 1996). The second step adds glycerol-3-phosphate to the newly synthesized CDP-DG, yielding a molecule of PG phosphate (PGP) catalyzed by PGP Synthase (PGS1, formerly PEL1, in yeast) (Poorthuis *et al.*, 1976; McMurray *et al.*, 1978). Subsequently, PGP phosphatase rapidly removes the phosphate group to produce PG. The PGP intermediate does not accumulate in the cell. We have recently demonstrated that expression of both murine CDS-2 and PGS1 may be regulated by peroxisome proliferator-activated receptor α (Jiang *et al.*, 2004). In the final step of the CL biosynthetic pathway, PG is condensed with another molecule of CDP-DG to form a molecule of CL catalyzed by CL synthase (hCLS1) (Hostetler *et al.*, 1972). The synthesis of CL proceeds on the inner leaflet of the inner mitochondrial membrane (Schlame *et al.*, 1993). In yeast expression of PGS1 and CL synthase (CDR1 in yeast) were shown to be regulated by factors that guide mitochondrial growth (Gaynor *et al.*, 1991; Jiang *et al.*, 1999).

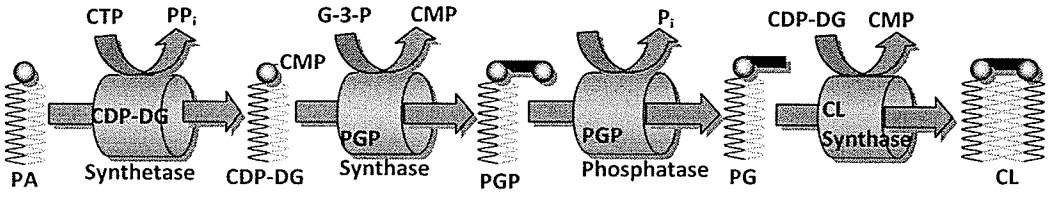

Figure 1-2: *De novo* synthesis of mammalian CL.

Condensation of PA with CTP to form CDP-DG with release of inorganic phosphate is catalyzed by CDP-DG Synthetase (CDS-1 or -2). The committed step adds Glycerol-3-phosphate (G-3-P) to CDP-DG yielding PGP catalyzed by PGP Synthase (PGS1). The phosphate is removed by PGP phosphatase. The final step is the condensation of PG and CDP-DG to form CL with release of CMP catalyzed by CL Synthase.

1.2.1.2. Cardiolipin remodeling

Newly synthesized CL is rapidly remodeled in mammalian tissues to generate CL species enriched in linoleic acid (**Figure 1-3**) [reviewed in (Hatch, 2004)]. The CL synthase was shown to have no preference in the species of fatty acyl chains utilized for *de novo* CL biosynthesis (Hostetler *et al.*, 1975; Rustow *et al.*, 1989). In addition, the molecular composition of CL was shown to be influenced by the composition of fatty acids ingested (Hoch, 1992; Valianpour *et al.*, 2003). The resulting tetra-acylated structure can adopt a number of different acyl chain combinations, however, the most biologically prevalent structure is L_4-CL [reviewed in (Schlame *et al.*, 2000)]. In the rat liver, endogenous mitochondrial associated phospholipase A_2 (PLA_2) was shown to catalyze acyl chain removal from both PG and CL *in situ* [(Hostetler *et al.*, 1978), reviewed in (Hostetler, 1982)]. The resulting monolysocardiolipin (MLCL) was reacylated by monolysocardiolipin acyltransferase (MLCL AT), utilizing linoleoyl-Coenzyme A [(Schlame *et al.*, 1990; Ma *et al.*, 1999)]. We purified to homogeneity a MLCL AT activity from pig liver mitochondria, characterized its activity, and determined that thyroid hormone modulated its expression (Taylor *et al.*, 2003). Thyroxine has been shown to be involved in mitochondrial synthesis, and respiration as well as increasing levels of CL, and this reacylation process may act as a regulation point further indicating the importance of specific acyl species of CL in mitochondrial function (Mutter *et al.*, 2000; Taylor *et al.*, 2002; Webster *et al.*, 2005).

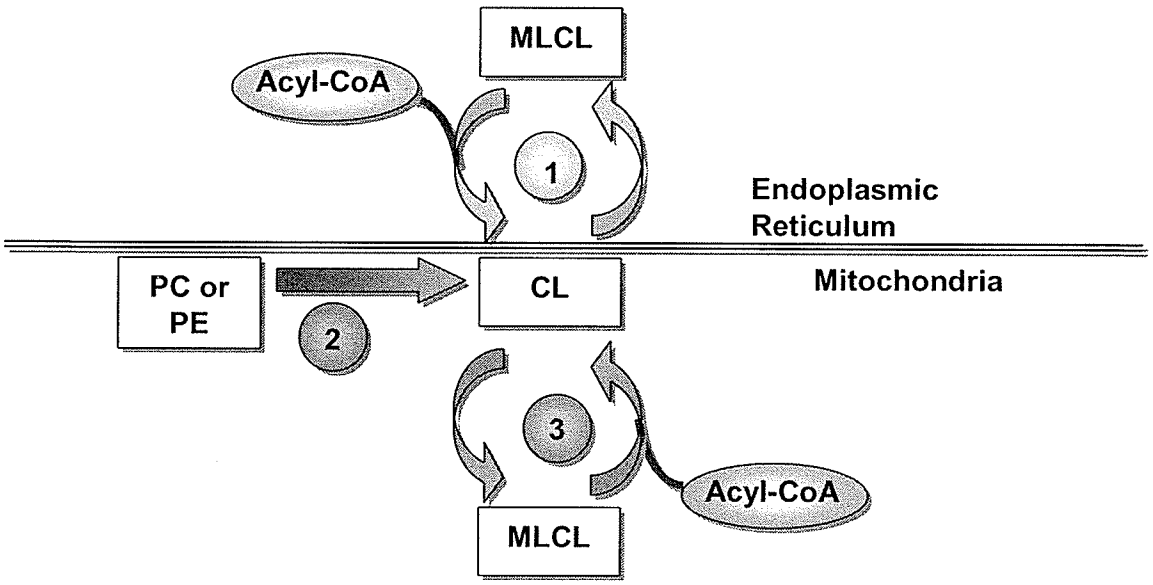

Figure 1-3: Pathways of Mammalian CL Remodeling.

Three enzymes responsible for CL resynthesis have been characterized to date. ALCAT1 (1) is localized to ER. Acyl-Coenzyme A acts as cofactor in the transfer of an acyl chain to MLCL. Mitochondrial CL transacylase (2) requires PC or PE as an acyl chain donor. Mitochondrial MLCL AT (3) requires acyl-Coenzyme A as a cofactor in the transfer of an acyl chain to MLCL. In (1) and (3) CL is first deacylated to MLCL via phospholipase A_2.

Recently a CL transacylase activity in crude mitochondrial fractions prepared from rat liver was shown to transfer acyl moieties, in conjunction with adenine nucleotides or Coenzyme-A, from phosphatidylethanolamine (PE) or phosphatidylcholine (PC) to CL or MLCL *in vitro* (Xu *et al.*, 2003a). More recently, a murine gene encoding an acyl-CoA:lysocardiolipin acyltransferase-1 (ALCAT1) with catalytic activity similar to the pig liver mitochondrial enzyme has been identified (Cao *et al.*, 2004). The ALCAT1 exhibited a preference for linoleoyl-CoA and oleoyl-CoA substrates and was localized to the endoplasmic reticulum (ER), suggesting a link between the ER and mitochondria in CL remodeling. CL has been established to localize to the outer mitochondrial membrane and regions of contact between ER and mitochondria have been demonstrated (Hostetler, 1982; Rusinol *et al.*, 1994). Hence, ALCAT1 may be involved in the remodeling of outer mitochondrial membrane CL. The above observations indicated that there are likely several acyltransferase activities involved in mammalian CL remodeling.

1.2.2. Function of Cardiolipin

It has been suggested that CL is the glue that holds the electron transport chain together (Zhang *et al.*, 2002). The essential components of the respiratory chain (Complexes III and IV) form a supercomplex in the mitochondrial membrane, through which protons and electrons move to generate ATP. This supercomplex required a strong association with CL for formation. The ADP/ATP translocator has also been shown to be tightly associated with six molecules of CL and was this required for translocator activity (Beyer *et al.*, 1985; Hoffmann *et al.*, 1994). Interestingly, in that model the specific acyl composition of CL was determined not to be the source of its high affinity for the

ADP/ATP carrier (Schlame *et al.*, 1991). In addition to its key role in support of mitochondrial oxidative phosphorylation, CL metabolism has been implicated in mitochondrial mediated apoptosis [reviewed in (McMillin *et al.*, 2002; Degli Esposti, 2004; Wright *et al.*, 2004; Fariss *et al.*, 2005)].

To investigate the role of CL in mitochondrial function, cellular models have been developed where the production of CL was diminished by inhibiting PG synthesis. In each case a distinct attenuation of cell growth was observed. A Chinese hamster ovary (CHO) cell line defective in PG synthesis (PGS-S), due to a mutation in the PGP synthase gene, exhibited a temperature sensitive reduction in the amount of PG and CL produced (Ohtsuka *et al.*, 1993). This mutant exhibited morphological and functional alteration in mitochondria when grown at the non-permissive temperature. These included reduction in ATP production, oxygen consumption and an increase in glycolysis. These alterations were the result of disruption of the electron transport chain, with the rotenone-sensitive NADH-ubiquinone reductase (Complex I) complex exhibiting the greatest dysfunction. These abnormalities were reversed upon transfection of the mutant CHO cells with PGS1 cDNA (Kawasaki *et al.*, 1999).

A PGS1 inducible *S. cerevisiae* strain has also been developed in which the PGS1 gene, under the control of a doxycycline promoter, was transfected into PGS1 deficient yeast, allowing for a controlled level of CL synthesis interruption (Ostrander *et al.*, 2001b). These *pgs1Δ* cells exhibited defective translation of mitochondrial electron transport chain components. The authors suggested that the reduced translation may have accounted for the reduction in mitochondrial function.

A CL synthase mutant (YDL142c, *crd1Δ*) has been developed in *S. cerevisiae* to examine the effect of cells deficient in CL on mitochondrial function (Jiang *et al.*, 1997). These mutants exhibited defective growth at the restrictive temperature. The yeast model carries the added benefit of growth utilizing either fermentable or non-fermentable energy sources. This allows for comparison of the differences between aerobic respiration, utilizing the mitochondrial electron transport chain, and fermentation. In the absence of a fermentable carbon source, these mutants exhibited slower growth and reached a lower cellular density even at the optimal temperatures (Jiang *et al.*, 1997; Jiang *et al.*, 1999; Jiang *et al.*, 2000). The *crd1*Δ *S. cerevisiae* mutant exhibited decreased viability associated with a reduction in the function of the ADP/ATP translocator, reduced mitochondrial membrane potential and increased leakiness leading to an overall reduction in the capacity for oxidative phosphorylation (Jiang *et al.*, 2000; Koshkin *et al.*, 2000, 2002; Zhong *et al.*, 2004). Interestingly, *crd1*Δ mutants that were able to produce high levels of PG, even under higher temperatures for extended periods of time, were shown to be more resistant to the growth retarding effects observed in the mutants producing less PG (Zhang *et al.*, 2003). These studies implicated a supportive role for PG in mitochondrial function. The apparent reliance on PG levels may have been due to the ability of PG to partially restore the function lost due to the lack of the CL under the conditions where levels of PG were elevated. In addition, mitochondrial PE biosynthesis was shown to compensate for a lack of CL, which may explain why these *crd1*Δ mutants were not lethal (Gohil *et al.*, 2005b). In that study, when both CL and PE mitochondrial synthetic pathways were deleted in *S. cerevisiae* the result was lethal. Finally, other studies have indicated that although CL was not required to maintain

stability of mitochondrial DNA, it was necessary for the organization of complexes III and IV in the *crd1*Δ mutants (Zhang *et al.*, 2003; Zhang *et al.*, 2005).

The principal molecular species of CL in yeast is $(16:1)_2(18:1)_2$ (Iverson *et al.*, 2004). The effect of alteration of the molecular species composition of CL on oxidative phosphorylation has been examined in the *S. cerevisiae crd1*Δ mutant. Oxidative phosphorylation did not appear to require CL specific molecular species definitively (Koshkin *et al.*, 2000, 2002). Rather, specific molecular species of CL were required to improve the efficiency of the respiratory chain enzymes and maintain the stability of the mitochondrial membrane against osmotic challenges.

1.3. Lipid alterations in Barth Syndrome

Lipid abnormalities including decreased cholesterol, docosahexaenoic acid (DHA) and arachidonic acid (AA) in BTHS plasma and erythrocytes have been reported [reviewed in (Barth *et al.*, 2004)]. In addition, the phospholipid composition of mitochondrial membranes in BTHS cultured fibroblasts and patients with a primary defect in the respiratory chain have been examined (Vreken *et al.*, 2000). These studies indicated that only the BTHS patients had a reduction in CL levels, demonstrating that the decrease in CL was not a result of mitochondrial dysfunction. Incorporation of [1,3-^{3}H]glycerol into CL was unaltered in these cells indicating that CL *de novo* biosynthesis was unaffected. In contrast, incorporation of [1-^{14}C]linoleic acid into CL and its immediate precursor PG was reduced in only the BTHS fibroblasts indicating a defect in CL remodeling.

The above study was supported by several studies in which a deficiency in L_4-CL in myocardium, skeletal muscle, thrombocytes, cultured skin fibroblasts and platelets was observed (Schlame *et al.*, 2002; Valianpour *et al.*, 2002a; Valianpour *et al.*, 2002b; Schlame *et al.*, 2003). In one of these studies the phospholipid abnormalities of BTHS were compared to other BTHS-like diseases (Schlame *et al.*, 2003). These authors found that only the true BTHS patients exhibited a CL deficiency. CL was the only phospholipid decreased in all tissues examined and an alteration in the CL molecular species was observed. Linoleic acid levels in CL were decreased in all BTHS tissues examined, whereas, in the heart linoleic acid levels were increased in both PC and PE. However, the degree of CL deficiency did not correlate to the disease severity or genotype. Another study compared the acyl composition of major mitochondrial phospholipids, PC, PE and CL in BTHS lymphoblasts (Xu *et al.*, 2005). The most dramatic effect was a shift in CL containing linoleic (18:2) and palmitoleic (16:1) acid to palmitic (16:0) and stearic (18:0) acid. In addition, a shift from the use of oleic acid ($18:1\Delta^9$) to vaccenic acid ($18:1\Delta^{11}$) as the predominant acyl group in CL from BTHS lymphoblasts was observed. Palmitoleic acid (16:1) was increased in PC of the BTHS lymphoblasts, at the expense of oleic acid ($18:1\Delta^9$). PE exhibited little alteration, except that there was a small increase in palmitoleic acid. Fluorescence and electron microscopy revealed abnormal proliferation of the mitochondria in these cells. In addition, using a fluorescent probe, JC-1, a 30% reduction in the mitochondrial membrane potential of BTHS lymphoblasts was observed. However, the rate of ATP formation in the mitochondria of these cells was not dissimilar from controls. This was attributed to an ability of the increased mitochondrial mass to compensate for a partial uncoupling of

oxidative respiration in the BTHS lymphoblasts. Finally, only a small increase in MLCL accumulation was observed in BTHS platelets and fibroblasts, which may have accounted for the lack of detection in another study (Valianpour *et al.*, 2005). In that earlier study significant accumulation of MLCL was shown to occur in lymphoblasts, lymphoblasts and skeletal as well as cardiac myocytes.

A *S. cerevisiae* mutant was developed that contained a null mutation in the homologue of the human G4.5 gene, *taz1* YRP140w (Gu *et al.*, 2004). The yeast *taz1*Δ mutant exhibited many of the same characteristics as the *crd1*Δ yeast mutants, including temperature sensitivity with ethanol as the sole carbon source. In addition, an accumulation of MLCL and a reduction in the unsaturated fatty acid species in CL similar to that demonstrated in BTHS was observed (Gu *et al.*, 2004; Valianpour *et al.*, 2005). Despite an increased apparent rate of CL synthesis, it was noted that the expression of the *CRD1* and *PGS1* genes were not altered (Gu *et al.*, 2004). Furthermore, the *taz1*Δ mutant exhibited a decrease in total CL content as well as defective acylation of CL, as identified in BTHS. These studies indicated that *de novo* synthesis of CL may be affected by alterations in CL acylation. In addition, an increased amount of PE and phosphatidylserine (PS) in the *taz1*Δ mutants and a decreased level of PA were observed. The *taz1*Δ mutant also exhibited instability of the mitochondrial membrane under elevated temperatures and hypotonic conditions (Ma *et al.*, 2004). Another study, utilizing the *taz1*Δ yeast model, compared the ability of the different possible human TAZ splice variants to restore the normal lipid composition in the yeast *taz1Δ* mutant (Vaz *et al.*, 2003). Only the human TAZ variant lacking exon-5 was able to fully restore CL levels to control values. However, it should be noted that the full human

TAZtranscript was able to partially restore the reduced CL levels observed in the *taz1*Δ mutant.

A more recent study described a reduction in the ability to form a proper respiratory chain complex when *taz1* was deleted in *S. cerevisiae* (Brandner *et al.*, 2005). The electron transport chain components, cytochrome bc_1 (complex III) and cytochrome c oxidase (complex IV) normally associate as homodimers, which assemble a stable supercomplex, III_2IV_2, within the mitochondria (Cruciat *et al.*, 2000; Gohil *et al.*, 2004). In these *taz1* deficient cells, a selective release of complex IV monomers from the III_2IV_2 complex was observed (Brandner *et al.*, 2005). Moreover, assembly analysis revealed a reduced ability to incorporate complex IV monomers into supercomplexes. In addition, as expected with a defective CL reacylation process, accumulation of MLCL was observed in these *taz1* deficient cells (Vaz *et al.*, 2003; Gu *et al.*, 2004). Together these studies in yeast have indicated the necessity of *taz1* in the remodeling of CL, which, in turn, acts to stabilize the respiratory chain complex for optimal mitochondrial respiration.

1.4. The TAZ Acyltransferase

The identification of the enzymatic activity of the protein encoded by the TAZ gene has been the focus of many studies since the recognition of a high level of sequence homology with the glycerolipid acyltransferase family of enzymes (Neuwald, 1997). Strong anecdotal evidence has indicated that the function of TAZ was to mediate phospholipid acylation, specifically linoleic acid addition to CL or MLCL (Vreken *et al.*, 2000; Xu *et al.*, 2003b). In addition, accumulation of MLCL in various cell types lacking

TAZ further indicated that its primary target was CL (Schlame *et al.*, 2003; Valianpour *et al.*, 2005). However, the precise biochemical function of TAZ has not been elucidated to date.

In mammals, at least two acyltransferases are known to be involved in CL remodeling (Taylor *et al.*, 2002; Cao *et al.*, 2004) (**Figure 1-3**). The molecular mass of the major protein products of *in vitro* expressed human TAZ do not correspond to the reported 74 kDa molecular mass of the purified Pig liver MLCL AT (Taylor *et al.*, 2003; Vaz *et al.*, 2003), indicating that this acyltransferase is not TAZ. Recent evidence indicated the presence of an acyltransferase activity capable of facilitating CL remodeling in yeast (Testet *et al.*, 2005). The yeast homologue corresponding to human TAZ, Ypr140w, was shown to have a lysophosphatidylcholine acyltransferease activity capable of catalyzing the acylation of CL without the need for acyl-Coenzyme A. Although ER retention signals were found on this protein, analysis of the *S. cerevisiae* mitochondrial proteome indicated that the protein may also reside within mitochondria (Sickmann *et al.*, 2003).

As previously indicated, a MLCL AT, ALCAT1, has been characterized in the mouse (Cao *et al.*, 2004). ALCAT1 exhibited a preference for linoleoyl-Coenyzme A and oleoyl-Coenzyme A as acyl donors and utilized MLCL and dilysocardiolipin species as substrates. The expression of ALCAT1 was highest in liver and heart, supporting a role for maintenance of heart function. However, ALCAT1 was localized to the ER. A previous study in yeast had reported a mitochondrial localization of the expressed epitope-tagged human TAZ (Ma *et al.*, 2004). In addition, a recent study confirmed this observation (Brandner *et al.*, 2005). In that model the expressed protein in yeast was determined to be an integral outer mitochondrial membrane protein, although its

connection to the membrane was not as strong as Tim22, a protein which spans the membrane several times. Several lines of evidence indicated that the C-terminal portion of the protein was localized to the intermembrane space, with the N-terminus acting as membrane anchor in the outer mitochondrial membrane.

As previously indicated a CL transacylase activity with the ability to resynthesize CL using PC and PE as the linoleoyl donor has been demonstrated in rat liver (**Figure 1-3**) (Xu *et al.*, 2003b). The activity of this CL transacylase was significantly reduced in BTHS lymphoblasts compared to controls. However, activity of the enzyme in lymphoblasts was low and was reduced by only 50% in the BTHS lymphoblasts. A complete loss of enzymatic activity in BTHS lymphoblasts might have been expected if this transacylase was TAZ.

Finally, conserved protein sequence homology between five regions critical for the function of human AGPAT-1 and -2 and human tafazzin have been observed (Lu *et al.*, 2004). Mutations within these regions of TAZ1 in BTHS patients have been observed. In addition, the recent observation of multiple molecular forms of mammalian AGPATs exhibiting differing molecular masses have been reported (Lu *et al.*, 2005). These observations might suggest the involvement of a yet to be identified AGPAT in BTHS. Alternatively, the possibility remains that TAZ does not correspond to any protein kinetically characterized to date.

1.5. Outlook for the future

Much information has been gathered regarding the nature of the TAZ gene, its protein product and the characterization of the lipid defects observed in BTHS. The understanding of the nature of the defect in BTHS will help to more rationally develop therapies to treat the disease. Additionally, knowledge gained from the *in vivo* metabolism and function of CL will help us to better understand the role of CL in mammalian cells.

Current evidence supports CL remodeling as a major defect observed in BTHS. Remodeling of CL and PG were decreased with a corresponding increase in linoleic acid content of PC and PE in BTHS cells. In addition, mutations in TAZ resulted in an accumulation of MLCL within BTHS cells. Other phospholipid alterations affected by mutations in TAZ included increased levels of total phosphatidylserine and PE with a decreased level of PA. The phenotype and morphologic similarities between CL in *taz1*Δ yeast and BTHS cells, combined with the ability of the wild-type human TAZ gene to restore function to the *taz1*Δ yeast model further support the role of TAZ in CL metabolism. However, the precise mechanism for the reduction of linoleic acid in CL in TAZ deficient cells is still poorly understood and the specific enzyme(s) responsible has not been identified. In fact, the number of functional TAZ gene protein products has not been universally agreed upon to date.

Within the cellular models of BTHS, a significant amount of growth retardation is observed. The mechanism or importance of this finding is still not clear. Ultimately, the lack of correlation between genotype and phenotype in BTHS indicates that a deficiency

in TAZ results in a far more complicated effect on metabolic processes than originally anticipated.

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Chapter 2: Cholesterol

Chapter 2: Cholesterol

The recent trend has been to use dietary, lifestyle changes and ultimately pharmacological approaches to lower CH levels as much as possible. The motive for this is based on a number of studies which followed large cohorts of people over several years, then correlated the factors that seemed to be associated with the incidence of cardiovascular events (ie, stroke, atherosclerotic lesions, chronic heart disease, etc) (Keys, 1975; Simons, 1986b, a), Framingham, (Anderson *et al.*, 1987), International Heart Study (INTERHEART) (Yusuf *et al.*, 2004), Multiple Risk Factor Intervention trial (MRFIT) (Kannel *et al.*, 1986; Stamler *et al.*, 1986), Lipid Research Clinics trial (LRC) (Lipid Research Clinics Program, 1984; The Collaborative Lipid Research Clinics Program Family Study. I. Study design and description of data, 1984; Green *et al.*, 1984a; Green *et al.*, 1984b; Greenberg *et al.*, 1984; Namboodiri *et al.*, 1984a; Namboodiri *et al.*, 1984b; Wallace, 1984). All of these studies revealed a correlation between CH, in particular, low density lipoprotein (LDL) and risk of cardiovascular disease (Hornung, 2002). In addition, clinical studies of statin therapies have shown a reduction in CH, which has resulted in the National Cholesterol Education Program (NCEP)'s mandate to target LDL CH as a primary preventative measure against atherosclerosis (Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report, 2002). Since then the aim has been to lower CH at all costs (Grundy, 1998; Laufs *et al.*, 2004), even though more recent studies have suggested no further benefit is obtained beyond a lowering of 20-25% (Pfeffer *et al.*, 1995; Shepherd, 1995; Shepherd *et al.*, 1995; Tonkin, 1995). Some studies even exhibited a lack of reduced

mortality with LDL lowering (Bays, 2002; Gagne *et al.*, 2002; McKenney *et al.*, 2006). But what is the cost? Does CH have a role in a healthy body? The casual observer these days might say no, but in fact, CH has a large role to play in the healthy functioning of the human body. This is not to imply CH is healthy even in excess, but to remind one that, as with most things in life, CH is required in moderation. A balance between its good effects and its pathological effects must be attained. In fact, CH biosynthesis is absolutely essential for the proliferation and viability of mammalian cells (Dahl *et al.*, 1988; Yeagle, 1993). The NCEP cites the ability for neonates to survive off serum CH concentrations of 0.78 mmol/L as an indication that only very low levels of CH are required by the human body (Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report, 2002). However, a neonate has very little in the way of a functioning immune system, which requires CH, as will be discussed later (***Cholesterol and immunity***). In addition, the neonate very rapidly increases their CH levels within the first year of life to levels closer to 4.27 mmol/L (Berenson *et al.*, 1979; Carlson, 1991). Those that don't increase their serum CH, as in Smith-Lemli-Opitz Syndrome (SLOS; MIM 270400), where CH levels remain at about 0.26 mmol/L, due to a defect in CH synthesis, experience many neurological, developmental, and physical abnormalities (Witsch-Baumgartner *et al.*, 2000; Waterham, 2002). Though the literature largely seems to ignore the fact that CH plays a necessary role in the healthy functioning of the human body, there is a multitude of data suggesting that an optimal CH level exists and to surpass it in our efforts to lower LDL cholesterol is not only futile, but may be detrimental. [Reviewed by (Jacobs, 1993; Ravnskov *et al.*,

2006)]. It, therefore, becomes obvious that a deficiency in CH could have detrimental effects on these important pathways. In fact, low levels of CH have been shown to lead to apoptosis in NIH3T3 cells via a p38 mitogen activated protein kinase (MAPK) mediated process (Calleros *et al.*, 2006a; Calleros *et al.*, 2006b). Contrary to this idea of increased apoptosis with CH lowering, recent evidence has even suggested an increased risk of cancer as a result of ezetimibe-statin therapy, which inhibits dietary CH uptake and CH synthesis, respectively, as compared to the statin alone (Drazen *et al.*, 2008a). This is very new evidence, however, and there is not enough information to determine if this was the result of the further 27% of CH lowering observed by this combination, or some other effect of the drug combination. Moreover, this drug combination has not yet revealed a decrease in mortality, nor even a decrease in the rate of atherosclerotic disease progression (Drazen *et al.*, 2008b). It does, however, suggest that aggressive CH lowering by multiple drug therapies is not a responsible action with our lack of understanding of the importance of circulating CH.

2.1. Function of Cholesterol

Cholesterol is known to be important in a number of different processes. One role that has been known for decades is the role CH plays in membrane fluidity. Due to the rigid structure of CH, membrane fluidity can be maintained under conditions of extreme temperature. The hydroxyl group on carbon 3 makes CH amphipathic (Bloch, 1983), allowing it to intercalate between the lipid tails of the membrane bilayer, while the hydroxyl group mingles with the aqueous phase. This incorporation influences the gel to liquid-crystalline transition temperature (T_m) of the bilayer by restricting the movement

of the hydrophobic tails under physiologic conditions [reviewed in (Ohvo-Rekilä *et al.*, 2002)]. This increased order within the membrane leads to a more densely packed, less permeable membrane. In this way, CH can also affect the properties of proteins within the membrane. Some proteins are directly regulated by binding CH with sterol sensing domains, while others are altered by the properties of the local membrane, known as membrane or lipid rafts. Recently, the role of cholesterol in the aggregation and maintenance of lipid rafts (Incardona *et al.*, 2000), has become recognized as important for signaling cascades in the cell surface [reviewed in (Simons *et al.*, 2002)]. By bringing important cofactors together within the same vicinity, rafts can facilitate the formation of signaling complexes. In addition, caveolae are important membrane features which bring together the appropriate components of signaling effectors and are strongly associated with CH [reviewed in (Ikonen *et al.*, 2004)].

Cholesterol is known to be the precursor to many biologically active molecules, such as Vitamin D, bile acids, and steroid hormones, [reviewed in (Ohvo-Rekilä *et al.*, 2002)]. In addition, CH intermediates such as farnesyl and garnesyl are important for post-translational modifications to 0.2-5% of cellular proteins (Epstein *et al.*, 1991; Prendergast *et al.*, 2000). This step is so important that if all CH synthesis was inhibited, the cell would not survive (Wong *et al.*, 2007). The biological relevance of prenylation will be discussed further, later in this thesis (***Isoprenoid Biosynthesis***).

2.1.1. Cholesterol and immunity

Several studies have established that immunity requires CH for proper functioning. One such series challenges years of work, which considers LDL CH to be a major cause in the generation of atherosclerosis. These studies looked at the gram negative bacterial lipopolysaccharide (LPS), which is the main pathogenic factor. Not only was LPS able to bind lipoproteins, with a preference for LDL, this LDL-LPS complex was no longer able to stimulate an effective immune response, as determined by *in vitro* cytokine secretion (Cavaillon *et al.*, 1990; Weinstock *et al.*, 1992; Flegel *et al.*, 1993). A similar effect was identified with the *Staphylococcus aureus* α-toxin (Bhakdi *et al.*, 1983). Though this could act to inhibit an immune response to a potential pathogen, it is more likely a protective effect, acting to prevent the overstimulation of the immune system. As LPS can often remain long after the pathogen has been dealt with, causing a pyrogenic response. This 'mopping up' effect would serve to subdue any further reponses. Furthermore, men with low LDL levels (mean LDL 2.287mmol/L) were shown to have fewer circulating lymphoblasts than hypercholesterolemic men (mean LDL 4.805mmol/L) (Muldoon *et al.*, 1997b). Thus, it is possible that the hypocholesterolemia observed in BTHS could be correlated with the neutropenia often evident in BTHS. However, the correlation doesn't tell us whether the low CH is a result of low lymphoblast count or the lack of lymphoblasts result from low CH. Further investigation is required on this matter.

In addition to BTHS, hypocholesterolemia has been associated with a variety of other disease states. The severity of meningococcal sepsis in children entering emergency rooms was found to be greater with decreased levels of total, LDL, and high density

lipoprotein (HDL) CH (Vermont, 2005). Though all emergency admissions had low CH levels, the survivors had higher circulating CH levels on admission, and their CH levels started to recover sooner than non-survivors.

2.2. Temporal effects

Cholesterol requirements in the human body change over time. The liver is the predominant site for differential regulation. Alterations in sterol concentrations in the diet can cause the liver to respond by increasing or decreasing CH production to maintain a balance between supply and demand. In addition, CH production in the liver is increased each day, predominantly by an increase in the activity of the major rate-limiting step, 3-hydroxy-3-methylglutaryl-CoA (HMGR), during the mid-dark portion of the circadian cycle (Polo *et al.*, 1999). Interestingly, this diurnal variation is independent of CH ingestion.

Throughout the human life span, maintaining appropriate levels of CH in the brain is important (Waterham, 2002; Waterham, 2006). As a component of lipid rafts, which are very important structures for the maintenance of cellular signaling, CH is important not only for neuronal development but also for their maintenance. Recent studies have characterized the need for CH in the nervous system. CH is required for both neurite outgrowth and repair, as well as synaptogenesis (Koudinov *et al.*, 2001; Mauch *et al.*, 2001; Hayashi *et al.*, 2004). In addition, CH may need to be tightly regulated, as different cell types and even axonal versus dendritic processes require differing optimal amounts of CH (Ko *et al.*, 2005).

The demand for CH begins early on, even before birth (Roux *et al.*, 2000). Embryongensis and neuronal development both have a strong dependence on CH. Correspondingly, the first year of life is when serum CH levels undergo the greatest alterations (Frerichs *et al.*, 1976; Berenson *et al.*, 1979; Waterham, 2006). As will be discussed, the CH synthetic pathway produces isoprenoids (***Isoprenoid Biosynthesis***), which are important in cell survival and signal transduction, among others. These intermediates of CH metabolism are so important that mutations inhibiting the activity of the isoprenoid pathway are embryonic lethal (Ohashi *et al.*, 2003). Beyond the first year of human life, massive growth spurts occur. During preschool years, the CH levels become more stable, and reach near adult levels (Berenson *et al.*, 1978; Frerichs *et al.*, 1978). As previously stated, CH acts as a precursor for steroidal synthesis (***Function of Cholesterol***). It stands to reason then, that higher demands for CH synthesis would be placed on the body during times of increased steroid production. As a result, serum CH levels peak in young males just before puberty (4.70 mmol/L), and slowly decline to a low mean value at the age of 17 (4.33 mmol/L), when a new adult homeostasis is reached (Abraham *et al.*, 1978; Berenson *et al.*, 1981). It should be noted that CH levels in females and males of Caucasian and African decent all revealed different ranges, but the trends remain the same. Perhaps not surprisingly, the years between the CH demands of development and puberty, approximately 4-11 years old, corresponds to the time many BTHS patients refer to as the "honeymoon period", when many of the symptoms spontaneously resolve, and some patients are even well enough to go off medications. However, indefinitely, near the age of puberty, symptoms once again worsen, and the patient deteriorates, sometimes to a level worse than before (Kelley, 2002).

2.3. Cholesterol and the Barth Syndrome Defect

Hypocholesterolemia is an often overlooked characteristic of BTHS. First reported in the early 90's (Kelley *et al.*, 1991b), hypocholesterolemia in BTHS has been little more than a noted anomaly, garnering no further insight or investigation, until recently. A report published in the summer of 2006, by Spencer *et al* was the first attempt to systematically identify secondary symptoms that might affect disease outcome (Spencer *et al.*, 2006). Though they were unable to identify any modifying factors to the severity of BTHS, their work is ongoing, and will be a useful tool in systematically characterizing BTHS. What they did find was that not all patients studied had low serum CH, identified as total CH less than 2.84 mmol/L (110 mg/dL). In fact, of the 25 patients tested, only 24% exhibited a reduction in total serum CH. Furthermore, HDL was only reduced in 16% of the patients (<1.036mmol/L), though, LDL levels were reduced in 56% of the patients considered (<1.554mmol/L). Under no conditions was there a correlation between CH levels and cardiac ejection fraction. However, the ages of study participants ranged from 1.2–22.6 years (n = 34), it is well known that CH demand changes over time (Abraham *et al.*, 1978; Berenson *et al.*, 1981; Webber *et al.*, 1991; Jolliffe *et al.*, 2006), and it is possible that hypocholesterolemia only has an impact under times of higher cholesterol demand. A previous study observed that radioactive acetate incorporation into CH, as well as HMGR enzyme activity, were unaltered in BTHS fibroblasts cultured under normal serum containing conditions (Gibson *et al.*, 1991). In this study they were unable to identify any conditions under which CH metabolism was altered. However, this study combined the results of 3 BTHS patients, which resulted in a large variation, making a

statistical change very difficult to detect. Therefore, we have chosen to report our results from each BTHS patient separately.

As described previously (***Temporal effects***), during the course of human development, the demand for CH increases (Berenson *et al.*, 1978; Berenson *et al.*, 1979; Berenson *et al.*, 1981). Reports of what is termed a "honeymoon period" occurs in BTHS patients between the ages of approximately 4 and 11 years old (Kelley, 2002), during which time, symptoms are often known to improve and sometimes even resolve altogether. Throughout the time coincident with the onset of puberty, symptoms once again worsen. Thus, it is possible that CH may, in fact, be a modifying factor that is temporally affected. Under conditions of high CH demand, or low supply, CH biosynthesis may be taxed further, causing increased stress on the already distressed BTHS system.

Studies by Ness and Chambers revealed that thyroid hormone can increase hepatic HMGR levels by both increasing transcription and increasing the transcript stability (Ness *et al.*, 2000). It could be postulated then, that a lack of thyroxine may result in a decrease in HMGR activity. In addition, a lack of thyroid hormone can, in fact, result in a reduction in CL levels [reviewed in (Schlame *et al.*, 2000)]. This suggests a further potential link between CH and CL and thus predicts the lack of CL in BTHS may be a possible mechanism for the hypocholesterolemia observed in these patients.

Chapter 3: Biochemistry

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We have used several different radiolabeled precursors to investigate the biochemistry of lymphoblasts deficient in TAZ, as compared to a wild type counterpart. In doing so, we have targeted the three major routes of anabolism into cholesterol, as well as a plethora of other products, namely other neutral and phospholipids (PL). Most of these biochemical pathways have a common intermediate step in which the mitochondria is an important player. In particular, we have used [1-^{14}C]acetic acid, D-[^{14}C(U)]glucose and [^{14}C(U)]palmitic acid as precursors representing; a mitochondrial membrane permeable precursor, glycolysis, and β-oxidation, respectively. In addition, we examined the incorporation of [2-^{14}C]pyruvate as an alternative to glucose, due to the broad number of potential products glucose can be involved in. Here we will explore the role of the mitochondria for each pathway.

Eukaryotic cells have the ability to utilize multiple different sources of energy and carbon for biosynthesis. Different cells in the mammalian body have different requirements, and thus different preferences for energy and carbon sources. One common intermediate in eukaryotic metabolism is frequently the mitochondria. In a resting muscle cell, and many other peripheral cells, preference is given to fatty acids as sources of β-oxidation, to be discussed later (***β-oxidation***). Upon the increased energy demands of exercise, muscle cells can no longer derive energy from fatty acids fast enough to supply the demands. Initially phospho-creatine stores can cover the deficit, but the stores quickly run out, and a better source is required. In this case, glycolytic oxidative phosphorylation becomes one of the potential sources for energy. Glucose is processed in a number of cytosolic reactions, known as glycolysis, to form 2 molecules of pyruvate. Further processing of

pyruvate occurs within the mitochondria, and will be discussed further (***Mitochondrial Biochemistry***). Alternatively, oxaloacetate, downstream of pyruvate, can be reconverted to glucose. The last step in glycolysis, catalyzed by pyruvate kinase (EC 2.7.1.40), is highly exergonic, and thus, cannot be reversed under physiologic conditions. Therefore, if gluconeogenesis is to proceed, oxaloacetate must be decarboxylated back to phosphoenolpyruvate via the enzyme, phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.49) (Murray *et al.*, 1996). Glycogen is also an effective way to store energy for use under situations of high energy demand. In addition, multiple different metabolic intermediates and storage molecules can be utilized when necessary, but, as they are not the predominant sources under normal, fully fed circumstances, they will not be further discussed here.

Lymphoblasts, on the other hand, revealed a strong preference for glucose or glutamate as an energy source (Newsholme, 2001). This is predominantly due to the high energy demands lymphoblasts have in maintaining immunity. As a result, β-oxidation does not play a large role in metabolism within these cells. When glucose supplies are not sufficient, lymphoblast activation cannot occur, and cellular growth is halted (Doughty et al., 2006). This inhibition is true even if an alternate energy source is provided (Jacobs *et al.*, 2008). Glucose uptake is a rate limiting step in the ability of the cell to utilize glucose. One of the major transporters responsible for glucose uptake into the lymphoblast is the glucose transporter (GLUT1) (Wofford *et al.*, 2008). Alterations in glucose uptake and glucose utilization have been demonstrated to alter immune function [reviewed in (Calder *et al.*, 2007; MacIver *et al.*, 2008)]. It is clear that glucose metabolism is very important for lymphoblast metabolism, and would be the main source

of carbon for many products. Therefore, if this pathway were altered, CH metabolism may be correspondingly affected.

3.1. Mitochondrial Biochemistry

The mitochondria is an important part of the cell, as it allows for aerobic generation of ATP via oxidative phosphorylation, and it provides a reservoir for apoptotic factors that can be released upon stimulation by the appropriate signaling cascade. However, the mitochondria is also the cellular source for acetyl-CoA production (**Figure 3-1**Figure 3-1), a precursor for many biological products, including CH (Sabine, 1977). Glucose is converted to pyruvate in the cytosol and is then transported into the mitochondria by the pyruvate translocator, which is known to require CL for proper functioning (Paradies *et al.*, 1988, 1989). Once pyruvate has passed the outer and inner mitochondrial membranes, it is available for processing to acetyl-CoA and can proceed into the tricyclic acid (TCA) or Kreb's cycle. From here, intermediates are funneled into the electron transport chain to complete oxidative phosphorylation energy generation. Alternatively, various intermediates can be exported to the cytosol for use in other metabolic processes. As citrate, acetyl-CoA is shuttled back to the cytosol, where it can be a source of carbon for CH synthesis. This citrate carrier, has also been demonstrated to function optimally only in the presence of CL (Palmieri *et al.*, 1993).

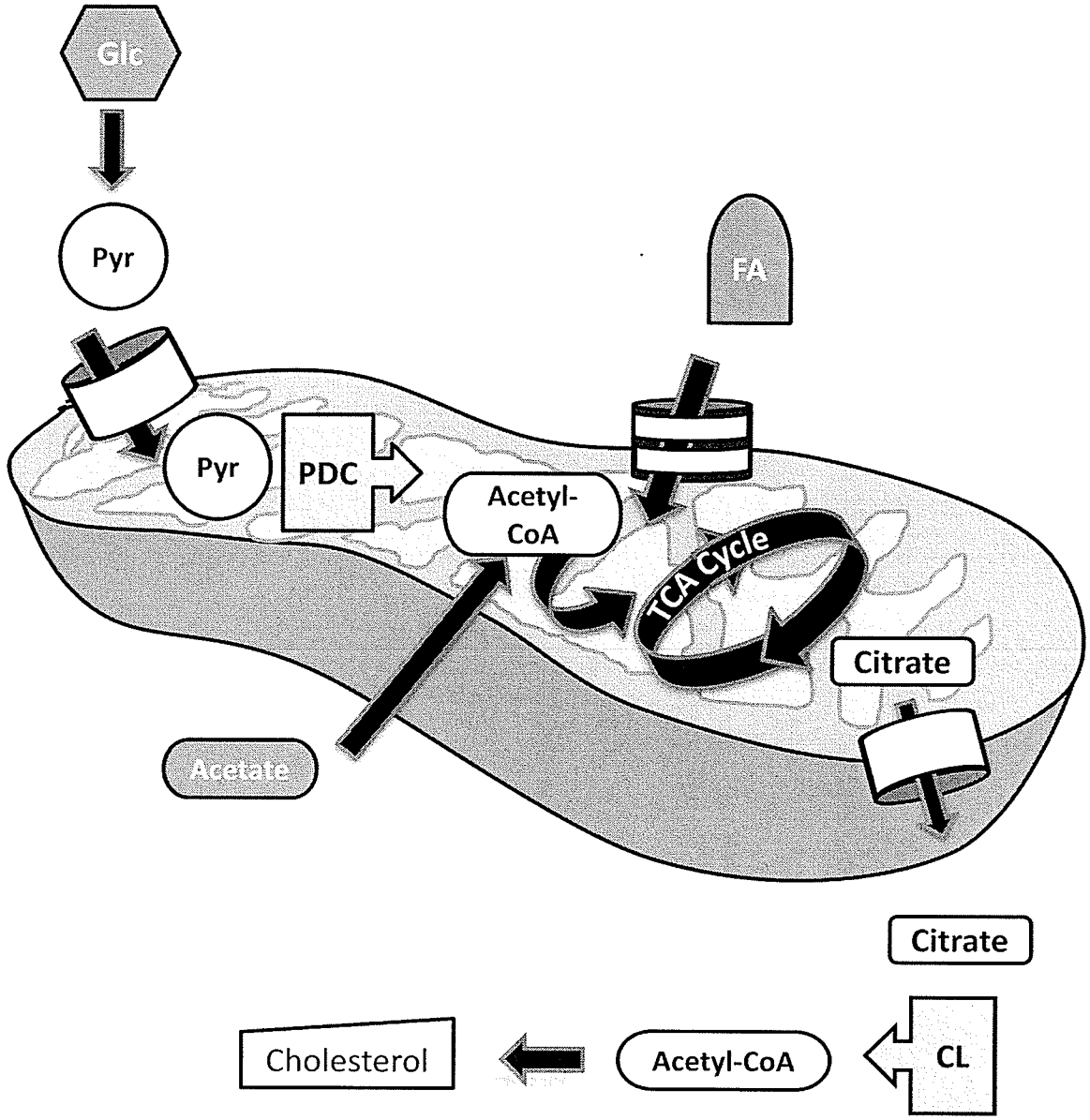

Figure 3-1: Sources of carbon for Cellular Anabolism

Most, if not all sources of carbon for anabolism are localized to the mitochondria for conversion of pyruvate, from glucose, acetyl-CoA, from fatty acids, and acetate to citrate. Pyruvate is imported into the mitochondria via the pyruvate translocator, which has optimal activity in the presence of CL. In the mitochondria, pyruvate conversion to acetyl-CoA is accomplished by the pyruvate dehydrogenase complex (PDC). Acetate is able to freely permeate the mitochondrial membranes, and is acylated by acetyl-CoA synthetase. Fatty acids enter the mitochondria and undergo beta-oxidation, to be discussed later, resulting in Acetyl-CoA. Once the appropriate reactions have generated acetyl-CoA, it enters the tricyclic acid (TCA cycle), where citrate is formed. Citrate can then be exported by the citrate carrier, where acetyl-CoA can be reformed by citrate lyase and utilized as a precursor for multiple anabolic reactions, including CH synthesis.

Acetyl-CoA is formed, within the mitochondria, by the activity of the pyruvate dehydrogenase complex (PDC; EC 1.2.4.1). However, as animals are incapable of reforming glucose from acetyl-CoA, this complex is tightly controlled by the ATP/ADP ratio, which indicates energy demand, and by the levels of NADH, pyruvate, and acetyl-CoA (Garrett *et al.*, 2005). Phosphorylation by several isoforms of the pyruvate dehydrogenase kinase (PDK; EC 2.7.11.2) causes the PDC to be inactivated (Harris *et al.*, 2001; Harris *et al.*, 2002; Huang *et al.*, 2003). Glucose sparing for use by the brain is accomplished during starvation by activation of PDK2, while PDK4 is more responsive to alterations in lipid availability (Roche *et al.*, 2001). When energy demands are higher than the supply, pyruvate dehydrogenase phosphatase (EC 3.1.3.43) dephosphorylates the PDC, thereby activating it for the production of acetyl-CoA. Hypothyroidism, a pathology known to decrease CL levels [(Paradies *et al.*, 1989) reviewed in (Schlame *et al.*, 2000)], results in the increased protein expression of both PDK2 and 4 (Holness, Bulmer, Smith, & Sugden, 2003). Thus, it is reasonable to expect that pyruvate processing by the mitochondria may be inhibited by a lack of mature CL species.

CL plays a role in many of the proteins associated with the mitochondria. This is not surprising, given its relative abundance in the mitochondrial membrane, particularly the inner mitochondrial membrane [reviewed in (Joshi *et al.*, 2009)]. It should not, therefore, be surprising to imagine that an alteration in the quantity and/or the quality of CL, as demonstrated in BTHS, could have wide-ranging effects on the function of the mitochondrial proteins it associates with. Some proteins have already been considered, while others, such as the relationship of CL to cytochrome c (Cyt c) release during apoptosis, will be discussed later (***The role of CL in Cellular Replication and***

Apoptosis). A recent paper by Brandner *et al* demonstrated that the mitochondrial supercomplexes III and IV were destabilized in *Saccharomyces cerevisiae* lacking *Taz1* (Brandner *et al.*, 2005). The activity of multiple transport proteins in the mitochondrial membrane are reduced in models lacking CL, as in hypothyroidism. As discussed, the pyruvate translocator and citrate carrier (***Biochemistry***) require cardiolipin (Paradies *et al.*, 1989; Palmieri *et al.*, 1993). Furthermore, the mitochondria of BTHS lymphoblasts and fibroblasts demonstrated altered conformation with a lack of cristae. These BTHS mitochondria also demonstrated a reduced membrane potential, compared to wild type counterparts, yet they were able to maintain ATP production. (Xu *et al.*, 2005; Acehan *et al.*, 2006; Claypool *et al.*, 2006). ATP production was maintained in BTHS, similar to wild type, but it has been suggested that this is due to a compensatory increase in mitochondrial number. Thus, it is possible that mitochondrial functions, including those functions associated with processing intermediates in the CH biosynthetic pathway, are altered in BTHS.

3.1.1. β-oxidation

Fatty Acids are processed in the mitochondria matrix to acetyl-CoA via successive rounds of β-oxidation [reviewed in (Schulz, 2002)]. In the case of odd numbers of carbon atoms, propionyl-CoA remains and is converted to succinate, which can then enter the TCA cycle. The fatty acid must be activated by fatty-acyl thioester formation before β-oxidation can occur. Short and medium chain fatty acids are membrane permeable and can be acylated within the mitochondrial matrix. Longer chain fatty acids, however, require a thioester of the fatty acyl chain, formed at the cytosolic side of the

mitochondrial membrane by the ATP-dependent acyl-CoA synthetase (ACS; EC 6.2.1.3) followed by transport by a series of proteins (**Figure** 3-2). Carnitine is used to carry the acyl-chains across the outer mitochondrial membrane, therefore, the carnitine palmitoyltransferase I (CPT; EC 2.3.1.21), located on the outer mitochondrial membrane, is used to add carnitine to the fatty acid. The carnitine:acylcarnitine translocase (C:AT; NM_000387), generally thought to localize to contact sites between the inner and outer mitochondrial membranes, is responsible for the transport of acylcarnitine into the mitochondrial matrix, while carnitine is sent back out to CPTI. It is interesting to note that CL is considered to be important in the formation of membrane contact sites (Ardail *et al.*, 1990), and accordingly, it is not suprising that this complex was also demonstrated to be dependent on CL for full functioning (Noel *et al.*, 1986; Pande *et al.*, 1986). Once in the matrix, the carnitine moiety is removed and the thioester reformed by CPT II (EC 2.3.1.21) so the fatty acyl chain can finally enter the β–oxidation spiral. Generally there are four different enzymes associated with the β-oxidation cycle, the acyl-CoA dehydrogenase (AD; EC 1.3.99.13), the enoyl-CoA hydratase (EH; EC 4.2.1.17), the L-3-hydroxyacyl-CoA dehydrogenase (HD; EC 1.1.1.35) and the 3-ketoacyl-CoA thiolase (KT; EC 2.3.1.16). After carnitine is removed and the acyl-chain is active, it enters the cycle according to the length of its acyl-chain. The membrane associated very long chain dehydrogenase (VAD) dehydrogenates acyl-CoA to 2-*trans*-enoyl-CoA. This is now a substrate for the reversible hydration by the membrane associated EH to form L-3-hydroxyacyl-CoA. The soluble matrix enzyme, HD, catylzes the third reaction to 3-ketoacyl-CoA. Finally, the thiol is cleaved by KT, adding it back to the working end of the fatty acyl-chain. As a result, each round of the β-oxidation cycle shortens the fatty

acyl-CoA chain by two carbons, released as acetyl-CoA. As β-oxidation proceeds, the dehydrogenases with specifity for the acyl-chains change. The long chain dehydrogenase (LAD; EC 6.2.1.3), exhibits a preference for chains of at least 8 carbons, and the medium chain dehydrogenase (MAD; EC 6.2.1.2) exhibits a specificity for chains of 6-12 carbons. While the short chain dehydrogenase (SAD; EC 6.2.1.1) is most active on fatty acyl chains of 4-6 carbons [reviewed in (Schulz, 2002)].

The details differ somewhat in considering unsaturated fatty acids, as the double bond requires an enoyl-CoA isomerase or a dienoyl-CoA reductase, depending on whether the bond is at an even or an odd numbered carbon (Vance *et al.*, 2002). Once the double bond is close in proximity to the thioester group, these auxilary enzymes isomerize, dehydrogenate, reduce and isomerize the double bond, the resulting acyl-CoA can then enter back into the β-oxidation spiral.

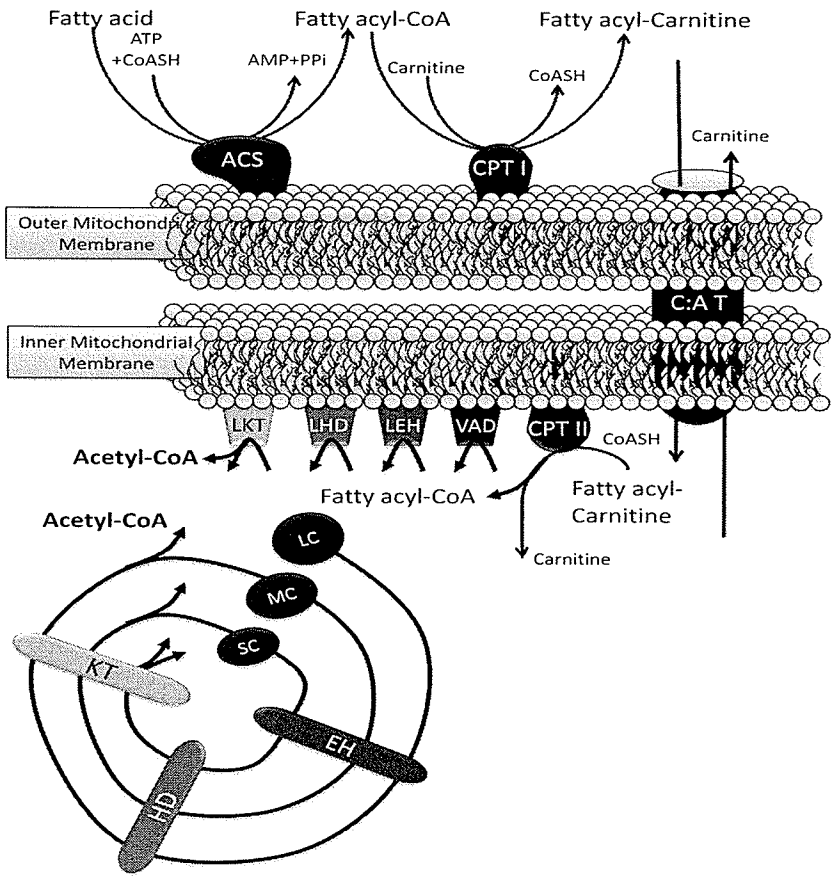

Figure 3-2: Long chain fatty acid activation and uptake into the mitochondria for β-oxidation

Long chain fatty acids are acylated by the ACS, which requires energy in the form of ATP. The CPT I adds carnitine to the activated fatty acid preparing it for transport through membrane contact points into the mitochondrial matrix via the C:AT, which also recycles the carnitine residue to the cytosol. Once in the matrix, carnitine is replaced with the thioester and can enter into the β-oxidation cycle at the enzyme appropriate for its chain length. A very long chain fatty acyl-CoA is dehydrogenated, hydrated, dehydrogenated and the acetyl-CoA released and a thioester bond reformed by the membrane-bound forms of the dehydrogenase, hydratase, dehydrogenase and thiolase. Once the chain is 8 carbons or less, it enters into the matrix associated forms of the enzymes, and spirals though the β-oxidation cycle, releasing 1 acetyl-CoA per turn until oxidation is complete. ACS (ATP-dependent acyl-CoA synthase); CPTI/II (carnitine palmitoyltransferase I/II); C:AT (carnitine: acylcarnitine translocase); VAD (very long chain acyl-CoA dehydrogenase); LEH (very long chain enoyl-CoA hydratase); LHD (very long chain L-3-hydroxyacyl-CoA dehydrogenase); LKT (very long chain 3-ketoacyl-CoA thiolase); LAD (long chain acyl-CoA dehydrogenase); MAD (medium chain acyl-CoA dehydrogenase); SAD (short chain acyl-CoA dehydrogenase); EH (enoyl-CoA hydratase); HD (L-3-hydroxyacyl-CoA dehydrogenase); KT(3-ketoacyl-CoA thiolase). Modified from (Schulz, 2002) © Elsevier (2002).

3.2. Unfolded Protein Response

The unfolded protein response (UPR) is a stress response pathway within the ER which generally acts to aid in appropriate protein folding when proteins accumulate. The UPR causes an induction of a number of molecular chaperone proteins, including the 78kDa glucose regulated protein (GRP78), also known as the immunoglobulin heavy chain binding protein (BiP), a member of the heat shock protein (HSP)70 family, which localizes to the mitochondria (Sun *et al.*, 2006). In addition, the UPR, specifically the chaperone GRP78, is induced by dephosphorylation, as a response to cellular acidosis (Sugawara *et al.*, 1993; Aoyama *et al.*, 2005). As a result of chronic ER stress, heart failure can develop (Fu *et al.*, 2008).

3.3. The role of CL in Cellular Replication and Apoptosis

Much work has gone into the role of CL in apoptosis, as we will discuss further. In addition, it has been established that CL, as well as a number of other lipids, are increased upon cell cycle progression (Cornell *et al.*, 1980). Whereas cell cycle progression can be haulted by a deficiency in CH (Brown *et al.*, 1974). Thus, we felt it imperitive to determine if cell cycle progression was inhibited by decreased mature CL, as observed in BTHS.

Programmed cell death, apoptosis, is a useful tool in complex organisms. The ability to force a cell to die in a non-violent manner allows infected and transformed cells to be eliminated without causing mass destruction of the surrounding tissue. In addition, it also has a significant role in development. The immune system has a very intricate balance of

life and death during its development, too much cell death, and the body will not have adequate protection from potential invaders. Not enough programmed cellular death and rogue cells in the immune system that recognize self as its target can cause autoimmunity. Recently CL was shown to play a role in regulating apoptosis (Kirkland *et al.*, 2002). However, caution should be used when interpreting the results of many of these studies, as a recent publication by Gohil *et al* suggests that binding of 10-N-nonyl acridine orange (NAO) can bind yeast deficient in CL production (*crd1*Δ), with an affinity equal to the wild type (Gohil *et al.*, 2005a). NAO is a dye frequently used to specifically bind, and quantify, CL in apoptotic studies. This suggests that a non-specific binding of NAO can occur, thus calling into question any results that suggested CL was unaltered.

During Fas, Fas-Ligand induced cell death, CL relocalizes to the outer surface of the cell membrane. The relocation of CL was associated with a the direct association of CL or MLCL with cytosolic Bid (Sorice *et al.*, 2004). In a previous study, we observed that a conversion of CL to MLCL was associated with apoptosis and was mediated by PLA_2 in rat heart cells stimulated with the pro-apoptotic, tumour necrosis factor (TNF)-α (Xu *et al.*, 1999). It has been suggested that Caspase-8 association with the mitochondria, necessary for the cleavage of Bid to its more effective mediator, tBid, requires mature CL (Gonzalvez *et al.*, 2008). The further association of CL with tBid seems to mediate Cyt c release from the mitochondrial membrane (Lutter *et al.*, 2000; Liu *et al.*, 2005). In the absence of apoptotic signals, Cyt c, is maintained on the inner mitochondrial membrane by its strong association with CL. In order to release Cyt c, this interaction needs to be disrupted. Upon Cyt c release, Bcl-2-associated X protein (Bax) is able to increase

membrane permeability (Ott *et al.*, 2002). More recently a study by Iverson *et al* disputed earlier studies, hypothesizing that CL is required for Bax-mediated pore formation rather than Cyt c release. They established this by exhibiting Cyt c release in the CL deficient *crd1*Δ yeast model. This confirmed their previously proposed two-step process of Cyt c release followed by Bax-induced permeability (Iverson *et al.*, 2004). Upstream of the Bid/Bax/CL interaction, Bcl-x(L) (of the B-cell lymphocytic-leukemia proto-oncogene 2 family) acts to control Cyt c release by preventing mitochondrial protein release and, hence, apoptosis (Kuwana *et al.*, 2002).

Interestingly, increased apoptosis of neutrophilic precursors has been described as the causative factor in many neutropenia disorders, including a down-regulation of Bcl-x in myelokathexis (Aprikyan *et al.*, 2000). To this end, G-CSF has been recommended as a viable therapy for neutropenia with various causes, including BTHS [Reviewed by (Stein *et al.*, 2003)]. Due to the neutropenia observed in some cases of BTHS, studies comparing the function of the Bcl-x/Bid pathway in BTHS and their normal counterparts have been ongoing. Unfortunately, the results have not been as clear cut as originally expected. Although MLCL, which accumulates in BTHS and is produced in Fas-induced cell death, seems to be the major target for tBid (Esposti *et al.*, 2003), a down regulation of the pro-apoptotic molecule Bid in BTHS has been described [Reviewed by (Esposti, 2004)]. In BTHS, surprisingly few studies have considered the viability of neutrophils. One report described the ability of neutrophils and eosinophils but not monocytes or lymphoblasts from 7 BTHS patients, to bind annexin-V. This was indicative of an increase in PS translocation to the outer membrane, although aminophospholipid translocase activity, which acts to maintain the PS concentration gradient, was not

inhibited. In light of an increase in total PS levels reported in the *taz1Δ S. cerevisiae*, the increased annexin-V binding may reflect an accumulation of PS (Gu *et al.*, 2004). Regardless, an additional study revealed that Bax translocation was not altered, nor were the BTHS neutrophils more likely to be phagocytosed when exposed to macrophages (Kuijpers *et al.*, 2004), suggesting that apoptosis was not increased in BTHS cells. Furthermore, a recent publication comparing Epstein Barr virus transformed BTHS lymphoblasts to Jurkat T cells, exhibited an accumulation of MLCL similar to the findings in the *taz1Δ* yeast (Gu *et al.*, 2004). Contrary to the predicted response, this accumulation of MLCL did not lead to apoptosis, as was demonstrated by the lack of PARP-cleavage by caspase 3, which prevents repair of DNA damage, and the normal localization of Bid and Cyt c. In addition, there was no apparent difference in sensitivity to Fas-mediated apoptosis between BTHS and control lymphoblasts (Valianpour *et al.*, 2005).

Although initial studies supported a role for CL deficiency leading to improper control over neutrophillic apoptosis, more recent evidence suggests a far more complex role for CL in BTHS. Therefore, we investigated the ability of our cells to enter S-phase of the cell cycle, indicating their ability to maintain cell growth and replication.

3.4. Lipid Biochemistry

3.4.1. Phospholipid Synthesis

Phospholipids are all derived from the common PL, PA. Glycerol-3-phosphate (G-3-P) and dihydroxyacetone phosphate (DHAP), by-products of the glycolysis pathway, can

each be used to generate PA by acyl-chain addition. Ligation of thioesters to activate fatty acids for addition to G-3-P by ACS (**Figure 3-3**), is followed by an acyltransferase reaction. With no preference for acyl substrates, the ER localized glycerol-3-phosphate O-acyltransferase (GPAT; EC 2.3.1.15) transfers the acyl-CoA to G-3-P, to form lysoPA. The mitochondrial form, however, is more specific, and may provide the PA pool for TG synthesis (Vance, 2003). Similarly DHAP is acylated, and then converted to 1-acyl-G-3-P, or lysoPA. Addition of a second fatty acyl chain to lysoPA is accomplished by AGPAT (EC 2.3.1.51), of which, multiple isoforms have been identified; however, the different characteristics of each have not yet been fully described.

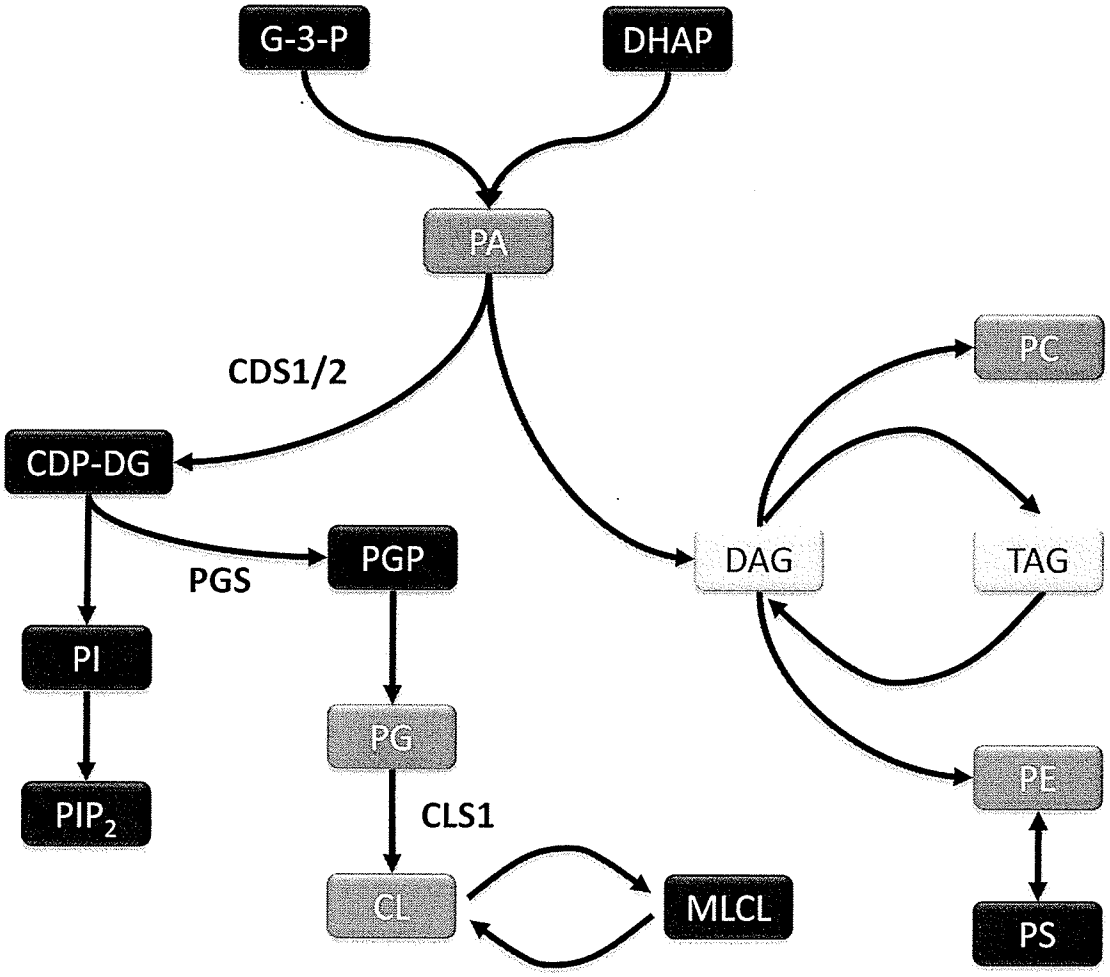

Figure 3-3: The Biosynthesis of Phospho- and Neutral Lipids in Mammalian Cells

G-3-P or DHAP are precursors for PA synthesis, which acts as the major branch point for lipid synthesis. DAG can be made from PA, and serves as the source for *de novo* synthesis of PC, PE and PS as well as TG. Alternatively, CDP-DG is formed from PA and can be used to make either PG or PI. The addition of a second moeity of CDP-DG to PG results in the formation of CL. G-3-P (glycerol-3-phosphate); DHAP (dihydroxyacetone phosphate); PA (phosphatidic acid); CDP-DG (cytidine- 5'-diphosphate-1,2-diacyl-sn-glycerol); PI (phosphatidylinositol); PIP_2 (phosphatidylinositol diphosphate); PGP (phosphatidylglycerol phosphate); PG (phosphatidylglycerol); CL (cardiolipin); MLCL (monolysocardiolipin); DG (diacylglycerol); TG (triacylglycerol); PC (phosphatidyl choline);PE (phosphatidyl ethanolamine); PS (phosphatidyl serine).

As the branch point for PL synthesis, PA leads to the synthesis of nearly all the other lipids. The next reaction is either the production of CDP-DG, the first point in the PG and CL synthetic pathway, as discussed in (***De novo biosynthesis of cardiolipin***), or the production of diacylglycerol (DG), as will be discussed (***Neutral Lipid Synthesis***). From DG, both PC and PE can be synthesized. In the Kennedy pathway of PC synthesis, DG is used as the acyl-glycerol building block for PC, by addition of the choline head group (Kennedy, 1956). Choline first needs to be phosphorylated by a choline kinase (CK; EC 2.7.1.32). Then cytidine 5'-triphosphate (CTP) is used to activate the choline moiety for the addition to DG by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CCPT; EC 2.7.8.2). The nucloetidyl treansfer to produce this CDP-choline substrate for CPT is catalyzed by CTP:phosphocholine cytidylyltransferase (CT; EC 2.7.7.15). Loading of macrophages with CH leads to an increase in production of PC, likely to expand the cellular membrane, in an attempt to prevent toxicity from excess CH (Tabas, 2002).

De novo production of PE is similar to that of PC; ethanolamine is activated via phosphorylation by ethanolamine kinase (EK; EC 2.7.1.82). CDP is added by a nucleotidyl group transfer with CTP:phosphoethanolamine cytidyldyltrasnferase (ET; EC 2.7.7.14). Finally, the substitution group transfer of ethanolamine to DG, by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (CEPT; EC 2.7.8.1) forms PE. PE can be synthesized from 2 other pathways in eukaryotes; however these conduits involve the decarboxylation or the reversible phospho group transfer of phosphatidylserine (PS). This reversible reaction is actually the mechanism by which

CDP-diacylglycerol-serine O-phosphatidyltransferase (EC 2.7.8.8) generates PS in the cell (Hubscher *et al.*, 1959).

Finally, PI can be generated by the substituted phospho-group transfer of inositol to CDP-DG by CDP-diacylglycerol-inositol 3-phosphatidyltransferase (EC 2.7.8.11).

3.4.2. Neutral Lipid Synthesis

Neutral lipids, including triacylglyceride (TG) and DG, are important energy stores and second messangers for the human body. Formation of TG from DG acts as an energy storage molecule (Vance *et al.*, 2002). DG, on the other hand, represents the branch point for TG, PC and PE synthesis, thus DG is an important step in the synthesis of PLs, in addition to its role in signalling. Conversion of PA to DG is accomplished by PA phosphatase (PAP; EC 3.1.3.4), an enzyme that is activated by translocation from the cytosol to the ER (Vance, 2002). As previously mentioned (***Phospholipid Synthesis***), both PC and PE can be formed from DG. While TG is formed by the further ligation of a third acyl-CoA chain to the DG moiety (Weiss *et al.*, 1960) by the enzyme diacylglycerol acyltransferase 1 and 2 (DGAT; EC 2.3.1.20). Conversely, the energy stored can be recaptured by cleavage of the acyl-chain by lipases, to reform DG. Alterations in TG metabolism, leading to an accumulation of TG, as in obesity or diabetes, result in cardiomyopathy, suggesting a toxic effect of excess TG [reviewed in (Lewin *et al.*, 2003)].

3.4.3. Cholesterol Metabolism

Cholesterol is derived from approximately a dozen reactions, utilizing 18 molecules of acetyl-CoA (Bloch, 1964; Lynen, 1964; Sabine, 1977; Raju, 1999). This process occurs in all nucleated cells of the human body, however, the liver is commonly thought of as the major source of CH production (Vance *et al.*, 2002). CH can be taken up from the pool provided by the liver, as very low density lipoprotein (VLDL) or LDL, as determined by the amount of associated TGs (Murray *et al.*, 1996). Dietary sources may also be used; these travel to the liver as chylomicrons. Transport and uptake of CH will be discussed further anon (***Cholesterol Import and Export***). In the absence of a sufficient supply of CH in the serum, synthesis of CH is increased, as will be discussed further (***Regulation of Cholesterol synthesis***). In cell cultures, we can harness this response, driving the synthesis of CH to increase, by withdrawing the serum source of extracellular CH (Fogelman *et al.*, 1977). This is advantageous to our studies, as we can look at CH synthesis in the presence and absence of serum and identify deficiencies under basal conditions and during heightened CH synthesis, respectively.

The first step in CH synthesis involves the condensation of acetyl-CoA, from mitochondrial sources, as discussed previously (***Mitochondrial Biochemistry***) and acetoacetyl-CoA, to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by the enzyme, HMG-CoA synthase (HMGS; EC 2.3.3.10). The second reaction is the major rate limiting enzyme of the CH biosynthetic pathway. HMG-CoA reductase (HMGR; EC 1.1.1.34) (**Figure 3-4**) catalyzes a redox reaction to produce mevalonate from HMG-CoA (Brown *et al.*, 1980). Next the mevalonate moiety is dually phosphorylated, by mevalonate kinase (EC 2.7.1.36) and phosphomevalonate kinase (EC 2.7.4.2), to form

mevalonate-5-pyrophosphate. Finally, the decaboxylation of mevalonate-5-PP by diphosphomevalonate decarboxylase (EC 4.1.1.33) results in the production of isopentenyl diphosphate, one of the first isoprenoids. As will be discussed (***Isoprenoid Biosynthesis***), isoprenoid assembly is required for so many processes that the cell requires it to live (Brown *et al.*, 1980; Siperstein, 1984). Farnesyl diphosphate synthase (EC 2.5.1.1) transfers an alkene to form farnesyl-PP. A second alkene transfer by squalene synthase (EC 2.5.1.21) results in squalene, which now comprises all 18 acetyl-CoA residues. It is interesting to note that squalene synthase is also regulated by cellular CH (Tansey *et al.*, 2000), thus it is important in the sparing of isoprenoid substrates in the presence of sufficient CH. The remainder of the reactions to synthesize CH involve a series of cyclations and reductions to form the final multi-ring structure of CH (Liscum, 2002).

HMGR is the major site of statin activity, although new evidence suggests that there is an important anti-inflammatory aspect to this class of drugs (Cutts *et al.*, 1989; Muldoon *et al.*, 1997a; Aprahamian *et al.*, 2006; Greenwood *et al.*, 2006). This enzyme was first localized to the ER, and more recently, a peroxisomal form, which is resistant to statins, has been identified (Liscum *et al.*, 1985; Olivier *et al.*, 2000). Furthermore, this peroxisomal form seems to be resistant to most, if not all, the known regulatory methods for its ER counterpart (Aboushadi *et al.*, 2000), possibly indicating a failsafe mechanism to maintain mevalonate production for isoprenoid synthesis.

An excess of CH in the cell can be toxic, so the cell has methods beyond the regulation of its synthesis to ensure a buildup of free CH doesn't occur. The predominant method of CH storage is to convert it to CH esters (CE) and store it in lipid droplets. This is

accomplished by an ER resident enzyme, acyl-CoA:cholesterol acyltransferase (ACAT; EC 2.3.1.26), (Buhman *et al.*, 2001). It is very important to note, however, that lymphoblasts do not seem to store CH as esters to any large extent (Gottfried, 1967), thus studies performed on lymphoblasts will not be able to detect any alterations in this aspect of CH metabolism.

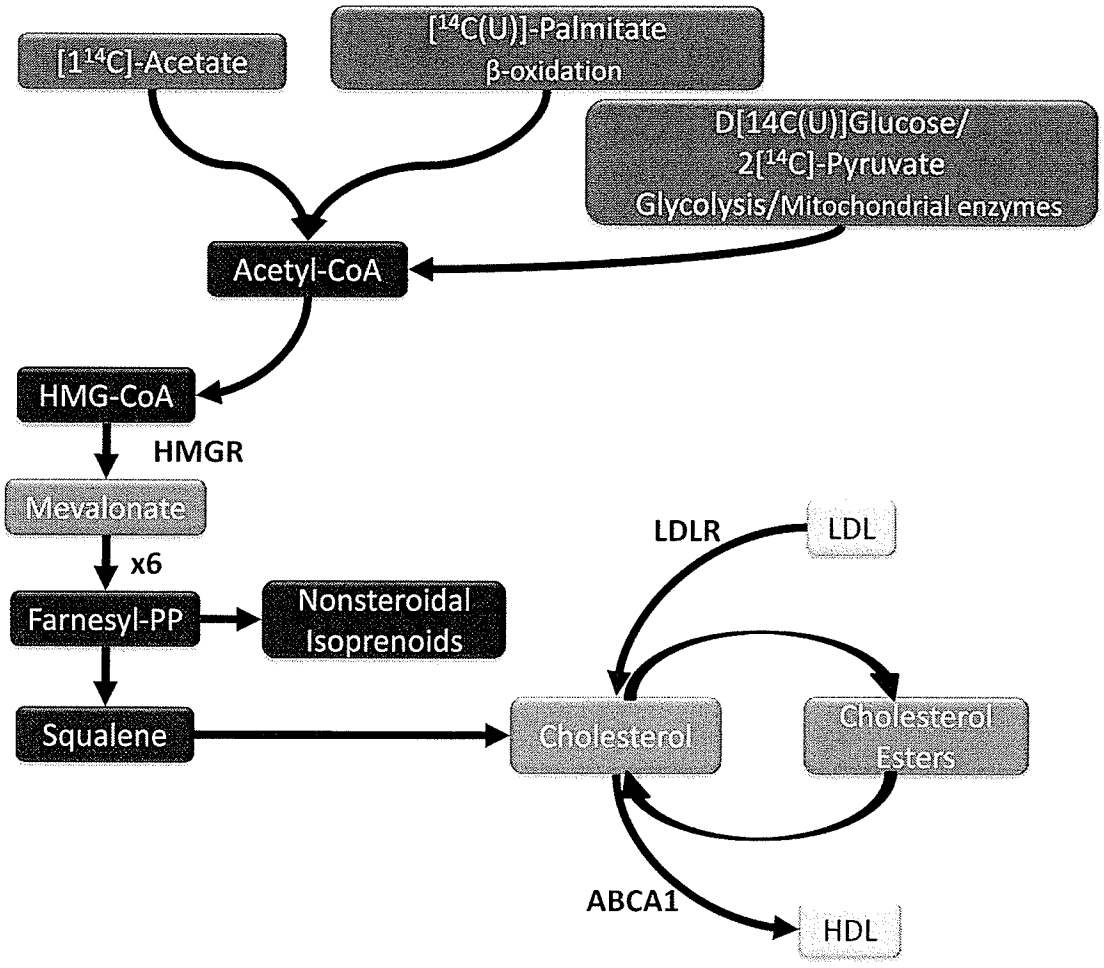

Figure 3-4: The Biosynthetic Pathway of Cholesterol and its Fates in the Mammalian Cell

CH metabolism is based on the simple 2-carbon molecule, acetyl-CoA, which is processed in the mitochondria. The conversion to mevalonate requires the major rate limiting enzyme, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). Isoprenoids are a very important byproduct of the CH synthetic pathway. Precursors utilized in the following studies are highlighted in light blue, while measured products are in red. Lipoproteins for CH transport, low density lipoprotein (LDL) and high density lipoprotein (HDL), are highlighted in green. (LDLR) LDL receptor; (ABCA1) ATP-binding cassette protein A1.

3.4.3.1. Regulation of Cholesterol synthesis

The first recognition that CH could control its own fate by virtue of a negative feedback pathway came in 1933, when Rudolph Schoenheimer noted that mice fed a low CH diet produced CH in a sufficient supply for the body's needs, while this synthesis was inhibited when CH was supplemented in the diet (Schoenheimer *et al.*, 1933). Since then, much work has gone into elucidating the mechanism of this feedback regulation and even earned Drs. Michael Brown and Joseph Goldstein the 1985 Nobel Prize in Physiology or Medicine, in addition to the 13 prior laureates related to CH[1] (Brown *et al.*, 1985; Raju, 2000). Now we know that both CH and oxysterols can regulate the process of CH synthesis, but each in a slightly different manner (Brown *et al.*, 2008). All of the known regulation is dependent on the localization of CH to the ER, where the target sterol sensing proteins reside (Brown *et al.*, 2002; Yabe *et al.*, 2002; Yang *et al.*, 2002). The sterol response element binding protein (SREBP) is a transcription factor with 3 known isoforms, 1a, 1c and 2 that bind to sterol response elements (SREs) in promoter regions to promote transcription of the target genes (Hua *et al.*, 1993). Isoforms SREBP-

[1] "Heinrich O. Wieland (1928), Adolf O.R. Windaus (1928), Leopold Ruzicka (1939), Robert Robinson (1947), and Otto P.H. Diels (1950) were awarded the Nobel Prize in Chemistry in part for work that led to the elucidation of the structure of cholesterol, a brilliant chapter in the history of organic chemistry. Konrad Bloch and Feodor Lynen were awarded the Nobel Prize in Medicine or Physiology in 1964 for their landmark studies of the cholesterol biosynthetic pathway, a complex sequence involving at least 30 steps. Robert B. Woodward, who pioneered the stereochemical synthesis of cholesterol, received the Nobel Prize in Chemistry in 1965 "for his outstanding achievement in the art of organic synthesis." Derek H.R. Barton and Odd Hassel were awarded the Nobel Prize in Chemistry in 1969 "for developing and applying the principles of conformation in chemistry," which included establishing the *a l l* chair conformation of cholesterol. John W. Cornforth, who collaborated with George Popjak to establish the orientation of all of the hydrogen atoms in the cholesterol molecule, received the Nobel Prize in Chemistry in 1975 "for his work on the stereochemistry of enzyme-catalyzed reactions."" Brown, MS & Goldstein, JL. (1985). **A Receptor-Mediated Pathway for Cholesterol Homeostasis.** In *Nobel Lectures in Physiology or Medicine 1981-1990*, ed. Lindsten, J, pp. 596. World Scientific Publishing Co., Singapore.

1a and -1c seem to be involved predominantly with the regulation of PL metabolism, and SREBP-1c is typically found in the liver (Vallett *et al.*, 1996; Shimano *et al.*, 1997; Liang *et al.*, 2002). While SREBP-2 is primarily involved with CH regulation, including, but not limited to increasing LDL receptor (LDLR), HMGR and its own synthesis, in an attempt to increase cellular CH levels, while maintaining a self-regulating balance (Horton *et al.*, 1998; Liscum, 2002; Horton *et al.*, 2003).

The transcription factor, SREBP-2, is bound to its escort protein, SREBP cleavage activating protein (SCAP), in the ER. When CH levels are in excess, this complex binds to insulin responsive gene (Insig)-1 (Yang *et al.*, 2002; Engelking *et al.*, 2005), which alters the conformation of SCAP, thus preventing its association with the COPII containing vesicles (Nohturfft *et al.*, 2000; Brown *et al.*, 2002; Espenshade *et al.*, 2002; Sun *et al.*, 2005; Sun *et al.*, 2007; Brown *et al.*, 2008). Without this COPII association, the SREBP-2/SCAP complex is retained in the ER (Nohturfft *et al.*, 2000; Espenshade *et al.*, 2002; Yang *et al.*, 2002; Sun *et al.*, 2005). In addition, Insig-1 can target HMGR and facilitates accelerated degradation of the protein, further maintaining the delicate CH balance (Sever *et al.*, 2003a; Sever *et al.*, 2003b).

When sterol levels are limiting, however, Insig-1 releases its hold on the complex, allowing its association with the COPII vesicle. Thus, the SREBP-2/SCAP complex is transported to the Golgi apparatus, where the site-1 and site-2 proteases (S1P and S2P respectively) act on SREBP-2 to sequentially release the N-terminal portion (Sakai *et al.*, 1996; Rawson *et al.*, 1997; Rawson *et al.*, 1998; Brown *et al.*, 1999; Rawson *et al.*, 1999; Yang *et al.*, 2001; McPherson *et al.*, 2004; Brown *et al.*, 2008). The mature transcription factor translocates to the nucleus, where it increases the transcription of the

aforementioned CH metabolic genes. It is interesting to note that oxysterols, an oxygenated derivative of CH, are actually stronger inhibitors of this transcriptional regulation than CH itself (Goldstein *et al.*, 1990; Engelking *et al.*, 2005; Brown *et al.*, 2008). The reason for this is not yet fully understood.

3.4.3.2. Cholesterol Import and Export

As previously mentioned, CH can be trafficked throughout the body, though it is not known to cross the blood-brain-barrier [Reviewed by (Dietschy *et al.*, 2004)]. However, CH is a very hydrophobic molecule, thus a transport system is required to solubilize CH for transport though the blood and lymph. As a result, lipoprotein complexes are formed, utilizing CH and PLs as a membrane barrier to surround esterified CH and TG (Oncley, 1954; Green *et al.*, 1960; Brown *et al.*, 1970; Fredrickson, 1974; Kang *et al.*, 2000). These lipoproteins are targeted to specific receptors by apolipoproteins (Brown *et al.*, 1970; Fredrickson, 1974). The main peripheral receptor is the LDLR, which binds to apolipoprotein B (ApoB) (Goldstein *et al.*, 1977), and receptor mediated-endocytosis results in the lysosomal localization of LDL. From there, an acidic environment is required to remove the LDLR from the CH-apoB complex and recycle it to the plasma membrane, while the CH is trafficked to the ER (Sugii *et al.*, 2003; Soccio *et al.*, 2004; Radhakrishnan *et al.*, 2008). Over activity of the LDLR would be expected to result in hypocholesterolemia; however, it has been established that hypothyroidism is associated with a decrease in SREBP-2 activation and, therefore a reduction in LDLR (Shin *et al.*, 2003). Hypothyroidism is the same condition that is known to cause a decrease in CL (Paradies *et al.*, 1989; Paradies *et al.*, 1991; Paradies *et al.*, 1997a; Paradies *et al.*, 1997b).

Strangely, in BTHS, a reduction in CL seems to cause hypocholesterolemia, rather than the hypercholesterolemia this model would predict. Thus, this is not likely to be the mechanism responsible for the altered CH metabolism in BTHS, though this remains to be confirmed.

At the other end of the spectrum, CH export from the peripheral cell is conducted by the ATP binding cassette protein A1 (ABCA1). This export protein associates with the apolipoprotein, ApoA1, to package TG and CE into CH and PL membranes in HDL particles for transport to the liver (Fielding *et al.*, 1995; Vance *et al.*, 2002). A genetic mutation in the ABCA1 transporter is responsible for Tangier's Disease, which results in an accumulation of CH within the peripheral cells and a decrease in serum HDL CH (Nofer *et al.*, 2005). Interestingly, one study demonstrated an increase in CL and MLCL coincident with this defect in ABCA1 CH export (Fobker *et al.*, 2001). Though this pathology displays an increase in CL, where BTHS exhibits a decrease, it is reasonable to expect the increase in CL synthetic enzymes, in an attempt to overcome the decreased CL of BTHS, could associated with this CH storage. Thus, we will consider the expression of the ABCA1 receptor in BTHS and a cell model deficient in hCLS1.

3.4.3.3. Isoprenoid Biosynthesis

The CH pathway, subsequent to mevalonate formation by HMGR results in isoprenoids for a multitude of different functions within the mammalian cell [reviewed in (Goldstein *et al.*, 1990; Waterham, 2006)]. The isoprenoid pathway provides substrates for the 0.5-2% of proteins that require prenylation. Protein prenylation is commonly thought to add a lipid soluble component for membrane anchoring. In addition, prenylation may aid in

the sorting of proteins within the membrane, thus allowing an extra level of regulation in protein and associated signaling pathways (Vance *et al.*, 2002). A minimal functioning of the CH biosynthetic pathway is required to maintain the cellular levels of isoprenoid synthesis, as dietary CH cannot make up for a deficiency, and prenylation is required for cell survival (Brown *et al.*, 1980). This may be one way in which CH synthesis may be further reduced, as limited resources may be diverted to isoprenoid synthesis.

Chapter 4: Study Design

Chapter 4: Study Design

4.1. Cellular Models

For all of the studies described herein two different models of the BTHS defect were utilized. The first is BTHS lymphoblasts transformed with an Epstein-Barr virus and cultured in suspension. These cells were generously provided by Dr. Richard Kelley. They represent actual BTHS patients, and presumably share the same basic biochemistry as any other cell type found in an affected individual. Although this assumption is not entirely correct, as each cell type has a very unique role in the human body, and as such, has a unique biochemical requirement, it was a necessary compromise. The lymphoblast model we have used here is of major importance in aiding our understanding of the altered biochemistry and cellular functioning in BTHS. These cells provide a surrogate for BTHS patients in the absence of a viable animal model, the closest being a model of an X-linked cardiomyopathy that was determined not to be a defect in tafazzin (Leatherbury *et al.*, 2008). In addition, the harvesting of human lymphoblasts is far less invasive than many other cell lines. Unfortunately lymphoblasts do not represent the predominant cell in CH production in the human body; that is generally considered the function of the hepatocyte [reviewed in (Ott *et al.*, 1981)]. Though, all cells in the body have the capacity to perform some cholesterol metabolism, and can, therefore be used as a model of the basic CH synthesis, as evidenced in the work by Brown & Goldstein. Currently we attribute the bulk of what we know today about CH metabolism to the work of Brown and Goldstein (Anderson, 2003), and many of their studies were performed in fibroblast cells. Therefore, the BTHS lymphoblasts are a good representation of BTHS, but may have some deficiencies as a model for this work.

Since liver is the main source of VLDL and ultimately LDL, and more than half the BTHS males in the Spencer, *et.al.* cohort exhibited a reduction in LDL levels (Spencer et al., 2006), it is possible there are further defects in VLDL secretion that could not be observed in the lymphoblast model. In addition, only certain tissues are steroidogenic, and express the enzymes and transporters responsible for converting CH into hormones and other sterol products in the mitochondria. It is known that tetralinoeoyl CL forms contact points for movement of CH into the mitochondrial lumen for steroid production (Sugawara *et al.*, 1995; Gasnier *et al.*, 1998; Miller, 2007). Moreover, lymphoblasts do not produce any significant amount of CE for storage, as other cells do (Gottfried, 1967). As a result, any deficiencies in these pathways may not be observed in our cell model.

The major characteristics of each patient and their age at the time the lymphoblasts were harvested are summarized below (**Table 4-1**). Each of the three patients had different mutations in tafazzin, resulting in different types of mutations, including a frameshift and a missense mutation. Two of the three individuals had CH readings taken; however, the ages of the CH readings are at minimum 2 years prior to the harvesting. It is unknown whether these values would remain the same. In fact, it would be expected that these values would change over time. Unfortunately, the ages of CH testing do not chronologically correlate with each other either, so we will be unable to identify any conclusions regarding our results and severity of CH deficiency with this information.

Table 4-1: Genotype of the BTHS cell models

Cell Line Identifier	Phenotype	Age at Harvest (years)	Family	Proband	Tafazzin Mutation	Cholesterol (mg/dL) (age)	References
Control	Wild Type	10		None	None	181.8 [a] (9-11yr)	(Abraham et al., 1978)
ΔTAZ 1	BTHS	4	3	c. 109+5G>C (Intron 1)	Splice Site	107 (2yr)	[b, c]
ΔTAZ 2	BTHS	9	5	c. 171delA (exon 2)	Frameshift	61 (5mo)	[b, c, d, e]
ΔTAZ 3	BTHS	1	11	c. 635T>C (Exon 8)	Missense		[b]

[a]estimated according to documented averages of American males 9-11yrs old; [b](Johnston *et al.*, 1997; Schlame *et al.*, 2003; Gonzalez, 2008); [c](Kelley *et al.*, 1991b); [d](Gonzalez, 2005); [e](Schlame *et al.*, 2005)

As an alternative to the BTHS lymphoblasts, HeLa cells were transfected with small hairpin RNAs (shRNA) to human CLS1 in a plasmid donated to us from Frohman's lab (Choi *et al.*, 2007). This model allowed us to determine whether it is the lack of functioning tafazzin, or the lack of CL that is causing the decrease in CH observed in BTHS. However we were not able to obtain the reduction in CL levels reported in BTHS cells (Valianpour *et al.*, 2002a; Valianpour *et al.*, 2002b), thus a result obtained in the BTHS lymphoblasts that was not confirmed by the hCLS HeLa cells could have been the result of a CL dose response. If a minimum CL level is required to retain a function, it is possible the hCLS cells won't show the deficiency due to a lack of CL reduction. Therefore, only the functions most sensitive to CL reduction will be observed in these cells.

4.2. Hypothesis

As reports of hypocholesterolemia in BTHS have infused the literature for nearly three decades with no major attempts to further investigate the cause, we have decided to explore the metabolism of cholesterol in BTHS. This thesis focuses on characterizing the biosynthesis of cholesterol in two cell lines with decreased mature cardiolipin levels. We hypothesize that cholesterol biosynthesis requires a minimal level of mature CL for appropriate functioning.

4.3. Study Rationale

This thesis examines the role of CL in CH, and subsequently lipid, metabolism. We have identified four main means by which serum CH concentrations might be affected. The first, and most obvious, is due to a decrease in CH biosynthesis, there are a number of possible reasons this might occur. CH metabolism is predominantly regulated by the activity of HMGR, thus, we have investigated the expression and activity of this enzyme, as well as the total CH pools in cells lacking mature CL. Alternatively, if there is a global blockage in carbon metabolism, synthesis of CH, as well as a number of other metabolites, might be reduced. As CL is known to be required for full functionality of both the pyruvate transporter (Paradies *et al.*) and the CPTI (Noel *et al.*, 1986), we attempted to determine whether CH is reduced due to an alteration in glycolysis and/or β-oxidation. To investigate this, we utilized four different sources of radiolabeled carbon and identified their incorporation into various PLs. Alternatively, as suggested previously (***Isoprenoid Biosynthesis***), isoprenoid synthesis, which often takes priority over CH synthesis [reviewed in (Goldstein *et al.*, 1990)], may sequester CH intermediates. Thus, CH synthesis would be reduced as a result of insufficient resources. No attempt to study this final potential mechanism has been made in this body of work, but it does warrant further investigation. Alternatively, an increase in CH degradation, either as an increase in bile acid or steroid synthesis, could also limit the pool size of CH available. This was beyond the scope of the research, and won't be further discussed.

If CH synthesis and pool size is unaltered, it is possible that CH trafficking is affected. There are a few examples in the literature of conditions that alter the level of serum CH simply by inhibiting import and export. Familial hypercholesterolemia results from a

reduction of CH taken up by the peripheral cells by means of the LDLR [reviewed in (Brown *et al.*, 2008)], while serum LDL levels are decreased by polyphenol stimulation of the LDLR (Davalos *et al.*, 2006). Interestingly, this study also revealed that, the polyphenol supplementation was able to increase CH synthesis, via an increase in HMGR activity. Alternatively, the reduction in circulating HDL observed in Tangier's disease is the consequence of an inhibition of the peripheral CH export by the ABCA1 receptor. Therefore, the potential ability of CL deficient cells to take up and deliver CH to the extracellular milieu was examined in a cursory manner, in an attempt to fully explore the metabolism of CH.

As CL is known to increase with cellular division (Bergeron *et al.*, 1970), and a deficiency in CH is known to cause an inhibition in cellular growth and progression through S phase (Brown *et al.*, 1974; Cornell *et al.*, 1980; Wong *et al.*, 2007), we have included in this thesis a study that looks at CL in the progression to S phase of the cell cycle, and the effect of CL on cell cycle progression (Hauff *et al.*, 2009).

Chapter 5: Materials and Experimental Methods

Chapter 5: Materials and Experimental Methods

5.1. Materials

D-[^{14}C(U)]glucose, [1,3-^{3}H]-Glycerol , [1-^{14}C]-Acetic Acid, sodium salt, [^{14}C(U)]-Palmitic Acid, [^{14}C]Glycerol-3-phosphate, [5-^{3}H]CTP, and *methyl*-[^{3}H]thymidine were obtained from either Perkin-Elmer, Woodbridge, Ontario Dupont, Mississauga, Ontario, or Amersham, Oakville, Ontario, Canada. [^{14}C]PG was synthesized from [^{14}C]glycerol-3-phosphate (Hatch *et al.*, 1996) DMEM, fetal bovine serum and antibiotics were products of Canadian Life Technologies (GIBCO), Burlington, Ontario, Canada. Lipid standards were obtained from Serdary Research Laboratories, Englewood Cliffs, New Jersey, USA. Thin layer chromatographic plates (silica gel G, 0.25 mm thickness) were obtained from Fisher Scientific, Winnipeg, Canada. Ecolite scintillant was obtained from ICN Biochemicals, Montreal, Quebec, Canada. HeLa cells were obtained from American Type Culture Collection. Barth Syndrome Epstein-Barr virus transformed lymphoblasts (ΔTAZ1-3) were obtained from Dr. Richard Kelley, John Hopkins University, Baltimore Maryland and age-matched controls (3798) obtained from Coriell Institute for Medical Research, Camden, New Jersey. QIAGEN OneStep RT-PCR kit was used for PCR studies. All other chemicals were certified ACS grade or better and obtained from Sigma Chemical Company, St. Louis, USA or Fisher Scientific, Winnipeg, Manitoba, Canada.

5.2. Cell culture

Barth Syndrome Epstein-Barr virus transformed lymphoblasts (ΔTAZ1, ΔTAZ2, and ΔTAZ3) were obtained from Dr. Richard Kelley, John Hopkins University, Baltimore

Maryland and an age and sex-matched control (Control) was obtained from Coriell Institute for Medical Research, Camden, New Jersey. Lymphoblasts were maintained in Roswell Park Memorial Institute (RPMI)-1640 media + L-glutamine (Invitrogen 11875-119) containing 10% fetal bovine serum (FBS) and 100U Penicillin, 100µg Streptomycin, 0.25µg Amphotericin B (Invitrogen 15240-062). All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

HeLa cells were obtained from American Type Culture Collection. HeLa cells were transfected with plasmids containing shRNA to human CLS, generously donated by Dr. Micheal Frohman StoneyBrook University, NY USA (Choi *et al.*, 2007). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen 12800-017) containing 10% FBS and 100U Penicillin, 100µg Streptomycin (Invitrogen 15140-122). In addition, transfected cells had 10µg/ml Blasticidin (Invitrogen R210-01) in their media as a selective agent for propagation, but shared the same media as the untransfected cells (lacking in Blasticidin) during all experimental treatments.

5.3. DNA Replication Radiotracer studies

HeLa cells were enriched at the G_0/G_1 interface by 24 h serum starvation as described (Jackman *et al.*, 1998). Cells were incubated with 0.1 µM *methyl*- [^{3}H]thymidine (5µCi/dish) for up to 24 h, the medium removed and 2 mL of 10% trichloroacetic acid was added to each dish. The cells were transferred to glass tubes, centrifuged for 10 min at 12,000 x g and 200 µL 0.3N NaOH added to the pellet. The precipitate was collected

by filtration through Whatman GF/B glass microfiber filters, and after washing with 1mL of ethanol, the radioactivity in the dried filters was determined.

5.4. Real time PCR

RNA was isolated from all cells using the TRIzol method of phenol extraction (Invitrogen 15596-026) as per included protocol. Concentration of RNA was determined by UV spectrophotometer on the GeneQuant Pro. Appropriate amounts of RNA, or water in the no template control (NTC) blanks were added to thin-walled tube 96-well plates from Eppendorf (Eppendorf 951022003) with Qiagen RT-PCR master mix (Qiagen 204445), total reaction volume 25µl, and the appropriate primers, listed in **Table 5-1**, and sealed using a heat sealer and optically transparent thermal seal from Eppendorf (Eppendorf 951023019). Reverse transcription was performed immediately prior to polymerase chain reaction as part of the same cycler protocol. Three major cycler protocols were used for real time (qRT-PCR) on the Eppendorf Mastercycler ep Realplex 2, all consisted of a 30 min RT step at 50°C, followed by a 15 min Taq activation step at 95°C. In addition, all PCR programs were followed by a 1 min separation at 95°C and melting curve that increased in temperature incrementally from 60°C to 95°C over the course of 20 min, taking fluorescence readings throughout to determine product quality. The CDS 1 (400nM) and 2 (200nM) , as well as the PGS (200nM) and CLS (400nM) primers had a PCR program that cycled 40 times between a separation step of 94°C for 35 seconds followed by an annealing and elongation step of 55°C for 45 seconds. For Insig1 (400nM), LDLR (400nM), ABCA1 (400nM), AGPAT 1-6 & 9 (400nM) GPAM (400nM), GLUT1 (400nM), SREBP-2 (400nM) and HMGR (400nM) primers, the PCR

program ran 50 cycles of separation at 94°C for 15 seconds and annealing/elongation at 60°C for 45 seconds. While the remaining were similar, with the distinction that, PDK2 (400nM) and PDC (400nM) were annealed at 59°C for 1 min, and PEPCK (400nM) with PDK4 (400nM) were annealed at 60°C for 1 min.

Fluorescence readings were taken at the end of every elongation step. The housekeeping gene, 18srRNA (100nM), was run with the same PCR program as the primers of interest. The changes in gene expression were analyzed on an Eppendorf Mastercycler ep Realplex, software version 1.5.474, and the data presented as mean fold change ($2^{-\Delta\Delta Ct}$) (Livak *et al.*, 2001) in mRNA expression relative to 18s rRNA, a gene not affected by serum addition to quiescent cells (Schmittgen *et al.*, 2000).

Table 5-1: Table of primers for Real time RT-PCR.

Target	Ref. Seq. No.	Forward (5'-3')	Reverse (5'-3')
SREBP-2	NM_004 599	cggcCAA GAA AGT CTT CCA GTG C[FAM]G	GCG CTG GTC TTA GCT TCG TCT T
Insig1	NM_198 336.1	GGA CGA CAG TTA GCT ATG GGT GTT	GAG TCA TTT GTA CAG TCA GCC CGA
HMGR	NM_000 859	cggttGGA AGA GAC AGG GAT AAA C[FAM]G	GGG TAT CTG TTT CAG CCA CTA AGG
LDLR	NM_000 527.3	CTG CCT AAG TGG CGA GTG CA	CAG GTG GCC ACA GCG CAG TT
ABCA1	NM_005 502	CTA TGA ACA TGA ATG CCA TT	GCT TCA AGT TTG AGC TGG AT
AGPAT1	NM_006 411.2	CCA CCC TCT CCT TTT GCT C	ACT CCC TGC CTC TCA CTC
AGPAT2	NM_006 412	CAC AAG CTG CAT CAG GCT CTC T	ACA GTG CTG ACC CCA CAG AGT CTT
AGPAT3	NM_020 132.4	ACC ACT ACA CAA GAG CTA CTC GC	TGC TAA CTT ACA CTT CAG AGG CC
AGPAT4	NM_020 133.2	AGC ACT TTT CAC TTT ATC TCT GGC	CAT AGA AAG GGT ATT TTA GGC TGC
AGPAT5	NM_018 361.3	AAA CAC ACC CTT TTC TGA AAA A	TAA GAG CCG TAT ACC TAC CTG TAA G
AGPAT6	NM_178 819.2	GCT CTT GTC CTG CTT CCA AC	GGC TGT ACG GTG GAG TCA TT
GPAM	NM_020 918.4	ATC GCA GTT ATT TCA CAG TAC C	AGA TTC TTG ACC TTA TTT CTA AAC G
AGPAT9	NM_032 717.3	TGC TTA TGC TGT GTG AGT TGG	CTT TTT ATT CCC GAA TTT TCC C
CDS 1	NM_ 001263	HLUX3001921_FAM_CDS1_#100	Invitrogen HLUX3001921_CDS1_#100
CDS 2	NM_003 818	HLUX3014245_FAM_CDS2_#100	Invitrogen HLUX3014245_CDS2_#100
CLS	NM_019 095.3	CGA GAG ATG TAA TGT TGA TTG CTG	CGA ACC GTG GTG TTG GAA GAG TT[FAM]G
PGS1	NM_024 419	TCG GCC TCC AGC ACA TTA AG	cggtgAGT CAC TCA GGT TTG CAC[FAM]G
PDK2	NM_002 611	CCG CTG TCC ATG AAG CAG TT	TGC CTG AGG AAG GTG AAG GA
PDK4	NM_002 612.	CCC GAG AGG TGG AGC ATT T	GCA TTT TCT GAA CCA AAG TCC AGT A
PDH	NM_000 284	TGA TGG AGC TGC AGA CTT ACC GTT	TGC TGT TCA CCA TCC TGT CCT TGA
PEPCK	NM_002 591	TAT GAC AAC TGC TGG TTG GC	ATA ACC GTC TTG CTT TCGA TC
GLUT1	NM_006 516	ATC GTG GCC ATC TTT GGC TTT GTG	CTG GAA GCA CAT GCC CAC AAT GAA
18srRNA	X03205	CTC GGG CCT GCT TTG AAC AC	cgggTGC TCT TAG CTG AGT GTC C[FAM]G

5.5. Phospholipid Determination

5.5.1. Phospholipid Radiotracer Studies

HeLa and BTHS cells were incubated overnight with various radiolabeled precursors to determine the level of anabolism in these cells. Cells were then incubated with 0.1 mM [1,3-^{3}H]glycerol (10μCi/dish) or D-[^{14}C(U)]glucose (18μCi/dish) in the absence or presence of 10% FBS for up to 24 h. Media containing or deficient of FBS had 10μCi/ml [1-^{14}C]-Acetic Acid, sodium salt (Perkin Elmer NEC084H), [^{14}C(U)]-Palmitic Acid [Perkin Elmer NEC534, bound to bovine serum albumin (BSA) 1:1 molar ratio], 10μCi/ml [1,2,3-^{3}H]-Glycerol (Perkin Elmer NET022), or 16μCi/ml D-[^{14}C(U)]glucose (Perkin Elmer NEC042X) added, and cells were incubated for up to 24 h, as indicated. Cellular lipids were then isolated as described below, samples reconstituted with chloroform:methanol (2:1, v/v) were spotted onto TLC plates and radiolabel incorporation into neutral and phospholipids were determined as described (***Lipid Isolation***).

Mevalonate labeling of cells was modified from Roweïl et al., (Roweïl *et al.*, 1997). Cells were starved of mevalonate for 6 h in 3 ml depletion medium (DMEM, 10% FBS and 30 μM mevastatin, dissolved in acetone; Sigma M2537) per 1.5x10^{6}cells. Cells were briefly centrifuged to remove the media, then the cell pellet was resuspended in 3 ml labeling medium (depletion medium with 50 μCi [2-^{14}C]mevalonic acid added per 3ml). Labeling medium was prepared by evaporating RS-[2-^{14}C]mevalonic acid in toluene to dryness under dry nitrogen at 56^{O}C and resuspending the residue in fresh depletion medium, then filter sterilizing. Cells were metabolically labeled for 16 h at 37^{O}C in 5% CO_2.

5.5.2. Lipid Isolation

All isolation of lipids from cells are a modification of the Folch method (Folch *et al.*, 1957). After the initial treatment of cells, suspension cells were spun down at approximately 1200rpm in a swinging bucket rotor for 10 min, while adherent cells had as much media as possible removed from the cells, and then were scraped from the plates using a plastic scraper and the initial extraction medium, methanol: H_2O (1:1, v/v). A phosphate buffered saline (PBS) wash was not performed on the adherent cells due to potential loss of cells, so neither was it performed on the suspension cells. All cells were lysed in 2ml of the extraction medium, described above, transferred to a silanated glass tube, and an aliquot was taken for total radioactivity and protein (usually 25µl and 50µl respectively) determination as appropriate. An additional 0.5ml of H_2O and 2mls of chloroform were added to each sample to initiate phase separation, followed by brief vortexing, and samples were centrifuged at 2000rpm in a swinging bucket rotor for 10min. The upper, aqueous layer was removed by suction, along with any protein, interphase. Addition of 2mls theoretical upper phase (methanol:0.9% NaCl:chloroform; 48:47:3, v/v) was followed by a second brief vortex and centrifugation at 2000rpm for 5 min.

Removal of the aqueous phase was followed by drying down of the organic phase with nitrogen gas. Samples were either used immediately or capped and stored at -20°C. Separation of CL and other PLs and determination of radioactivity was performed as previously described (Hatch, 1994; Hatch *et al.*, 1996).

Isolated lipids were subjected to one dimensional thin layer chromatography (TLC) for neutral lipids (cholesterol, esters, diacylglycerol, and triacylglycerol) and 2D TLC for

phospholipids (cardiolipin, phosphatidylethanolamine, phosphatidylglycerol, phosphatidic acid, phosphatidylcholine). A portion of the total sample was spotted onto silica TLC plates for one dimensional separation of the neutral lipids. These plates were developed for approximately an hour in Hexanes:Diethyl ether:glacial acetic acid (70:30:2, v/v) (**Figure AI- 1**). A second fraction of the sample was spotted onto 0.4M borate coated plates, (one sample per plate) and developed in the first dimension in chloroform:methanol:NH_4OH:H_2O (70:30:2:3, v/v) for up to an hour, and left to dry for up to 3 h at room temperature. The plates were rotated 90° counterclockwise, and developed for 15-20 min in the second dimension, chloroform:methanol:water (65:35:5, v/v) (**Figure AI- 2**). After exposure to iodine vapour, the plates were scraped and the samples scintillation counted to determine radiolabel incorporation.

5.5.3. Total Lipid content analysis

Cardiolipin and other lipids were isolated from 2D polar TLC plates, as above, and treated along with 0-200 nmol monopotassium phosphate (KH_2PO_4) as a standard. Perchloric acid (450µl) was added to the isolated lipids, and the solution was heated to approximately 120°C for 2 h. After cooling, 2.5 mls ddH_2O, 0.5mls each of 2.5% ammonium molybdate and 10% ascorbic acid were added, vortexing after each addition. The samples were then heated to approximately 95°C for 15 min. After cooling again, the samples were spun down at 2000rpm for 5 min, and the optical density was measured at 820nm.

5.5.4. Total cholesterol content

Cholesterol content of the cells and serum was determined by colourimetric reaction. Utilizing the oxidation of CH to form H_2O_2, the Amplex Red cholesterol assay kit from Invitrogen's Molecular Probes (Invitrogen A12216) detects total cholesterol. Cholesterol was isolated from cells via standard lipid isolation, a modification of the Folch method, as described previously. After isolation of cellular lipids, the lipid residue was reconstituted with 1% Triton X-100 in isopropanol, and CH assays were preformed as per protocol. All isolates were measured immediately after drying down with nitrogen, as fresh samples seemed to yield the best results.

5.6. *In Vitro* Enzyme Assays

5.6.1. Isolation of intracellular compartments

Mitochondria and microsomes were isolated from cell cultures grown to approximately $1x10^8$ cells, seeded at a density of approximately $5x10^5$cells/ml. Cultures were grown overnight in culture medium containing FBS or in absence of FBS.

Cells were isolated by centrifugation at 1,500rpm for 10min and cell pellets were resuspended on ice in 2 ml homogenizing buffer (10 mM Tris-HCL, pH 7.4, 0.25 M sucrose), followed by homogenization with 2x20 strokes of a Dounce A homogenizer. The homogenate was centrifuged at 4°C, 1,000 x g for 5 min and the supernatant centrifuged at 4°C, 10,000 x g for 15 min. The mitochondrial pellet was resuspended in 0.5 ml stabilizing buffer (0.05M Tris-Maleate, 10% Glycerol, 0.1M KCl, 0.01M $MgCl_2$,

0.5% TritonX-100) and used for assay of mitochondrial enzyme activities. CDS, PGS and hCLS1 enzyme activities were determined as described (Hatch *et al.*, 1996).

The resulting supernatant was centrifuged for 1.5 h at 4°C, 100,000xg. The microsomal pellets were resuspended in homogenizing buffer and kept on ice while protein content was determined.

5.6.2. *In Vitro* 3-Hydroxy-3-methylglutaryl-CoA Reductase Activity

In vitro HMG-CoA reduction assays were preformed essentially as described by Ohashi *et al.*, (Ohashi *et al.*, 2003). Reaction buffer containing, at final concentration, 5μM NADPH (made fresh), 10μM ethylenediamine-tetraacetic acid (EDTA), 10μM dithiothreitol (DTT), 100μM TrisHCl (pH 7.4), was added to 50μg microsomal protein per sample. The reactions were initiated by the addition of 4.5μCi DL-3-[glutaryl-3-^{14}C] hyroxy-3-methylglutaryl coenzyme A and 110nM cold HMG-CoA to samples, and incubated at 37°C for 30 min. Addition of HCl to 1M and incubation for a further 30 min at 37°C was required to lactonize the mevalonate formed. Samples were then stored in the freezer overnight. Dried silica TLC plates were used for sample separation. Spotted samples were subjected to approximately 2 h in TLC tanks containing acetone-benzene (1:1 v/v). The plates were exposed to iodine vapour and spots corresponding to Rf = 0.6-0.9 were scraped and scintillation counted to determine [^{14}C]mevalonate formation. HMG-CoA reductase activity is expressed as picomoles of [^{14}C]mevalonate formed per min per mg of protein.

5.7. Other determinations

Fluorescent activated cell sorting (FACS) analysis was performed using 5-bromo-2'-deoxyuridine (BrdU) (Sigma) and counterstained with 7-aminoactinomycin D (7-AAD) (Sigma), as previously described (Cann *et al.*, 2006).

All protein assays were done with BioRad's Protein assay (BioRad 500-0006), a variation on the Bradford method. BSA was used as a standard, in the microassay procedure for microtiter plates. Assays were all completed in duplicate and measured at 595nm on the BMG Labtech FLUOstar OPTIMA - high-performance multidetection plate reader.

5.8. Statistics

Student's t-test, one-way or two-way ANOVA with Bonferroni's post test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, **www.graphpad.com** and data is reported as the means ± SEM, unless otherwise stated. The level of significance was defined as $p<0.05$.

Chapter 6: On the Mechanism of the Elevation in Cardiolipin during HeLa cell Entry into the S Phase of the Human Cell Cycle

Chapter 6: On the Mechanism of the Elevation in Cardiolipin during HeLa cell Entry into the S Phase of the Human Cell Cycle

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Abstract

Cardiolipin (CL) is a key phospholipid involved in ATP generation. Since progression through the cell cycle requires ATP we examined regulation of CL synthesis during S phase in human cells and if CL or CL synthesis was required to support nucleotide synthesis in S phase. HeLa cells were made quiescent by serum depletion for 24 h. Serum addition resulted in substantial stimulation of [*methyl*-^{3}H]thymidine incorporation into cells compared to serum starved cells by 8 h confirming entry into the S phase. CL mass was unaltered at 8 h but increased 2-fold by 16 h post serum addition compared to serum starved cells. The reason for the increase in CL mass upon entry into S phase was an increase in activity and expression of CL *de novo* biosynthetic and remodeling enzymes and this paralleled the increase in mitochondrial mass. CL *de novo* biosynthesis from D-[^{14}C(U)]glucose was elevated and from [1,3-^{3}H]glycerol reduced upon serum addition to quiescent cells compared to controls and this was a result of differences in selection of precursor pools at the level of uptake. Triascin C-treatment inhibited CL synthesis from [1-^{14}C]oleate but did not affect [*methyl*-^{3}H]thymidine incorporation into HeLa cells upon serum addition to serum starved cells. Barth Syndrome lymphoblasts, which exhibit reduced CL, exhibited similar [*methyl*-^{3}H]thymidine incorporation into cells upon serum addition to serum starved cells compared with cells from normal aged matched controls. The results indicate that CL *de novo* biosynthesis is up regulated via elevated activity and expression of CL biosynthetic genes and this accounted for the doubling of CL demonstrated during S phase. However, normal *de novo* CL biosynthesis or CL itself is not essential to support nucleotide synthesis during entry into S phase of the human cell cycle.

Key words: cardiolipin, phospholipid synthesis, cell cycle, mitochondria, S phase, HeLa cells, enzyme activity, real time-PCR, human gene expression, Barth Syndrome lymphoblasts, triacylglycerol biosynthesis, glucose, glycerol, metabolism

Abbreviations: CL, cardiolipin; CDP-DG, cytidine-5'-diphosphate-1,2-diacylglycerol; CDS, CDP-DG synthetase; PA, phosphatidic acid; PG, phosphatidylglycerol: PGPS, phosphatidylglycerolphosphate synthase; CLS, CL synthase; hCLS1, human CLS1; TG, triacylglycerol; DG, diacylglycerol; FBS, fetal bovine serum, BTHS, Barth Syndrome, ALCAT1, acyllysocardiolipin acylatransferase-1; MLCL, monolysocardiolipin; MLCL AT, monolysocardiolipin acyltransferase; TAZ, tafazzin; PLA_2, phospholipase A_2

Short Title: Human cardiolipin synthesis in S phase

6.1. Introduction

Phospholipids are important structural and functional components of all biological membranes and delineate compartmentalization of organelles as well as the protective barrier that surrounds cells, the cell membrane (White, 1973). Mitochondrial phospholipids compromise a significant proportion of the entire phospholipid content of most eukaryotic cells and possess diverse roles in the regulation of varied mitochondrial processes. Cardiolipin (CL) or bis-(1,2-diacyl-*sn*-glycero-3-phospho)-1',3'-*sn*-glycerol is a key mitochondrial membrane phospholipid involved in ATP generation, mitochondrial mediated apoptosis, diabetes and Barth Syndrome, a rare and often fatal X-linked genetic disorder in young boys associated with cardiomyopathy (Hostetler, 1982; Daum *et al.*, 1986; McMurray, 1986; Hoch, 1992; Dowhan, 1997; Schlame *et al.*, 2000; Esposti, 2002; Hatch, 2004; Hauff *et al.*, 2006; Han *et al.*, 2007). CL modulates the activity of a number of key mitochondrial membrane enzymes involved in the electron transport chain including cytochrome c oxidase, carnitine palmitoyltransferase, creatine phosphokinase, pyruvate translocator, mono-, di-, and tricarboxylate carriers, glycerol-3-phosphate dehydrogenase, phosphate transporter, ATP/ADP translocase, and ATP synthase [reviewed in (Hoch, 1992; Hatch, 1998)]. CL is considered to be the "glue" that holds this mitochondrial respiratory chain together (Zhang *et al.*, 2002). CL is an absolute requirement for some of these respiratory enzymes and its interaction with mitochondrial proteins is specific since substitution with other phospholipids does not fully reconstitute their enzymatic activity. For example, the activity of delipidated rat liver cytochrome c oxidase is reconstituted by the addition of CL (Yamaoka-Koseki *et al.*, 1991). Under experimental conditions in which CL is removed and digested away from mitochondrial

respiratory chain proteins by phospholipases, denaturation and complete loss in activity was observed. Thus, since CL may regulate ATP generation in cells, preservation of appropriate CL content in mitochondria is essential for proper mammalian cell function.

Mammalian CL is synthesized *de novo* via the cytidine-5'-diphosphate-1,2-diacylglycerol (CDP-DG) pathway (Hatch, 1994). In the first step of this pathway, phosphatidic acid (PA) and CTP are converted to CDP-DG by CTP:PA cytidylyltransferase or CDP-DG synthetase (CDS) (Kiyasu *et al.*, 1963). Pulse-chase heart perfusion studies have indicated that one of the rate-limiting steps of CL biosynthesis in the heart is the conversion of PA to CDP-DG (Hatch, 1994; Cheng *et al.*, 1995). Two CDS enzymes, CDS-1 and CDS-2, have been cloned and characterized in human, mouse, rat and pig (Heacock *et al.*, 1996; Lykidis *et al.*, 1997; Saito *et al.*, 1997; Weeks *et al.*, 1997; Halford *et al.*, 1998; Volta *et al.*, 1999; Mercade *et al.*, 2007). In the second and third steps of the pathway CDP-DG condenses with *sn*-glycerol-3-phosphate to form phophatidylglycerolphosphate (PGP) and then phosphatidylglycerol (PG), catalyzed by (PGP) synthase (PGPS) and PGP phosphatase, respectively (Kiyasu et al., 1963). PGP does not accumulate in tissues. In the last step of the CL biosynthetic pathway, PG is converted to CL by condensation with CDP-DG catalyzed by CL synthase (CLS) (Hostetler *et al.*, 1971). The gene encoding human CLS (hCLS-1) was recently identified and characterized (Chen *et al.*, 2006; Houtkooper *et al.*, 2006; Lu *et al.*, 2006). Once synthesized *de novo*, CL is remodeled by deacylation/reacylation or by the CL transacylase tafazzin (TAZ) [reviewed in (Hauff *et al.*, 2006)].

Eukaryotic cell reproduction involves duplication of cellular components, including biological membranes and DNA content, resulting in a doubling in size and then division

into two components. The cell cycle is composed of four phases: G_1, S, G_2 and mitosis (Horton *et al.*, 2003). In the absence of growth factors (e.g. serum starvation) cells will not divide but enter into a quiescent state known as G_0. Cells depleted of serum in G_0 may be triggered to enter into the S phase by the addition of serum. A previous study in P815Y mast cells revealed that the phospholipid content doubled during S phase (Bergeron *et al.*, 1970). In addition, doubling of phospholipid content is not DNA synthesis dependent, and is a cell cycle-regulated process rather than a growth factor triggered event (Jackowski, 1994; Jackowski, 1996). In fission yeast mitochondrial growth and DNA synthesis may occur in the absence of nuclear DNA replication (Sazer *et al.*, 1990). Progression through the cell cycle requires ATP and serum addition to quiescent mammalian cells increases mitochondrial respiration (Herzig *et al.*, 2000). Since CL is essential for mammalian mitochondrial respiration its biosynthesis during entry into the S phase of the eukaryotic cell may need to be maintained. CL *de novo* biosynthesis during entry into the S phase of the human cell cycle had never been examined. Thus, the purpose of this study was to examine if CL *de novo* biosynthesis was altered upon serum addition to quiescent HeLa cells and if normal CL levels or CL biosynthesis was required for nucleotide synthesis during entrance into S phase. We hypothesize that CL synthesis was elevated in HeLa cells during entry into the S phase and this was due to an up regulation in activity and expression of CL synthetic genes. However, normal CL biosynthesis is not required for nucleotide synthesis during S phase in HeLa cells. In addition, normal CL levels are not required to support nucleotide synthesis in Barth Syndrome lymphoblasts.

6.2. Materials and Methods

6.2.1. *Materials*

[^{14}C]Glycerol-3-phosphate, [5-^{3}H]CTP, D-[^{14}C(U)]glucose, [1,3-^{3}H]glycerol, [1-^{14}C]oleate, [1-^{14}C]linoleoyl-CoA and [*methyl*-^{3}H]thymidine were obtained from either Dupont, Mississauga, Ontario, Perkin-Elmer, Woodbridge, Ontario, or Amersham, Oakville, Ontario. [^{14}C]PG was synthesized from [^{14}C]glycerol-3-phosphate (Hatch *et al.*, 1996). DMEM, fetal bovine serum (FBS) and antibiotics were products of Canadian Life Technologies (GIBCO), Burlington, Ontario, Canada. Lipid standards were obtained from Serdary Research Laboratories, Englewood Cliffs, New Jersey, USA. Thin layer chromatographic plates (silica gel G, 0.25 mm thickness) were obtained from Fisher Scientific, Winnipeg, Canada. Ecolite scintillant was obtained from ICN Biochemicals, Montreal, Quebec, Canada. HeLa cells were obtained from American Type Culture Collection. Barth Syndrome (BTHS) Epstein-Barr virus transformed lymphoblasts (596) were obtained from Dr. Richard Kelley, John Hopkins University, Baltimore, Maryland, and age-matched controls (3798) obtained from Coriell Institute for Medical Research, Camden, New Jersey. QIAGEN OneStep RT-PCR kit was used for PCR studies. All other chemicals were certified ACS grade or better and obtained from Sigma Chemical Company, St. Louis, USA or Fisher Scientific, Winnipeg, Manitoba, Canada.

6.2.2. Cell culture, radiotracer studies, cell harvesting

Cell culture was performed in a sterile environment, in a laminar flow hood, and working surfaces were cleaned with 70% ethanol. HeLa cells were grown in DMEM with 10% FBS and 100U Penicillin, 100μg Streptomycin in 60 mm-diameter dishes and were incubated at 37°C in a humidified atmosphere of 5% CO_2. HeLa cells were enriched at

the G_0/G_1 interface by 24 h serum starvation as described (Jackman *et al.*, 2003). Cells were then incubated with D-[^{14}C(U)]glucose (18μCi/dish) or 0.1 mM [1,3-^{3}H]glycerol (10μCi/dish) in the absence or presence of 10% FBS for up to 16 or 24 h, respectively. Subsequent to incubation the medium was aspirated and dishes washed twice with 2 mL of ice-cold phosphate-buffered saline. Cells were harvested in 2 mL of methanol:water (1:1, by vol) and 25 μL aliquots were taken for protein determination and measurements of total radioactivity. Subsequently, 0.5 mL of water and 2 mL chloroform were added to initiate phase separation. Samples were centrifuged at 2000 rpm for 10 min and the upper phase was aspirated. Two ml of theoretical upper phase (methanol:0.9% NaCl:chloroform) (48:47:3, by volume) was added and centrifugation was repeated for 5 min. The organic phase was removed and dried under nitrogen gas. Separation of CL and other phospholipids and determination of radioactivity was performed as previously described (Hatch *et al.*, 1996). In other experiments, cells were incubated as above with 0.1 mM [1-^{14}C]oleate (3 μCi/dish) bound to albumin (1:1 molar ratio) in the absence or presence of 4 μM triacsin C for 24 h. In other experiments, cells were incubated as above with 0.1 μM [*methyl*-^{3}H]thymidine (5μCi/dish) for up to 24 h, the medium removed and 2 mL of 10% trichloroacetic acid was added to each dish. The cells were transferred to glass tubes, centrifuged for 10 min at 12,000 x g and 200 μL 0.3N NaOH added to the pellet. The precipitate was collected by filtration through Whatman GF/B glass microfiber filters, and after washing with 1mL of ethanol, the radioactivity in the dried filters was determined. In other experiments, BTHS Epstein-Barr virus transformed lymphoblasts (596) and age-matched controls (3798) were grown in RPMI-1640 medium supplemented with 10% FBS and 100U Penicillin, 100μg Streptomycin, 250 ng

Amphotericin B in suspension and were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cells were serum starved for 24 h and then incubated in the absence or presence of 10% FBS for 16 h and radioactivity incorporation determined as above.

6.2.3. *Isolation of subcellular fractions and enzyme assays*

Quiescent HeLa cells were incubated in the absence or presence of 10% FBS for 24 h. Subsequently, the cells were washed twice with ice cold PBS and harvested with 2 ml homogenizing buffer (10 mM Tris-HCL, pH 7.4, 0.25 M sucrose). Cells were homogenized with 40 strokes of a Dounce A homogenizer. The homogenate was centrifuged at 1,000 x g for 5 min and the supernatant centrifuged at 10,000 x g for 15 min. The pellet was resuspended in 0.5 ml stabilizing buffer (0.05M Tris-Maleate, 10% Glycerol, 0.1M KCl, 0.01M $MgCl_2$, 0.5% Triton X-100) and used for assay of mitochondrial enzyme activities. The supernatant was centrifuged at 100,000 x g for 60 min, resuspended in 0.5 ml stablilizing buffer and used as the source of microsomal fraction. CDS, PGPS and CLS enzyme activities were determined as described (Hatch *et al.*, 1996). Mitochondrial monolysocardiolipin acyltransferase (MLCL AT) was determined as described with modifications (Van *et al.*, 2007). 25 µg of HeLa cell mitochondria was incubated in 50 mM Tris-HCL, pH 8.0 in the presence of 300 µM MLCL and 20 µM [1-^{14}C]inoleoyl-CoA (approximately 70,000 dpm/nmole) for 10 min at 37°C. Mitochondrial phospholipase A_2 (PLA_2) activity was determined as described (Van *et al.*, 2007). Mitochondrial citrate synthase activity was determined as described with modifications (Williams *et al.*, 1998). 5 µg of HeLa cell mitochondria was incubated in 0.9 ml of 50 mM potassium phosphate pH 7.4, at 21°C in the presence of 100 µM

dithiobis(nitro benzoic acid) and 100 μM acetyl CoA. The citrate synthase reaction was initiated by the addition of 100 μM oxaloacetate and monitored by the spectrophotometric analysis of thionitrobenzoate which is formed when dithiobis(nitrobenzoic acid) reacts with sulfhydryls in CoASH (measured at 405 nm). The concentration of citrate was determined using the molar absorption coefficient for thionitrobenzoate (1/13.6 mM,cm). ALCAT1 activity was determined as described with modifications (Van *et al.*, 2007). 50 μg of HeLa cell microsomal fraction was incubated in 50 mM Tris-HCL, pH 8.0 in the presence of 300 μM MLCL and 20μM [1-^{14}C]linoleoyl-CoA (approximately 70,000 dpm/nmole) for 60 min at 37°C.

6.2.4. *Real time-PCR mRNA analysis*

Quiescent HeLa cells were incubated in the absence or presence of 10% FBS for 8-24 h and mRNA analysis of CDS-1 and -2, PGPS, hCLS-1 and TAZ determined. The primers used for real time-PCR were Invitrogen's D-LUX Fam-labeled primer sets for human CDS-1 and -2 (HLUX3001921_FAM CDS1 and HLUX3014245_FAM_CDS2), PGPS (NM_024419.3, reverse primer cggtgAGTCACTCAGGTTTGCACc[FAM]G, forward primer TCGGCCTCCAGCACATTAAG), hCLS1 (NM_019095.3, reverse primer CGAACCGTGGTGTTGGAAGAGTT[FAM]G, forward primer CGAGAGATGTAATGTTGATTGCTG) and 18srRNA (X03205.1 reverse primer cgggTGCTCTTAGCTGAGTGTCC[FAM]G, forward primer CTCGGGCCTGCTTTGAACAC). TAZ primers were (NM_000116.2 , reverse primer tct ggt aga cgc cat ctc ct, forward primer, ctc cca ctt ctt cag ctt gg) PCR conditions included a 20 min reverse transcriptase step at 50°C followed by activation of the Qiagen

Hot Start polymerase at 95°C for 15 min. Forty cycles of a 35 second denaturation step, at 94°C and a 45 second annealing and extension step were followed by a standard melt curve analysis of products. The changes in CDS-1 and -2, PGPS, hCLS-1 and TAZ were analyzed on an Eppendorf Mastercycler ep Realplex, software version 1.5.474, and the data presented as mean fold change ($2^{-\Delta\Delta Ct}$) in mRNA expression relative to 18s rRNA, a gene not affected by serum addition to quiescent cells (Luciakova *et al.*, 1992).

6.2.5. Other determinations

Protein was determined as described (Lowry *et al.*, 1951). Cells were serum starved for 24 h then incubated in the absence or presence of 10% FBS for up to 24 h and then phospholipid phosphorus content were determined as described (Rouser *et al.*, 1970). Fluorescent activated cell sorting analysis was performed using 5-bromo-2'-deoxyuridine (BrdU) and counterstained with 7-aminoactinomycin D, as described (Cann *et al.*, 2006). Student's t-test, or two way ANOVA with Bonferroni's post test was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com and data is reported as the means ± SEM, unless otherwise stated. The level of significance was defined as $p<0.05$.

6.3. Results

The regulation of CL *de novo* biosynthesis during entrance into the S phase of the human cell cycle had never been examined. HeLa cells were enriched at the G_0/G_1 interface by serum starvation for 24 h (Jackman *et al.*, 2003). Fluorescent activated cell sorting analysis of HeLa cells post serum addition confirmed that there was a significant shift in the population of serum replete cells from G_1 to S phase by 16 h (data not shown). These cells exhibited >95% Trypan blue exclusion comparable with serum containing cell cultures. Subsequently, serum starved HeLa cells were incubated with or without 10% FBS for up to 24 h and incorporation of [*methyl*-^{3}H]thymidine into cells was examined as a measure of nucleotide synthesis during entry into S phase. [*Methyl*-^{3}H]thymidine incorporation was significantly elevated in serum treated cells and was maximum by 16 h compared to serum starved cells (**Figure 6-1**). Thus, entrance into the S phase of the human cell cycle was induced under these incubation conditions.

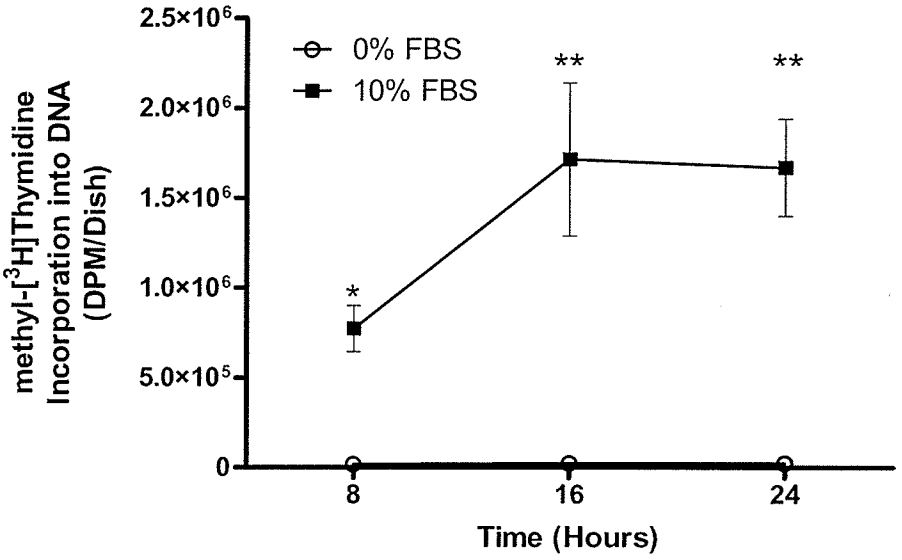

Figure 6-1: Incorporation of [*methyl*-^{3}H]thymidine into DNA of HeLa cells during induction of cell division.

HeLa cells were serum starved for 24 h, then incubated for up to 24 h with *methyl*-[^{3}H]thymidine in the absence (open circles) or presence (closed squares) of 10% FBS and the radioactivity incorporated into DNA was determined as described in Materials and Methods. Data represent the mean ± SEM of three experiments. (**$p<0.001$).

HeLa cells were then serum starved for 24 h and subsequently incubated with or without 10% FBS for up to 24 h and the pool size of CL and other glycerophospholipids determined. The CL pool size was unaltered at 8 h post serum addition but approximately doubled to a maximum by 16 h of serum addition to quiescent HeLa cells (**Figure 6-2A**). In addition, the pool size of all other glycerophospholipids were elevated by 16 h post serum addition (**Figure 6-2B**). Thus, entrance into S phase and DNA synthesis coincided with the increase in CL content. The reason for the increase in the CL pool size was then examined. To examine if there was an increase in mitochondrial mass, the activity of citrate synthase was determined. Citrate synthase activity was elevated 40% by 16 h and 51% by 24 h post serum addition to quiescent HeLa cells (**Table 6-1**). Thus, the increase in CL content paralleled the increase in mitochondrial mass during HeLa cell entrance into S phase. To examine if the increase in CL upon serum addition was a result of an increase in gene expression of CL biosynthetic enzymes, mRNA levels of CDS-1 and -2, PGPS and hCLS-1 were examined. HeLa cells were serum starved for 24 h and subsequently incubated with or without 10% FBS for up to 24 h. Subsequent to total RNA isolation, real time-PCR was performed. CDS-2, PGPS and hCLS-1 mRNA levels were elevated 2- to 3-fold in serum-treated cells compared to serum starved cells relative to the constitutive expression of 18s rRNA, by 24h (**Figure 6-3**). CDS-1 was not significantly elevated upon serum addition (**Figure 6-3A**). The level of hCLS-1 and CDS-2 appeared to increase at 16 h post serum addition but this was not statistically significant. HeLa cells were then serum starved for 24 h and subsequently incubated with or without 10% FBS for 16 or 24 h and CDS, PGPS and CLS enzyme activities determined (**Table 6-1**). CDS activity was increased 28% by 16 h and PGPS activity

increased 65% by 24 h. CLS activity increased 31% by 16 h and 2-fold by 24 h in serum treated cells compared to serum starved cells, respectively. Thus, the increase in the CL pool size was supported by an increase in the activities and expression of the CL *de novo* biosynthetic enzymes.

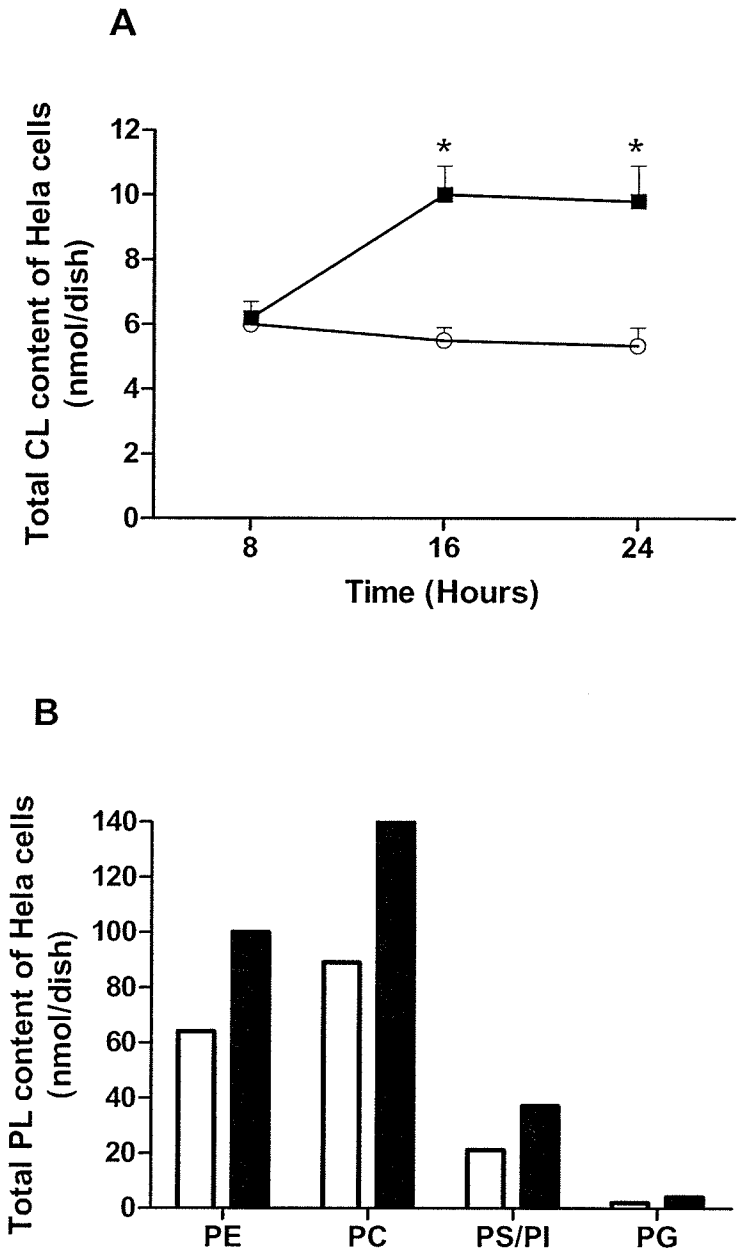

Figure 6-2: Pool size of CL and phospholipids in HeLa cells upon entry into S phase.

HeLa cells were serum starved for 24 h then incubated for up to 24 h in the absence (open circles) or presence (closed squares) of 10% FBS and the CL pool size determined as described in Materials and Methods. Data represents the mean ± SEM of three experiments (*$p<0.01$). **B**. HeLa cells were serum starved for 24 h then incubated for 16 h in the absence (open bars) or presence (closed bars) of 10% FBS and phospholipid pool size determined. Data represents the mean of two experiments.

Table 6-1: Activities of CL *de novo* biosynthetic enzymes and citrate synthase in quiescent HeLa cells incubated with serum.

HeLa cells were serum starved for 24 h then incubated for 16 or 24 h in the absence or presence of 10% FBS, mitochondrial fractions prepared and CDS, PGPS CLS and citrate synthase activities determined as described in Materials and Methods. Results are the mean ± SEM of at least three experiments (*p<0.05, **p<0.01).

Enzyme Activity	16 h		24 h	
	- Serum	+ Serum	- Serum	+ Serum
	pmol/min/mg protein			
CDS	53.2 ± 4.5	68.1 ± 4.3*	49.0 ± 3.5	63.0 ± 2.3*
PGS	117 ± 12.2	139.7 ± 10.5	95.5 ± 8.1	157.5 ± 15.9**
CLS	2.1 ± 0.5	3.6 ± 0.4*	2.5 ± 0.3	5.1 ± 0.4**
	nmol/min/mg protein			
Citrate Synthase	83.7 ± 4.7	117.9 ± 4.4**	89.8 ± 4.3	135.5 ± 7.2**

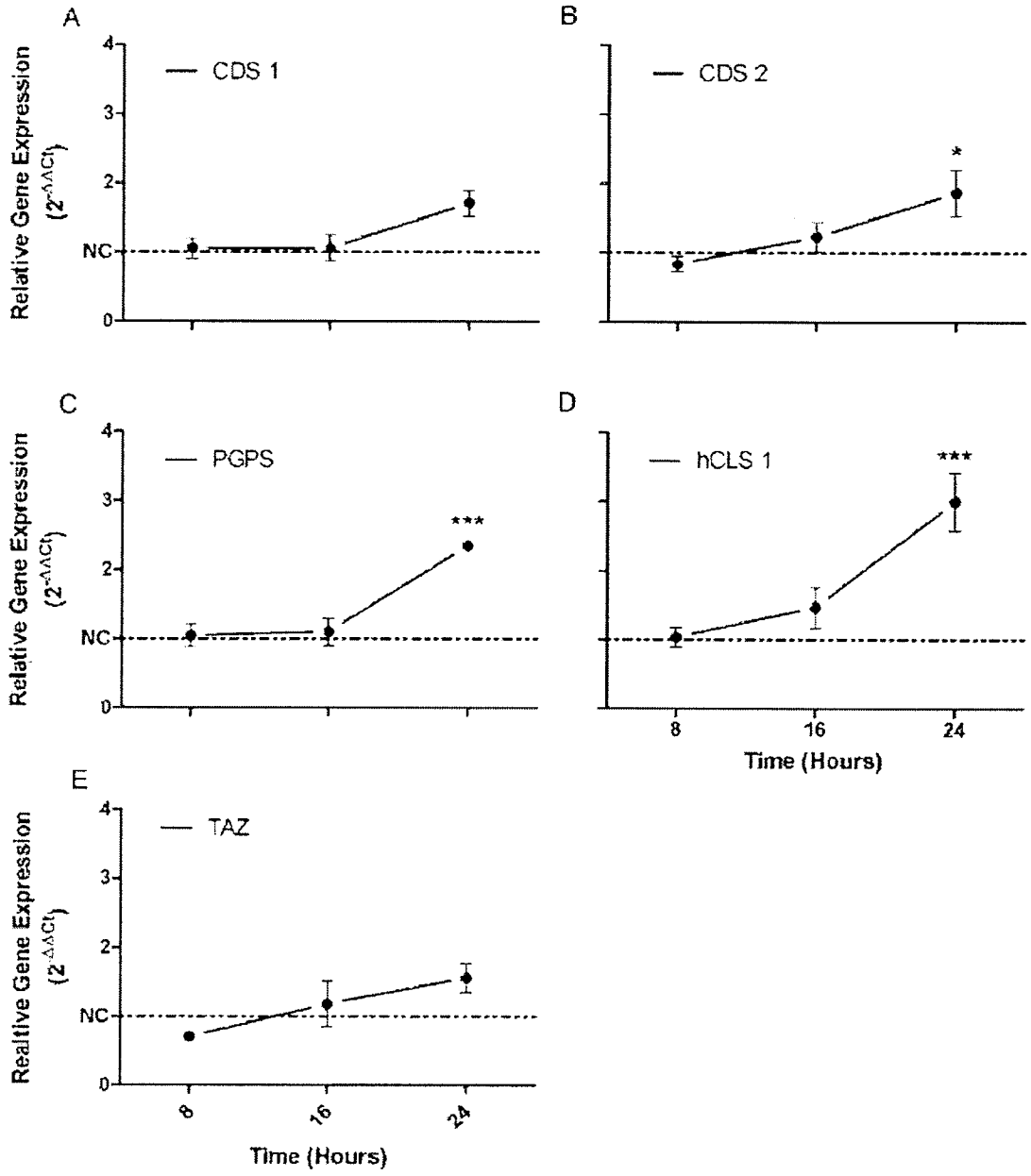

Figure 6-3: mRNA expression of *de novo* cardiolipin biosynthetic genes and TAZ in HeLa cells upon entry into S phase of the eukaryotic cell cycle.

HeLa cells were serum starved for 24 h, then incubated for up to 24 in the absence or presence of 10% FBS. Total RNA was isolated and real time-PCR performed as described in Materials and Methods. Data was expressed as fold increase in mRNA of CDS1 (**A**), CDS2 (**B**), PGPS (**C**) hCLS1 (**D**) and TAZ (**E**) relative to 18s rRNA. A value of 1 indicates no change (NC) in gene expression compared to the serum deprived control. Data represent the mean ± SEM of three experiments. (*$p<0.05$, ***$p<0.001$).

Once synthesized *de novo*, CL is rapidly remodeled by a deacylation-reacylation pathway catalyzed by PLA_2 and MLCL AT or by the CL transacylase TAZ [reviewed in (Hauff *et al.*, 2006)]. mRNA levels of TAZ and enzyme activities of mitochondrial MLCL AT and ALCAT1, an endoplasmic reticulum MLCL AT, were examined in HeLa cells serum starved for 24 h and then subsequently incubated with or without 10% FBS for up to 24h. TAZ mRNA levels seemed to increase in serum-treated cells compared to serum starved cells by 24 h, but this was not statistically significant (**Figure 6-3E**). Mitochondrial MLCL AT, ALCAT1 and PLA_2 activities were elevated 61%, 91%, and 46%, respectively by 24 h in serum-treated cells compared to serum starved cells (**Table 6-2**). The results indicate that the CL *de novo* biosynthetic and remodeling enzymes are elevated upon serum addition to quiescent cells and this likely accounted for the increase in CL mass observed in HeLa cells during S phase of the human cell cycle.

Table 6-2: Activities of CL remodeling enzymes in quiescent HeLa cells incubated with serum.

HeLa cells were serum starved for 24 h then incubated for 24 h in the absence or presence of 10% FBS, mitochondrial fractions prepared and PLA_2, MLCL AT and ALCAT1 activities determined as described in Materials and Methods. Results are the mean ± SEM of at least three experiments (*$p<0.05$, **$p<0.01$).

Enzyme Activity	- Serum	+ Serum
	nmol/min/mg protein	
PLA_2	0.71 ± 0.09	1.04 ± 0.08*
	pmol/min/mg protein	
MLCL AT	195.4 ± 27.7	315.7 ± 43.7
ALCAT1	54.6 ± 6.1	105.1 ± 14.4**

The carbon backbone of CL is readily synthesized from [1,3-^{3}H]glycerol and D-[^{14}C(U)]glucose precursors in HeLa cells (Wylie *et al.*, 1997) To examine if there was a preference for either precursor in CL *de novo* biosynthesis during S phase, HeLa cells were serum starved for 24 h and subsequently incubated with or without 10% FBS for up to 16 h in the presence of D-[^{14}C(U)]glucose or for up to 24 h in the presence of [1,3-^{3}H]glycerol and radioactivity incorporated into CL determined. D-[^{14}C(U)]Glucose incorporation into CL increased with time up to 16 h in both serum treated and serum starved cells (**Figure 6-4A**). However, a 1.5-fold ($p<0.001$) greater incorporation of D-[^{14}C(U)]glucose was observed upon serum addition at 16 h compared to serum starved controls. Incorporation of D-[^{14}C(U)]glucose into the CL precursor PG was elevated to a comparable extent in serum treated cells compared to serum starved cells. After a 16 h incubation period, serum treated cells exhibited a 50% increase (245 dpm/mg to 367 dpm/mg) in D-[^{14}C(U)]glucose incorporation into PG compared to serum starved cells. In addition, a greater incorporation of D-[^{14}C(U)]glucose into all other phospholipids was observed at 16 h upon serum addition (data not shown). Finally, total uptake of D-[^{14}C(U)]glucose into HeLa cells was elevated in the serum treated cells compared to the serum starved cells. Following a 16 h incubation period, serum treated cells exhibited a 2-fold (2.2×10^{5} dpm/mg to 4.6×10^{5} dpm/mg) increase in D-[^{14}C(U)]glucose uptake compared with serum starved cells. Analysis of neutral lipids revealed that D-[^{14}C(U)]glucose incorporation into triacylglycerol (TG) was increased (**Figure 6-5A**) and into diacylglycerol (DG) decreased (**Figure 6-5B**) in the serum treated cells compared to the serum starved cells indicating up regulation of HeLa cell TG biosynthesis during S phase.

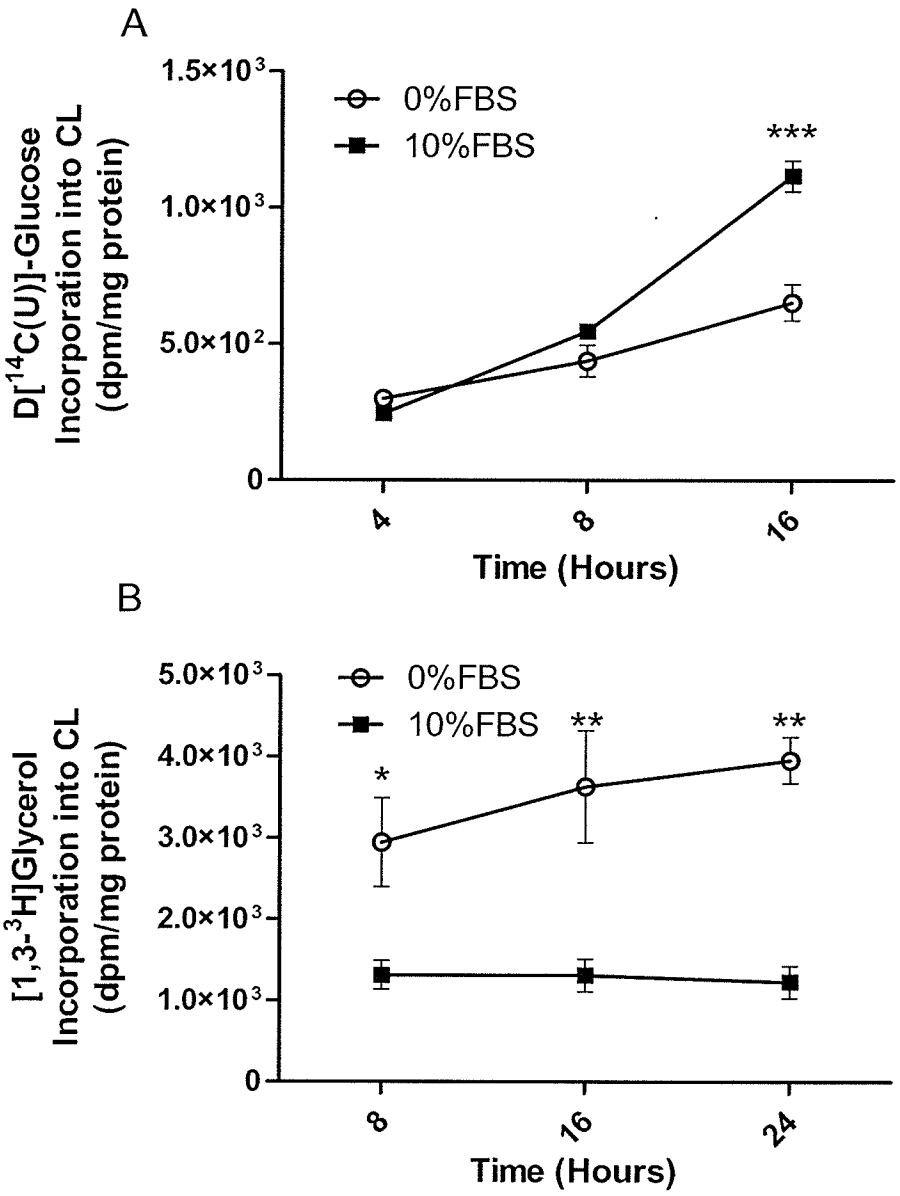

Figure 6-4: Incorporation of D-[^{14}C(U)]glucose and [1,3-^{3}H]glycerol into CL in HeLa cells during induction of cell division.

HeLa cells were serum starved for 24 h, then incubated for up to 16 h with D-[^{14}C(U)]glucose (**A**) or up to 24 h with [1,3-^{3}H]glycerol (**B**) in the absence (open circles) or presence (closed squares) of 10% FBS and the radioactivity incorporated into CL was determined as described in Materials and Methods. Data represent the mean ± SEM of three experiments. (*$p<0.05$; **$p<0.01$, ***$p<0.001$).

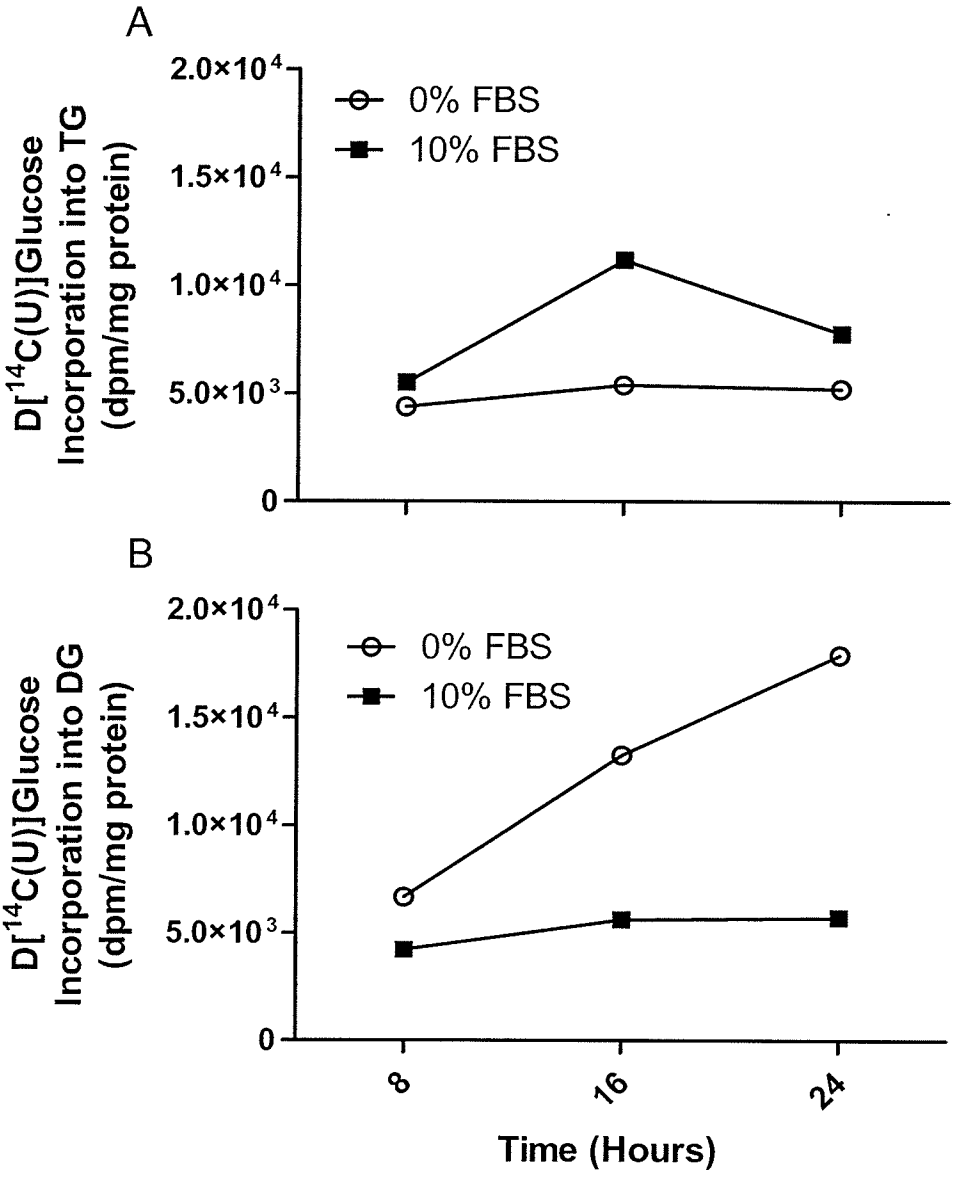

Figure 6-5: Incorporation of D-[^{14}C(U)]glucose into TG and DG in HeLa cells during induction of cell division.

HeLa cells were serum starved for 24 h, then incubated for up to 24 h with D-[^{14}C(U)]glucose in the absence (open circles) or presence (closed squares) of 10% FBS and the radioactivity incorporated into TG (**A**) and DG (**B**) determined as described in Materials and Methods. Data represent the mean of two experiments performed in triplicate. Results did not differ by more than 10%.

Surprisingly, [1,3-^{3}H]glycerol incorporation into CL was higher in serum starved cells compared to serum treated cells at all times examined (**Figure 6-4B**). In addition, incorporation of [1,3-^{3}H]glycerol into PG was reduced to a comparable extent in serum treated cells compared to serum starved cells. After a 16 h incubation period, serum treated cells exhibited a 33% decrease (367 dpm/mg to 245 dpm/mg) in [1,3-^{3}H]glycerol incorporation into PG compared to serum starved cells. Finally, total uptake of [1,3-^{3}H]glycerol into HeLa cells was lower in the serum treated cells compared to the serum starved cells. Following a 16 h incubation period, serum treated cells exhibited a 64% (2.2×10^{5} dpm/mg to 4.6×10^{5} dpm/mg) decrease in 1,3-[^{3}H]glycerol uptake compared to serum starved cells. These data indicate that there appears to be a difference in selection of precursor pools utilized for *de novo* CL and lipid biosynthesis at the level of precursor uptake during entry of HeLa cells into the S phase of the human cell cycle.

Progression through the cell cycle requires ATP and serum addition to quiescent cells increases mitochondrial respiration (Herzig *et al.*, 2000). Since CL is essential for mammalian mitochondrial respiration, we examined if reduced *de novo* biosynthesis of CL or reduced levels of CL itself would inhibit nucleotide synthesis upon serum addition to quiescent cells. Incubation of human cancer cell lines with triascin C, an inhibitor of long chain fatty acyl-CoA synthetase, has been established to inhibit *de novo* CL biosynthesis (Mashima *et al.*, 2005). To examine if normal CL *de novo* biosynthesis was required for entry into S phase, HeLa cells were serum starved for 24 h then incubated for 24 h with [1-^{14}C]oleic acid and 10% FBS plus or minus 4 μM triacsin C and incorporation of [1-^{14}C]oleic acid into CL and phospholipids examined. Oleic acid was used since it is the major fatty acid species found in HeLa cell phospholipids (Wylie *et*

al., 1997). Incorporation of [1-^{14}C]oleic acid into CL was reduced 32% ($p<0.05$) in the presence of triascin C (**Figure 6-6A**). Incorporation of [1-^{14}C]oleic acid into PG was elevated 40% ($p<0.01$) in the presence of triascin C (**Figure 6-6B**). In contrast, triascin C did not affect [1-^{14}C]oleic acid incorporation into other phospholipids (**Figure 6-6C**). Thus, triascin C inhibits CL synthesis in HeLa cells. HeLa cells were then serum starved for 24 h then incubated for 24 h with [*methyl*-^{3}H]thymidine and 10% FBS plus or minus 4 μM triascin C and incorporation of [*methyl*-^{3}H]thymidine into cells examined. Triascin C did not affect [*methyl*-^{3}H]thymidine into HeLa cells upon serum addition (**Figure 6-6D**). The data indicate that normal CL synthesis is not likely required for nucleotide synthesis during S phase in HeLa cells.

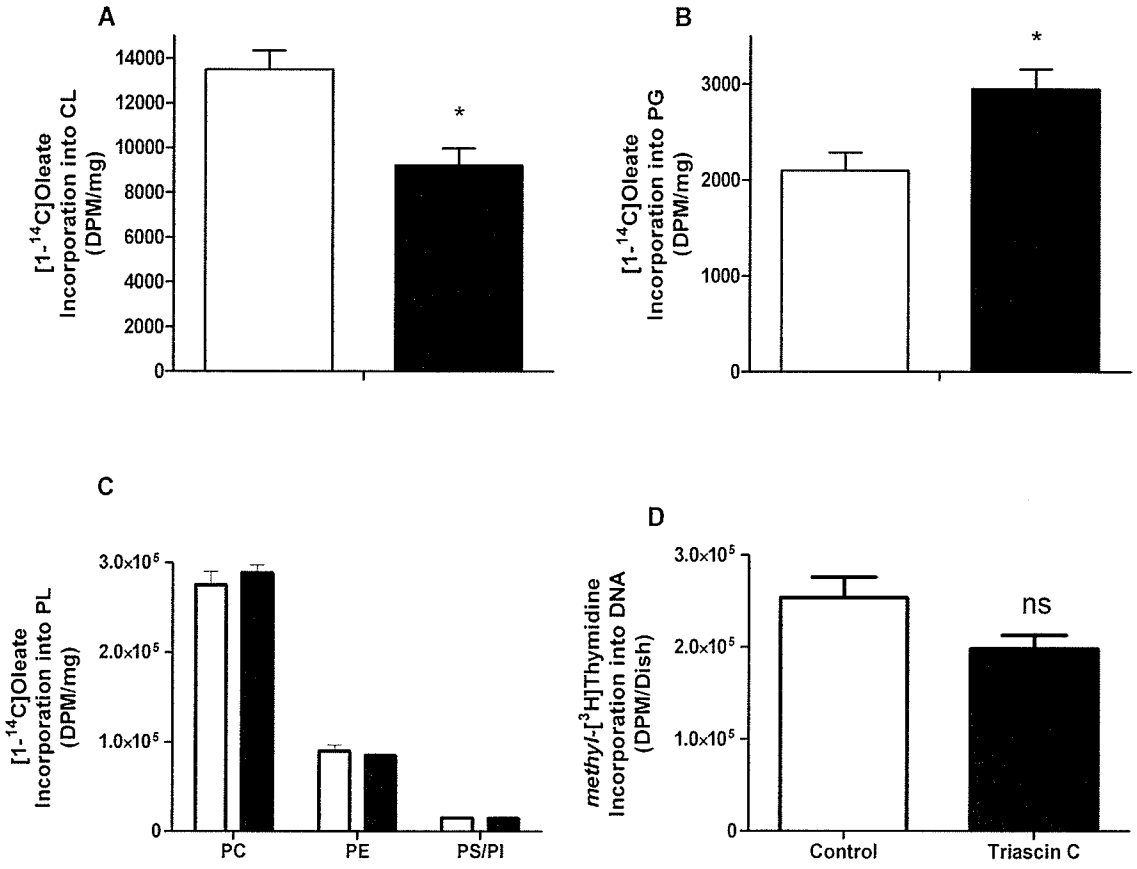

Figure 6-6: [*Methyl*-^{3}H]thymidine incorporation and [1-^{14}C]oleate incorporation into phospholipids in triascin C-treated HeLa cells.

HeLa cells were serum starved for 24 h, then incubated for 24 h with [1-^{14}C]oleate and 10% FBS in the absence (open bar) or presence (closed bar) of 4 μM triascin C and incorporation of [1-^{14}C]oleate into CL (**A**), PG (**B**) and other phospholipids (**C**) determined as described in Materials and Methods. PC, phosphatidylcholine, PE, phosphatidylethanolamine, PS/PI, phosphatidylserine/phosphatidylinositol. **D**. HeLa cells were serum starved for 24 h, then incubated for 24 h with [*methyl*-^{3}H]thymidine and 10% FBS in the absence (open bar) or presence (closed bar) of 4 uM triascin C and incorporation of [*methyl*-^{3}H]thymidine into cells determined as described in Materials and Methods. Data represents the mean ± SEM of three experiments (*$p<0.05$; ns, not significant).

BTHS lymphoblasts exhibit an 80% reduction in CL level compared with their age-matched controls (Valianpour *et al.*, 2005). To examine if normal levels of CL was required for entry into S phase, BTHS lymphoblasts and age-matched control lymphoblasts were serum starved for 24 h and then incubated with [*methyl*-^{3}H]thymidine in the absence or presence of 10% FBS for 16 h, and the pool size of CL and radioactivity incorporated into cells determined. The pool size of CL in BTHS lymphoblasts (596) was approximately one fifth of that of age-matched control lymphoblasts (3798) (**Figure 6-7A**). The pool size of CL was elevated approximately 50% upon serum addition to serum starved cells and was similar between both cell lines. Incorporation of [*methyl*-^{3}H]thymidine into both control and BTHS cells was elevated upon serum addition and was similar between both cell lines (**Figure 6-7B**). The data indicate that normal CL levels are not likely required for nucleotide synthesis in S phase in human lymphoblasts.

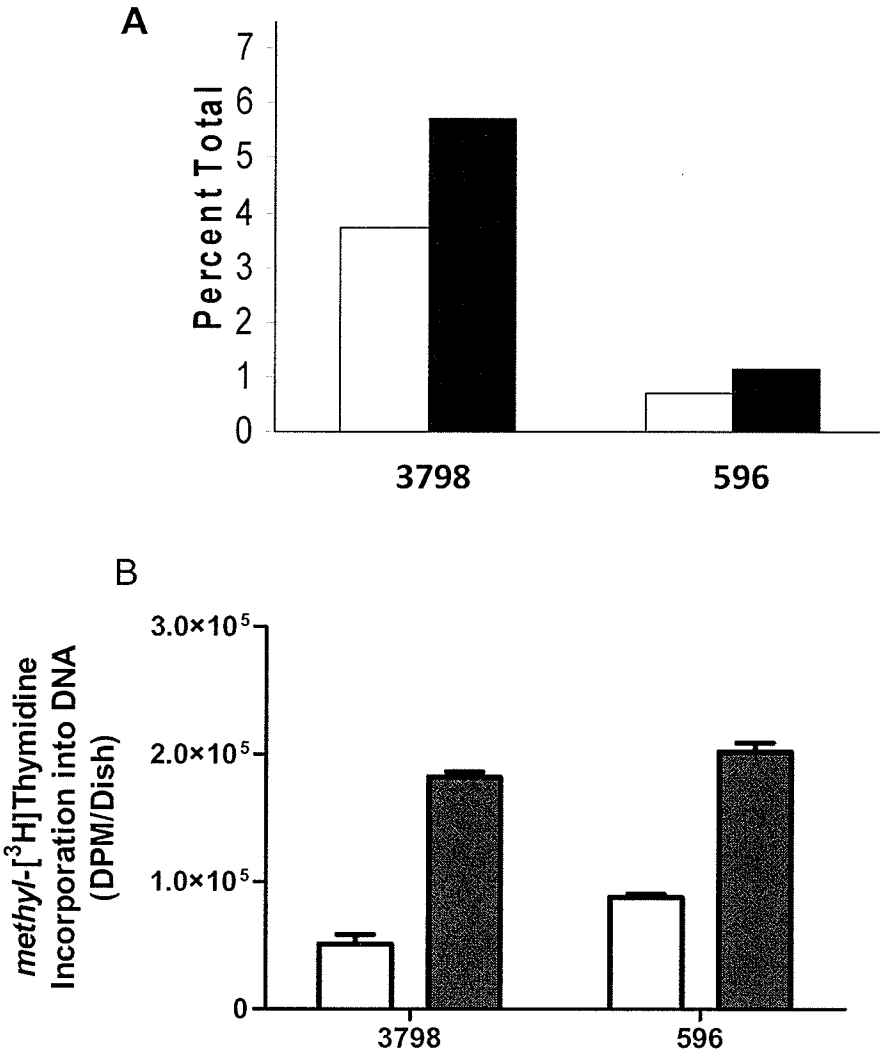

Figure 6-7: CL pool size and [*methyl*-^{3}H]thymidine incorporation in BTHS lymphoblasts.

A. Epstein-Barr virus transformed lymphoblasts from a BTHS patient (596) or age-matched control (3798) were serum starved for 24 h, then incubated for 16 h in the absence (open bars) or presence (closed bars) of 10% FBS and the pool size of CL, expressed as percent of total phospholipid, was determined. **B.** Epstein-Barr virus transformed lymphoblasts from a BTHS patient (596) or age-matched control (3798) were serum starved for 24 h, then incubated for 16 h with [*methyl*-^{3}H]thymidine in the absence (open bars) or presence (closed bars) of 10% FBS and incorporation of [*methyl*-^{3}H]thymidine into cells determined as described in Materials and Methods. Data represents the mean ± SEM of three experiments.

6.4. Discussion

CL plays a key role in the regulation of ATP generation, an important requirement for S phase of the eukaryotic cell cycle. We hypothesized that since CL is essential for mitochondrial respiration its biosynthesis during entry into the S phase of the cell cycle may need to be maintained. The current study addressed if *de novo* CL biosynthetic enzymes were up regulated during human cell entry into S phase to account for the doubling in mass of CL and if normal CL *de novo* biosynthesis and CL levels were essential for nucleotide synthesis in human cell S phase. The main findings of our study are; 1. The activity and mRNA expression of CL *de novo* biosynthetic and remodeling enzymes are up regulated upon serum addition to quiescent HeLa cells and this likely accounts for the doubling of CL observed during HeLa entry into the S phase of the cell cycle, 2. Glucose uptake and incorporation into CL is elevated whereas glycerol uptake and incorporation into CL is reduced during HeLa entry into the S phase of the cell cycle, and 3. normal CL *de novo* biosynthesis and normal CL levels are not likely required to support nucleotide synthesis during S phase of the cell cycle.

To study CL biosynthesis upon entrance into S phase of the human cell cycle, HeLa cells were enriched at the G_0/G_1 interface by 24 h serum starvation rendering the cells quiescent (Jackman *et al.*, 2003). The use of serum deprivation verses chemical inhibitors ensured cells entered into quiescence without the disadvantage of stressing the cells, which may have induced apoptosis or altered cellular responses (Horton *et al.*, 2003; Jackman *et al.*, 2003). There was significant elevation in incorporation of [*methyl*-^{3}H]thymidine into HeLa cells upon serum addition compared to serum starved cells confirming entrance into S phase of the eukaryotic cell cycle. Doubling of phospholipid

content occurs during S phase and has been established to be a cell cycle-regulated process rather than a growth factor triggered event (Jackowski, 1994; Jackowski, 1996). The phospholipid mass of CL, as well as other phospholipids, determined by phospholipid phosphorus analysis was approximately doubled by 16 h post serum addition during HeLa cell S phase and paralleled an increase in mitochondrial mass as determined by elevated activity of the mitochondrial marker enzyme citrate synthase. Interestingly, serum starved cells incorporated some (approximately $2x10^5$ dpm/dish) radioactivity at 8 h post [*methyl*-^{3}H]thymidine addition. This was likely due to the presence of isoleucine in the medium which results in maintenance of some nucleotide synthesis (Tobey *et al.*, 1970). A previous study had shown that the CL content in quiescent liver C9 cells doubled upon serum addition but the mechanism for this was not examined (Martinez-Diez *et al.*, 2006). In that study, the fluorescent probe 10-*N*-nonyl acridine orange was used to quantitate the CL content. It has been shown that 10-*N*-nonyl acridine orange is non-specific for CL and does not provide a reliable estimate of CL mass determination (Jacobson *et al.*, 2002; Gohil *et al.*, 2005a).

The reason for the increase in CL mass upon serum addition to quiescent HeLa cells was the observed increase in activity by 16 h and the expression by 24 h post serum addition of the *de novo* CL synthetic enzymes. Interestingly, the increase in enzyme activity of CDS and CLS at 16 h preceded the increase in mRNA expression of these enzymes. It is likely that the small, but not statistically significant, increase in expression of CDS-2 and hCLS1 mRNA at 16 h post serum addition accounted for the increase in CDS and CLS enzyme activity. It is well documented that these enzymes regulate the amount of CL in mammalian cells (Hostetler, 1982; Daum *et al.*, 1986; McMurray, 1986; Hoch, 1992;

Dowhan, 1997; Schlame *et al.*, 2000; Hatch, 2004). Interestingly, CDS-1 mRNA expression appeared elevated at 24 h but this was not statistically significant. CDS mRNA is present in all tissues examined but there are differences in amount and expression between CDS-1 and CDS-2 isoforms (Heacock *et al.*, 1996; Lykidis *et al.*, 1997; Saito *et al.*, 1997; Weeks *et al.*, 1997; Halford *et al.*, 1998; Volta *et al.*, 1999; Mercade *et al.*, 2007). For example, CDS-1 was not expressed in human heart whereas CDS-2 was highly expressed in that tissue. Peroxisome proliferator-activated receptor alpha activation mediated by clofibrate treatment of H9c2 rat cardiac myoblast cells resulted in elevated mRNA expression of CDS-2 but not CDS-1 (Jiang *et al.*, 2004). In addition, the CDS-1 and CDS-2 genes are localized to different chromosomes (Mercade *et al.*, 2007). Thus, the two isoforms are likely modulated differently under various cellular conditions and their expression may be regulated differently in S phase in HeLa cells. Once synthesized *de novo*, CL is remodeled by a deacylation-reacylation cycle or by the mitochondrial CL transacylase TAZ [reviewed in (Mercade *et al.*, 2007). Although CL transacylase activity is low in HeLa cells (Van *et al.*, 2007), mRNA levels of TAZ appeared elevated by 24 h post serum addition to quiescent cells but this was not statistically significant. In addition, mitochondrial PLA_2, MLCL AT and microsomal ALCAT1 activities were elevated at 24 h post serum addition compared to serum starved cells. The elevated activities of the CL remodeling enzymes are likely required to support remodeling of the increased newly synthesized CL.

Previous studies from our laboratory have demonstrated that glucose and glycerol precursors may be readily utilized as the carbon backbone for CL *de novo* biosynthesis in HeLa cells (Wylie *et al.*, 1997; Van *et al.*, 2007). In the current study, incorporation of

[^{14}C(U)]glucose into CL in HeLa cells was elevated upon serum addition to quiescent HeLa cells initially indicating up regulation of glucose utilization for the carbon backbone of CL in S phase. Surprisingly, [1,3-^{3}H]glycerol incorporation into CL was reduced upon serum addition to quiescent HeLa cells initially indicating that, in S phase, there appears to be preferential utilization of glucose over glycerol for CL *de novo* biosynthesis. However, the elevation in [^{14}C(U)]glucose and reduction in [1,3-^{3}H]glycerol incorporated into CL were likely due in part to an elevation in [^{14}C(U)]glucose uptake and reduction in [1,3-^{3}H]glycerol uptake into cells upon serum addition. We had previously shown that metabolic alteration of H9c2 cells with the carnitine palmitoyltransferase-1 inhibitor etomoxir resulted in distinct and differential channeling of labeled glycerol and fatty acid precursor pools into CL (Xu *et al.*, 2003a). Thus, selective preferential utilization of substrate precursors for CL biosynthesis may be dependent upon not only the metabolic state of the cell but in addition the periodicity of the cell within the eukaryotic cell cycle at the level of precursor uptake.

Is normal CL *de novo* biosynthesis essential for nucleotide synthesis during entry into S phase of the human cell cycle? We tested this directly by examining CL synthesis from [1-^{14}C]oleate and [*methyl*-^{3}H]thymidine incorporation into cells upon serum addition to serum starved HeLa cells incubated with triascin C. Triascin C is an inhibitor of long chain fatty acyl-Coenzyme A synthetase and was shown to inhibit *de novo* CL biosynthesis in human cancer cell lines (Mashima *et al.*, 2005). In that study, although total phospholipid was reduced by triacsin C-treatment in growing SF268 cells the reduction in CL mass was much greater than the reduction in total phospholipid content. In our study, triascin C-treatment inhibited CL synthesis from [1-^{14}C]oleate in HeLa

cells. In addition, HeLa cells treated with triascin C revealed a similar level of [*methyl*-^{3}H]thymidine incorporation compared to control. This data indicated that normal CL *de novo* biosynthesis is likely not required to support nucleotide synthesis during entry into S phase of the human cell cycle. Interestingly, [1-^{14}C]oleate accumulated in PG in the presence of triacsin C. We previously reported that triacsin C did not affect the acylation of MLCL to CL in isolated rat heart mitochondrial fractions (Ma *et al.*, 1999). This observation, coupled with the current study, suggest that the triacsin C-mediated reduction in CL synthesis may in addition be at the level of inhibition of the CLS.

Is normal CL level essential for nucleotide synthesis during entry into S phase of the human cell cycle? We tested this directly by examining [*methyl*-^{3}H]thymidine incorporation upon serum addition to serum starved BTHS lymphoblasts and age-matched controls. Serum addition to BTHS cells, in which CL levels are reduced by 80% (Valianpour *et al.*, 2005), demonstrated a similar level of CL accumulation as well as [*methyl*-^{3}H]thymidine incorporation compared to age-matched control cells. This data indicated that normal CL levels are not required to support nucleotide synthesis during entry into S phase of the human lymphoblast cell cycle. However, it should be noted that these are Epstein-Barr virus transformed lymphoblasts and the transformed nature of these cells may serve to make other cell cycle control modulators redundant. A Chinese hamster ovary temperature sensitive mutant cell line defective in PGPS activity exhibited a 25% reduction in CL level when grown at the restrictive temperature (Ohtsuka *et al.*, 1993). The mutant exhibited abnormalities in mitochondrial morphology, mitochondrial respiration and growth. However, nucleotide synthesis during S phase was not examined in these cells. Moreover, the level of PG was reduced in these cells as well. Given that

mutant Chinese hamster ovary cells (Ohtsuka *et al.*, 1993) and Barth Syndrome lymphoblasts (Valianpour *et al.*, 2005) still contain CL, it is possible that a minimum amount of CL is indeed required to support nucleotide synthesis during S phase of the mammalian cell cycle. A previous study in *CRD1 S. cerevisiae* mutants, defective in CL biosynthesis, indicated that the null mutant could grow on both fermentable and non-fermentable carbon sources at lower temperatures but it could not form colonies at 37°C (Jiang *et al.*, 1999). Although these yeast mutants were completely devoid of CL they exhibited elevated PG levels.

Interestingly, D-[^{14}C(U)]glucose incorporation into TG was increased and into DG decreased upon serum addition to serum starved cells. These data indicated that there was an up regulation of HeLa cell TG biosynthesis during S phase. The observed decrease in DG is likely linked to utilization of DG for both TG biosynthesis and provision of DG for phosphatidylcholine and phosphatidylethanolamine biosynthesis through their respective CDP-choline and CDP-ethanolamine pathways during S phase (Lykidis *et al.*, 2001; Banchio *et al.*, 2003). In summary, induction of HeLa cell entrance into the S phase of the human cell cycle results in elevated *de novo* biosynthesis of CL and this is due to up regulation of expression of the CL *de novo* biosynthetic genes which likely account for the doubling of CL observed in S phase. In addition, a preferential utilization of glucose over glycerol for CL *de novo* biosynthesis at the level of precursor uptake occurs in HeLa cells during S phase. Finally, normal CL *de novo* biosynthesis or CL levels may not be essential to support nucleotide synthesis during S phase in of the human cell cycle.

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Chapter 7: Reduction in Cholesterol Synthesis in Human Barth Syndrome Lymphoblasts in Response to Serum Starvation

Chapter 7: Reduction in Cholesterol Synthesis in Human Barth Syndrome Lymphoblasts in Response to Serum Starvation

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Abbreviated Title: Cholesterol Synthesis in Barth Syndrome Lymphoblasts

Abstract

Barth Syndrome (BTHS) is a rare X-linked genetic disease in which a mild hypocholesterolemia is observed in some patients. We investigated cholesterol (CH) synthesis in lymphoblasts from two BTHS patients with different mutations (ΔTAZ1 and ΔTAZ2). When cultured in media containing serum, BTHS lymphoblasts exhibited no difference in total CH levels compared to age-matched normal human lymphoblasts. In contrast, when BTHS cells were incubated in serum free media, a condition in which CH *de novo* synthesis is normally upregulated, they were unable to maintain CH production levels compared to control. CH biosynthesis from 2-[^{14}C]pyruvate was stimulated 18-fold in control but only 6- to 7-fold in BTHS lymphoblasts and biosynthesis from [1-^{14}C]acetate was stimulated 8-fold in control but only 1.6- to 3-fold in BTHS lymphoblasts upon serum removal indicating a lowered ability of BTHS cells to upregulate CH biosynthesis. The reason was an inability of BTHS cells to increase hydroxymethylglutaryl-Coenzyme A reductase (HMGR) activity, which was already maximal in BTHS lymphoblasts, in response to serum removal. Insulin responsive gene-1 (Insig-1) mRNA expression was elevated in BTHS cells upon serum removal, opposite to what was observed in control cells, indicating the potential for accelerated degradation of HMGR. The reduced ability to up regulate HMGR enzyme activity in BTHS cells was not accounted for by a decrease in HMGR mRNA transcription, as HMGR expression was unaltered in ΔTAZ2 but reduced in ΔTAZ1 lymphoblasts compared to control. Together, the data indicate that CH levels may be maintained in BTHS lymphoblasts under normal culture conditions, but these cells have a diminished capacity to respond to increased demand for cholesterol biosynthesis.

Word count: 268

Keywords: Barth Syndrome, Tafazzin, cholesterol biosynthesis, hypocholesterolemia, HMG-CoA Reductase, insulin responsive gene-1, lymphoblasts, gene regulation, metabolism

Abbreviations: HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; CL, cardiolipin; BTHS, Barth Syndrome; Taz, tafazzin; LDL, low density lipoprotein; HDL, high density lipoprotein; LDLR, low density lipoprotein receptor; SREBP, sterol response element binding protein; Insig-1, insulin responsive gene-1; ER, endoplasmic reticulum;

7.1. Introduction

Barth syndrome (BTHS) is a rare X-linked genetic deficiency which results in mutation in a protein, or group of proteins known as tafazzins (taz) which remodel CL [reviewed in (Hauff *et al.*, 2006)]. The characteristic biochemical feature of this disease is a deficiency in tetralinoleoyl CL, a phospholipid known to be important in the proper functioning of the mitochondria, and therefore, ATP production [reviewed in (Hatch, 1998; Hauff *et al.*, 2006)]. Clinical defects, occurring in young BTHS males, include dilated cardiomyopathy, cyclic neutropenia, type II 3-methylglutatonic aciduria, moderate hypocholesterolemia, and failure to thrive (Barth *et al.*, 1999; Mazzocco *et al.*, 2001) [reviewed in (Gonzalez, 2005; Hauff *et al.*, 2006)]. Although BTHS is a rarely diagnosed disorder, the incidence may be as high as 1/100,000. The gene responsible for BTHS, known as *taz*, was localized to the Xq28.12 region (Bolhuis *et al.*, 1991; Ades *et al.*, 1993; Bione *et al.*, 1996; D'Adamo *et al.*, 1997). While greater than 90 mutations have been described, a significant correlation between genotype and disease severity in BTHS has not been observed to date (Johnston *et al.*, 1997). It is, therefore, likely that a modifying factor(s) is involved in altering the outcome of this disease. In fact, even within patients, symptoms seem to resolve for a certain period of time (Schlame *et al.*, 2006). This period, commonly referred to as the "honeymoon period", occurs between the ages of approximately 5-12 years (Kelley, 2002). Coincidentally, these are typically the years between peak cholesterol demand (Berenson *et al.*, 1981). Anecdotal reports of low cholesterol in BTHS patients have appeared in the literature for decades (Kelley *et al.*, 1991b; Barth *et al.*, 1999; Mazzocco *et al.*, 2001; Barth *et al.*, 2004), but until

recently, there has been no serious attempt to investigate the validity, impact, or cause of this hypocholesterolemia.

Cholesterol (CH) is important in many biological processes, including maintenance of cell membrane fluidity, steroid production, and embryonic, including brain, development (Waterham, 2002). Reduced levels of circulating total CH, low density (LDL) and high density lipoprotein (HDL) CH, and other lipoproteins, lead to a condition known as hypocholesterolemia. Hypocholesterolemia is associated with a number of pathological conditions, including Barth Syndrome (Kelley *et al.*, 1991a), meningococcal sepsis, septic shock and hypocortisolism (Vermont *et al.*, 2005). In addition, CL is imporatant in CH transport into the mitochondria, and in subsequent cleavage to innitiate steroid synthesis, in specialized steroidogenic tissues [reviewed in (Stocco, 2000; Miller, 2007; Houtkooper *et al.*, 2008)]. Futhermore, there have been reports of other pathological states suggesting a link between CH metabolism and CL. Recently a shotgun lipidomics approach was utilized to determine that the primary PL affected in diabetes is CL (Han et al., 2007). Tangier Disease fibroblasts, deficient in CH export due to mutations in the ATP-binding cassette (ABCA1) protein, have a 3-5 fold increase in CL and lysoCL (Fobker et al., 2001). These examples implicate a link between CL and CH metabolism in mammals. Therefore, it is important to understand how expression of the Barth Syndrome Gene, *taz*, can alter CH metabolism. With this knowledge we may begin to understand how mutations in *taz* affect metabolic enzyme systems and how alterations in these systems may be reversed or compensated for by the body.

De novo CH biosynthesis can occur in all nucleated cells (Vance *et al.*, 2002) by cleavage of the sterol response element binding protein (SREBP)-2 in response to low intracellular

sterol concentrations. Under conditions of high ER sterol concentration (Soccio *et al.*, 2004), the SREBP cleavage-activating protein/SREBP-2 complex undergoes a conformational change that allows it to bind to insulin responsive gene (Insig)-1, thereby preventing exit from the ER and further transcription of the genes involved in increasing intracellular CH concentration [reviewed in (Goldstein *et al.*, 2006; Espenshade *et al.*, 2007)]. One such product is the enzyme responsible for the major rate limiting step in *de novo* cholesterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) (Endo *et al.*, 1989).

It has been estimated that approximately two thirds of all cellular CH is from *de novo* biosynthesis (Endo *et al.*, 1989). Since the brain has a requirement for CH [reviewed in (Pfrieger, 2003)], and BTHS patients have displayed a mild cognitive and hypocholesterolemic phenotype (Mazzocco *et al.*, 2001; Barth *et al.*, 2004; Mazzocco *et al.*, 2007), we investigated CH biosynthesis in BTHS cells under conditions in which demand for CH *de novo* biosynthesis is upregulated. We examined the biosynthesis of CH in age-matched control and BTHS lymphoblasts under basal cell culture conditions and in response to serum removal. We have demonstrated that CH levels are maintained in BTHS lymphoblasts under normal culture conditions, but these cells have a diminished capacity to respond to an increased demand for CH biosynthesis.

7.2.Materials and Methods

7.2.1. Materials

[1-^{14}C]Acetate, sodium salt was obtained from Perkin-Elmer, Woodbridge, Ontario Dupont, Mississauga, Ontario, Canada. DL-3-[glutaryl-3-^{14}C] hydroxy-3-methylglutaryl Coenzyme A and [2-^{14}C]pyruvate were obtained from American Radiolabeled Chemicals, Inc, St. Louis, USA. RPMI, fetal bovine serum (FBS) and antibiotics were products of Canadian Life Technologies (GIBCO), Burlington, Ontario, Canada. Lipid standards were obtained from Serdary Research Laboratories, Englewood Cliffs, New Jersey, USA. Thin layer chromatographic plates (silica gel G, 0.25 mm thickness) were obtained from Fisher Scientific, Winnipeg, Canada. Ecolite scintillant was obtained from ICN Biochemicals, Montreal, Quebec, Canada. QIAGEN OneStep RT-PCR kit was used for PCR studies. All other chemicals were certified ACS grade or better and obtained from Sigma Chemical Company, St. Louis, USA or Fisher Scientific, Winnipeg, Manitoba, Canada.

7.2.2. *Cell culture and radiolabeling studies*

Barth Syndrome Epstein-Barr virus transformed lymphoblasts, ΔTAZ1 and ΔTAZ2, were obtained from Dr. Richard Kelley, John Hopkins University, Baltimore Maryland and previously reported as family 3 and 5, respectively (Johnston *et al.*, 1997). Epstein-Barr virus transformed lymphoblasts from an age-matched normal young male, control, were obtained from Coriell Institute for Medical Research, Camden, New Jersey. Lymphoblasts were maintained in RPMI media + L-glutamine containing 10% FBS and 100U/ml Penicillin, 100ug/ml Streptomycin, 0.25ug/ml Amphotericin B. All cells were

maintained at 37°C in a humidified atmosphere of 5% CO_2. Lymphoblasts ($1x10^6$ cells) were seeded to a density of 5 $x10^5$ cells/ml, and incubated for 16 h with various radiolabeled precursors. Media, containing or deficient in FBS, had 10 μCi/ml [1-^{14}C]acetate, sodium salt, or 14 μCi/ml [2-^{14}C]pyruvate, and cells were incubated for 16 h. Subsequent to incubation, radiolabel incorporation into CH was determined as described below.

7.2.3. Lipid Isolation and cholesterol determination

CH was isolated as described with modification (Folch *et al.*, 1957). Briefly, after the initial treatment of cells described above, cells in suspension were spun down at 1,200 rpm in a swinging bucket rotor for 10 min, supernatants were removed and cells were resuspended in 2 ml of methanol:H_2O (1:1, v/v). Cells were transferred to a silanated glass tube, and an aliquot was taken for total radioactivity and protein (usually 25 μl and 50 μl, respectively) determination. An additional 0.5 ml of H_2O and 2 ml of chloroform were added to each sample, followed by brief vortexing. Samples were then centrifuged at 2,000 rpm in a swinging bucket rotor for 10 min. The upper, aqueous layer was removed by suction, along with the protein interphase. Addition of 2 ml theoretical upper phase (methanol:0.9% NaCl:chloroform; 48:47:3, v/v) was followed by a second brief vortex and centrifugation at 2,000 rpm for 5 min. Removal of the aqueous phase this time was followed by drying down of the organic phase with nitrogen gas. Samples were capped and stored at -20°C. A portion of the total sample was spotted onto silica thin layer chromatrography plates for one dimensional separation of CH. Plates were developed for approximately an hour in Hexanes:Diethyl ether:glacial acetic acid

(70:30:2, v/v). Spots corresponding to CH were removed and radioactivity in the sample determined by liquid scintillation counting.

CH content of the cells was determined by colorimetric reaction of the Amplex Red CH assay kit from Invitrogen's Molecular Probes. After isolation of cellular lipids as described above, the lipid residue was reconstituted with 1% Triton X-100 in isopropanol, and cholesterol assays were preformed as per protocol. All isolates were measured immediately after drying down with nitrogen, as fresh samples seemed to yield the best results.

7.2.4. Real time PCR

BTHS lymphoblasts ($1x10^6$ cells) were seeded to a density of 5 $x10^5$ cells/ml, and incubated for 16 h with medium in the absence or presence of FBS. Subsequent to incubation, RNA was isolated from all cells using the TRIzol method of phenol extraction and stored at -80°C. RNA was combined with Qiagen RT-PCR master mix, total reaction volume 25 µl, and the appropriate primers, listed in (**Table 7-1**). Reverse transcription (RT) was performed immediately prior to polymerase chain reaction (PCR) as part of the same cycler protocol. The cycler protocol for real time PCR on the Eppendorf Mastercycler ep Realplex 2, consisted of a 30 min RT step at 50°C, followed by a 15 min Taq activation step at 95°C followed by a 1 min separation at 95°C and melting curve that increased in temperature incrementally from 60°C to 95°C over the course of 20 min, taking fluorescence readings throughout to determine product quality. The PCR program for the Insig-1 (400 nM), LDLR (400 nM) HMGR (400 nM), and

housekeeping gene, 18s rRNA (100 nM) primers ran 50 cycles of separation at 95°C for 15 seconds and annealing/elongation at 60°C for 45 seconds. Fluorescence readings were taken at the end of every elongation step. The changes in gene expression were analyzed on an Eppendorf Mastercycler ep Realplex, software version 1.5.474, and the data presented as mean fold change ($2^{-\Delta\Delta Ct}$) in mRNA expression (Livak *et al.*, 2001) relative to 18s rRNA, a gene not affected by short term incubation of cells under altered serum conditions (Schmittgen *et al.*, 2000).

Table 7-1: Primers used for real time RT-PCR

Target	Ref. Seq. No.	Forward (3'-5')	Reverse (3'-5')
HMGR	NM_000859	cggttGGAAGAGACAGGGATAAAC[FAM]G	GGGTATCTGTTTCAGCCACTAAGG
INSIG-1[1]	NM_198336.1	GGACGACAGTTAGCTATGGGTGTT	GAGTCATTTGTACAGTCAGCCCGA
LDLR[2]	NM_000527.3	CTG CCT AAG TGG CGA GTG CA	CAG GTG GCC ACA GCG CAG TT
18srRNA	X03205	CTCGGGCCTGCTTTGAACAC	cgggTGCTCTTAGCTGAGTGTCC[FAM]G

[1](Gharavi *et al.*, 2006); [2](Tveten *et al.*, 2006)

7.2.5. In vitro Enzyme Assays

Microsomes were isolated from cell cultures grown overnight to approximately 1 x 10^8 cells, seeded at a density of approximately 5 x 10^5 cells/ml. Cultures were grown for 16 h in culture medium in absence or presence of FBS. Cells were isolated by centrifugation at 1,500 rpm for 10 min and cell pellets were resuspended on ice in 2 ml homogenizing buffer (10 mM Tris-HCL, pH 7.4, 0.25 M sucrose), followed by homogenization with 2 x 20 strokes of a Dounce A homogenizer. The homogenate was centrifuged at 4°C, 1,000 x g for 5 min and the supernatant centrifuged at 4°C, 10,000 x g for 15 min. The resulting supernatants were then further centrifuged for 1.5 h at 4°C, 100,000 x g, and the resulting microsomal pellets were resuspended in homogenizing buffer and kept on ice while protein content was determined.

In vitro HMG-CoA reduction assays were performed as described (Ohashi *et al.*, 2003). Reaction buffer containing, at final concentration, 5 mM β-nicotinamide adenine dinucleotide phosphate reduced, tetrasodium salt (NADPH - made fresh), 10 mM Ethylenediamine-tetraacetic acid (EDTA), 10 mM dithiothreitol (DTT), 100 mM Tris-HCl (pH 7.4), was added to 50 µg microsomal protein per sample. The reactions were initiated by the addition of DL-3-[glutaryl-3-^{14}C] hyroxy-3-methylglutaryl coenzyme A (4.5 µCi/µmol), cold HMG-CoA to 110 µM, and incubation at 37°C for 30 min. Addition of HCl to 1N, and incubation for a further 30 min at 37°C was required to lactonize the mevalonate formed. Samples were then stored in the freezer overnight and then spotted onto dried silica thin layer chromatography plates. Plates were subjected to approximately 2 h in thin layer chromatorgraphy tanks containing acetone-benzene (1:1 v/v). The plates were exposed to iodine vapour and spots corresponding to Rf = 0.6-0.9

were removed into scintillation vials and radioactivity determined, i.e. [^{14}C]mevalonate formation. HMG-CoA reductase activity was determined and expressed as the percent difference of serum deprivation versus basal levels in picomoles of [^{14}C]mevalonate formed per min per mg of protein.

7.2.6. *Other determinations*

Protein assays were performed with BioRad's Protein assay, a variation on the Bradford method. BSA was used as a standard, in the microassay procedure for microtiter plates.

7.2.7. Statistics

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

7.3. Results

7.3.1. CH levels are reduced in BTHS lymphoblasts upon serum removal

Control of CH biosynthesis in BTHS cells had never been examined. We compared human lymphoblasts from two BTHS patients with different mutations (ΔTAZ1, ΔTAZ2) to those from a normal male of similar age (**Table 7-2**). Serum deprivation was used as a method to induce CH biosynthesis in lymphoblasts (Fogelman *et al.*, 1977). Control and BTHS lymphoblasts were incubated in the absence or presence of serum for 16 h and total CH content determined. Total CH levels were unaltered in BTHS cultured for 16 h in the presence of serum compared to controls (**Figure 7-1A**). Serum withdrawal for 16 h resulted in a mild reduction in total CH levels in both BTHS lymphoblast cell lines. When the data was expressed as a percent difference between +serum and –serum for each cell line the ΔTAZ1 lymphoblast demonstrated a statistically significant reduction (approximately 20%, $*p<0.05$) in total CH compared to the control lymphoblasts (**Figure 7-1B**).

Table 7-2. Description of Control and Barth Syndrome Lymphoblasts Used

Cell Line Identifier	Phenotype	Age at Harvest (years)	Family	Proband	Tafazzin Mutation	CH (mg/dL) (age)	Ref.
Control	Wild Type	10		None	None	181.8[a] (9-11yr)	[1]
ΔTAZ 1	BTHS	4	3	c. 109+5G>C (Intron 1)	Splice Site	107 (2yr)	[2, 3, 4, 5]
ΔTAZ 2	BTHS	9	5	c. 171delA (exon 2)	Frameshift	61 (5mo)	[2, 3, 4, 5, 6]

[a]-Average normal serum cholesterol levels reported for males aged 9-11 years old.
[1](Abraham *et al.*, 1978); [2](Gonzalez, 2008); [3](Johnston *et al.*, 1997); [4](Kelley *et al.*, 1991b); [5](Schlame *et al.*, 2003); [6](Gonzalez, 2005)

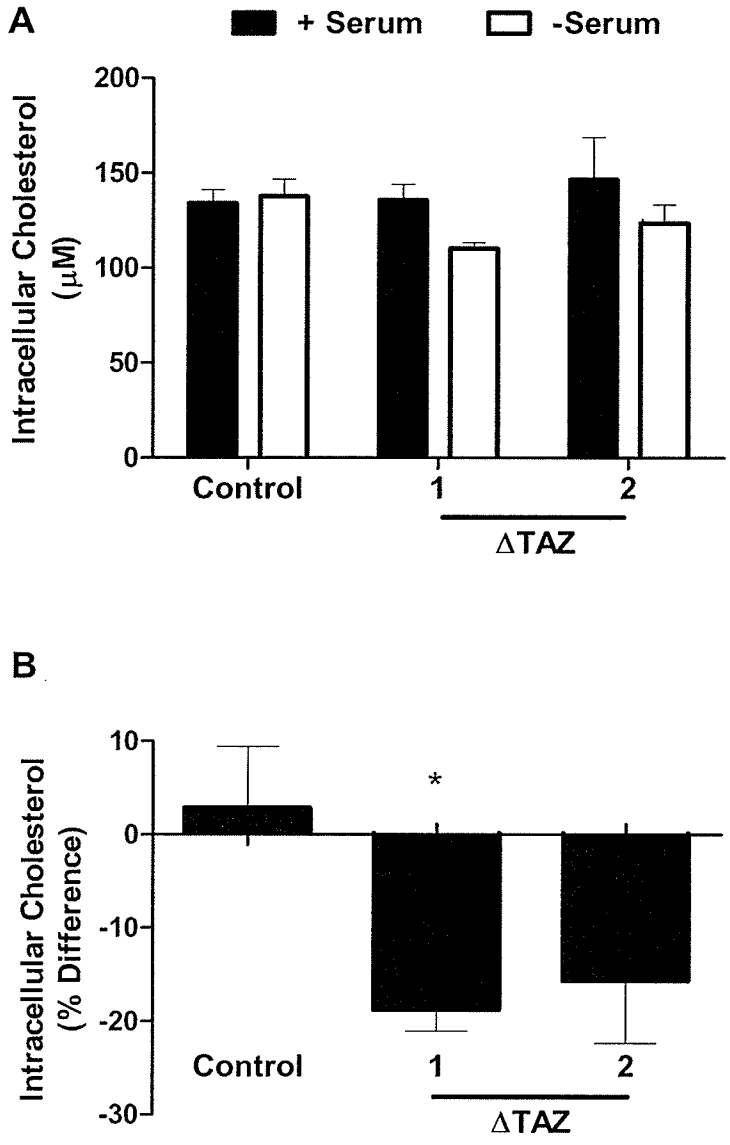

Figure 7-1: Total CH levels in BTHS lymphoblasts upon serum removal.

A. Control and BTHS lymphoblasts (ΔTAZ1, ΔTAZ2) were incubated in the absence or presence of serum for 16 h and total CH content determined as described in Material and Methods. **B**. The results from A are expressed as percent difference in CH levels between serum containing and serum depleted cells. Data represent the mean ± SEM, (n = 3 experiments). *, $p<0.05$ compared to plus serum.

7.3.2. Incorporation of [2-^{14}C]pyruvate or [1-^{14}C]acetate into CH is reduced in BTHS lymphoblasts upon serum removal

Glucose is a main source of carbon for lymphoblasts (Newsholme *et al.*, 1985; Calder *et al.*, 2007). Hence, we examined if a glycolytic intermediate, pyruvate, could be used for CH synthesis in lymphoblasts. Control and BTHS lymphoblasts were incubated with [2-^{14}C]pyruvate in the absence or presence of serum for 16 h and radioactivity incorporated into CH determined. Removal of serum resulted in an increased incorporation of [2-^{14}C]pyruvate into CH in control and BTHS cells (**Figure 7-2A**). When the data was expressed as a percent difference between +serum and –serum for each cell line an 18-fold increase in [2-^{14}C]pyruvate incorporation into CH was observed in control lymphoblasts deprived of serum, compared to serum supplemented cells (**Figure 7-2B**). In contrast, only a 6.5-7.5-fold increase in [2-^{14}C]pyruvate incorporation into CH was observed in BTHS lymphoblasts deprived of serum compared to serum supplemented cells indicating that the ability to up regulate CH synthesis from [2-^{14}C]pyruvate was significantly lower in BTHS lymphoblasts compared to control. These data indicate that [2-^{14}C]pyruvate may be used to examine CH synthesis in lymphoblasts. In addition, BTHS lymphoblasts have a reduced ability to up regulate CH *de novo* biosynthesis from glycolytic intermediates.

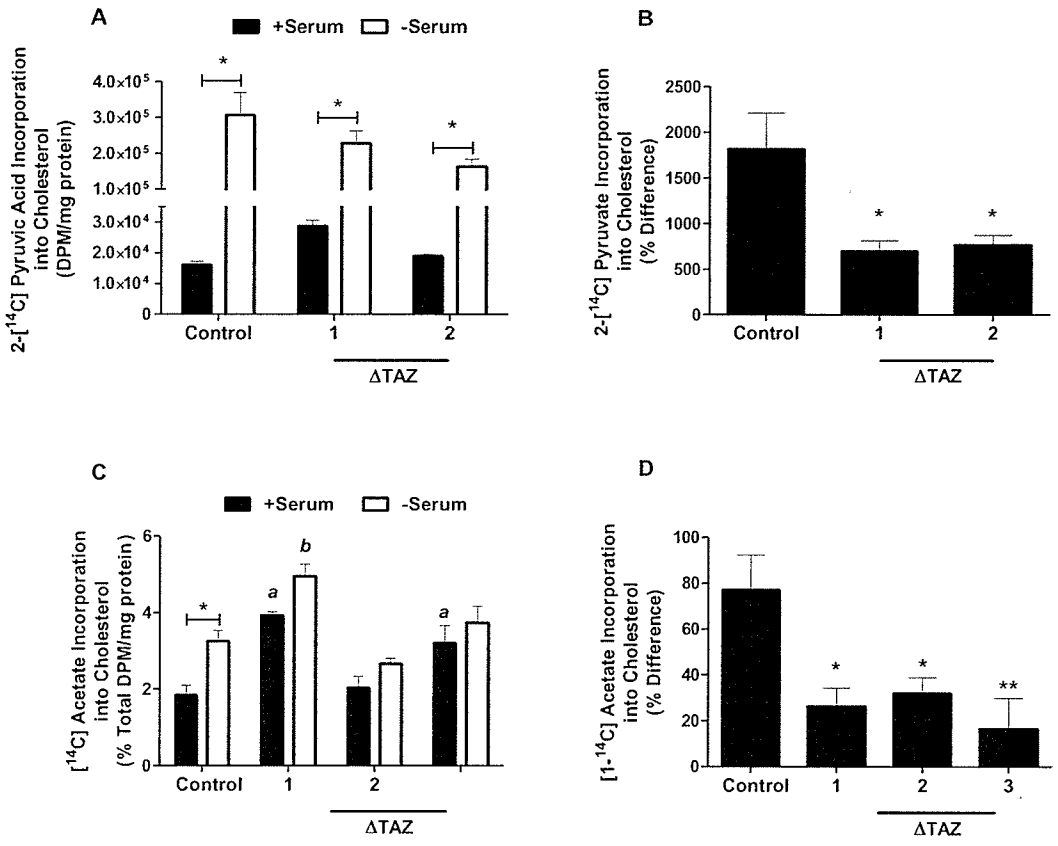

Figure 7-2: Incorporation of [2-^{14}C]pyruvate and [1-^{14}C]acetate into CH in BTHS lymphoblasts upon serum removal.

Control and BTHS lymphoblasts (ΔTAZ1, ΔTAZ2) were incubated for 16 h with [2-^{14}C]pyruvate (**A, B**), or [1-^{14}C]acetate (**C, D**) in the absence or presence of serum containing medium. Cells were harvested and radioactivity incorporated into CH determined as described in Materials and Methods. Data represent the mean ± SEM, (n=3 experiments). *, $p<0.05$ compared to plus serum; [a], $p<0.05$ compared to control plus serum; [b], $p<0.05$ compared to control minus serum.

Pyruvate transport into the mitochondria is known to be altered by a lack of CL (Paradies *et al.*, 1989, 1990). Thus, it was possible that the metabolic block in CH biosynthesis from [2-^{14}C]pyruvate was at the level of pyruvate uptake into the mitochondria. Acetate can passively diffuse across membrane barriers where it is acylated in the cytosol for use as the precursor acetyl-Coenzyme A in many biosynthetic pathways (Sabine, 1977; Murray *et al.*, 1996). To confirm that there was a reduced ability of BTHS cells to up regulate CH *de novo* biosynthesis, control and BTHS lymphoblasts were incubated with [1-^{14}C]acetate in the absence or presence of serum for 16 h and radioactivity incorporated into CH determined. Removal of serum resulted in an increased incorporation of [1-^{14}C]acetate into CH in control and BTHS cells (**Figure 7-2C**). When the data was expressed as a percent difference between +serum and –serum for each cell line, an 80% percent increase in [1-^{14}C]acetate incorporation into CH was observed in control lymphoblasts deprived of serum compared to serum supplemented cells (**Figure 7-2D**). In contrast, only a 15-30% increase in [1-^{14}C]acetate incorporation into CH was observed in BTHS lymphoblasts deprived of serum compared to serum supplemented cells. Thus, the ability to up regulate CH synthesis from [1-^{14}C]acetate was significantly lower in BTHS lymphoblasts compared to control. These data confirm that there was a reduced ability of BTHS cells to up regulate CH *de novo* biosynthesis and that the metabolic block is not at the level of pyruvate uptake into the mitochondria.

7.3.3. BTHS lymphoblasts fail to exhibit increased HMGR activity upon serum removal

The reason for the reduced ability of BTHS lymphoblasts to up regulate CH *de novo* biosynthesis from [1-^{14}C]acetate and [2-^{14}C]pyruvate was examined. Control and BTHS lymphoblasts were incubated in the absence or presence of serum for 16 h, microsomal fractions prepared and HMGR enzyme activity determined. The HMGR enzyme activity observed in these cells was within range of enzyme activity previously reported in lymphoid cell lines (Kayden *et al.*, 1976). HMGR *in vitro* activity was elevated 50% upon serum removal in control lymphoblasts (**Figure 7-3A**). In contrast, serum removal did not significantly increase HMGR *in vitro* activity in BTHS lymphoblasts. Interestingly, basal HMGR enzyme activity appeared higher in BTHS cells with ΔTAZ2 significantly increased compared to control. When the data was expressed as a percent difference between +serum and –serum for each cell line BTHS lymphoblasts failed to exhibit increased HMGR enzyme activity upon serum removal compared to control (**Figure 7-3B**).

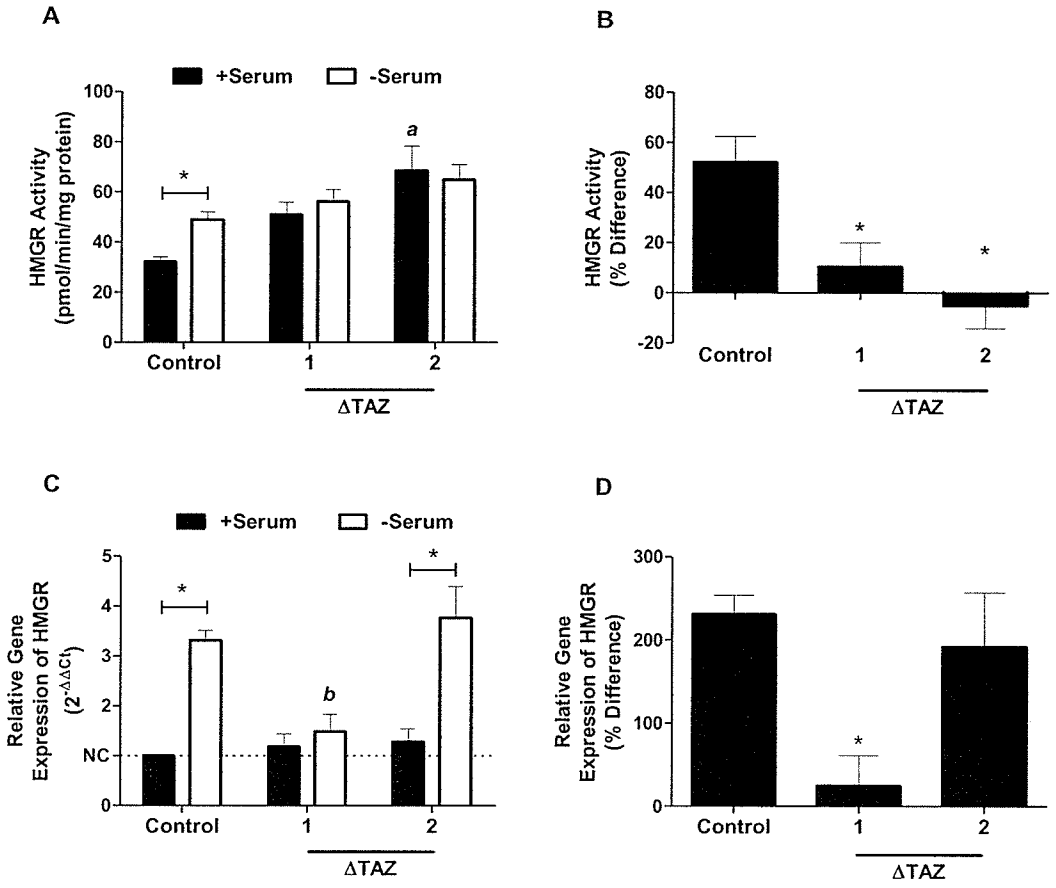

Figure 7-3: HMGR *in vitro* activity and mRNA expression in BTHS lymphoblasts upon serum removal.

A, B. Control and BTHS lymphoblasts (ΔTAZ1, ΔTAZ2) were incubated in the absence or presence of serum for 16 h, microsomal fractions were prepared and HMGR enzyme activity determined as described in Materials and Methods. **C, D**. Control and BTHS lymphoblasts (ΔTAZ1, ΔTAZ2) were incubated in the absence or presence of serum for 16 h, total RNA isolated and HMGR mRNA expression determined as described in Materials and Methods. Data represent the mean ± SEM, (n=3 experiments). *, $p<0.05$ compared to plus serum; [a], $p<0.05$ compared to control plus serum; [b], $p<0.05$ compared to control minus serum.

We next examined expression of HMGR mRNA in BTHS cells using real time-PCR. Control and BTHS lymphoblasts were incubated in the absence or presence of serum for 16 h, total RNA prepared and HMGR mRNA determined. Expression of HMGR mRNA was elevated 2.3-fold upon serum removal in control lymphoblasts (**Figure 7-3C**). ΔTAZ1 lymphoblasts demonstrated a lack of response of induction of HMGR mRNA expression upon serum removal, compared to control lymphoblasts. However, in ΔTAZ2 lymphoblasts mRNA expression was elevated similar to that of control upon serum removal. Similar observations occurred when the data was expressed as a percent difference between +serum and –serum for each cell line (**Figure 7-3D**).

7.3.4. *Expression of Insig-1 is elevated in BTHS lymphoblasts upon serum removal*

Insig-1 is known to bind to HMGR and facilitate its degradation (Goldstein *et al.*, 2006; Espenshade *et al.*, 2007). Control and BTHS lymphoblasts were incubated in the absence or presence of serum for 16 h, total RNA prepared and Insig-1 mRNA determined. Expression of Insig-1 mRNA appeared lower upon serum removal in control lymphoblasts but this was not statistically significant (**Figure 7-4A**). In contrast, in both ΔTAZ1 and ΔTAZ2 lymphoblasts expression of Insig-1 mRNA was elevated over 2-fold upon serum removal. A similar observation occurred when the data was expressed as a percent difference between +serum and –serum for each cell line (**Figure 7-4B**). These data suggest that HMGR degradation may be facilitated in BTHS cells upon serum removal.

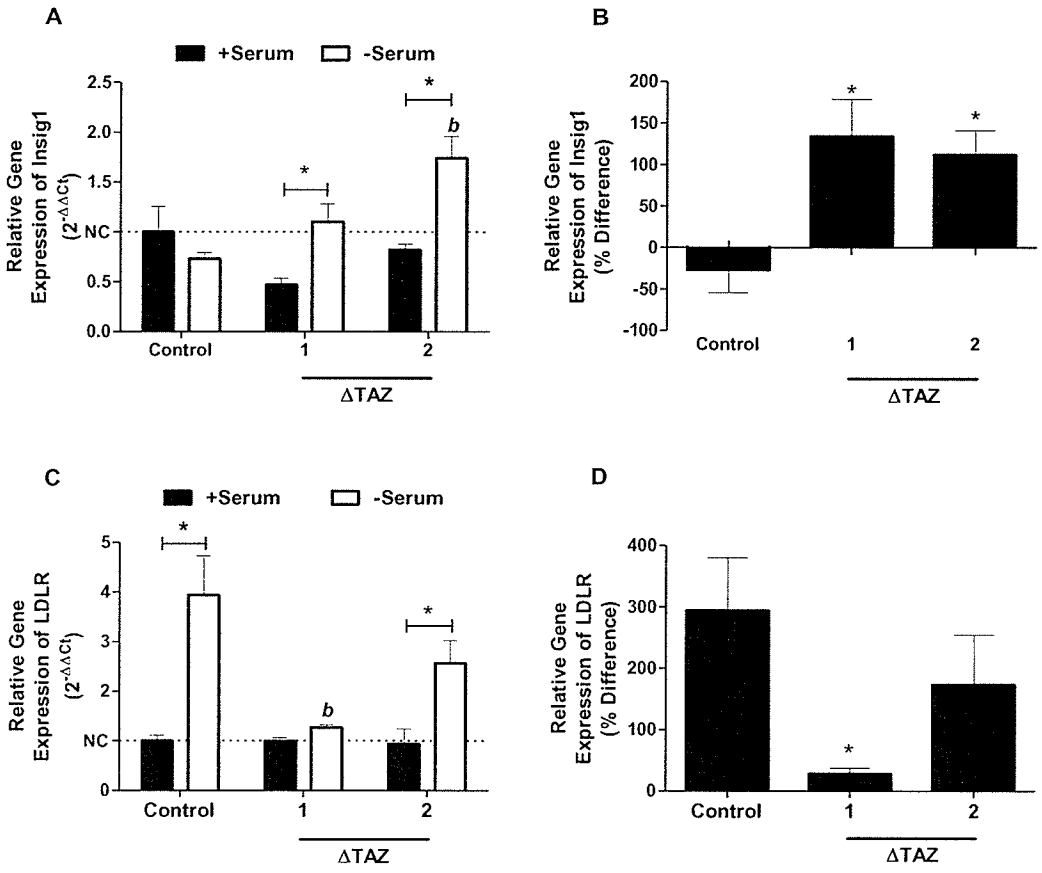

Figure 7-4: LDLR and Insig-1 mRNA expression in BTHS lymphoblasts upon serum removal.

Control and BTHS lymphoblasts (ΔTAZ1, ΔTAZ2) were incubated in the absence or presence of serum for 16 h, total RNA isolated and Insig-1 mRNA (**A, B**) and LDLR mRNA (**C, D**) expression determined as described in Materials and Methods. Data represent the mean ± SEM, (n=3 experiments). *, $p<0.05$ compared to plus serum; [b], $p<0.05$ compared to control minus serum.

Expression of LDLR should be regulated concomitantly with that of HMGR expression in normal cells (Brown *et al.*, 1999). Control and BTHS lymphoblasts were incubated in the absence or presence of serum for 16 h, total RNA prepared and LDLR mRNA determined. Expression of LDLR mRNA was elevated 4-fold upon serum removal in control lymphoblasts (**Figure 7-4C**). A lack of response to serum removal was observed in ΔTAZ1 lymphoblasts compared to control, similar to that observed with ΔTAZ1 HMGR mRNA expression. In contrast, expression of LDLR mRNA was elevated 2.5-fold upon serum removal in ΔTAZ2 lymphoblasts, similar to that observed with ΔTAZ2 HMGR mRNA expression. When the data was expressed as a percent difference between +serum and –serum for each cell line the level of LDLR mRNA expression was lower in both BTHS cells lines compared to control but only statistically significant in ΔTAZ1 cells. These data suggest that expression of LDLR is regulated concomitantly with that of HMGR expression in BTHS cells.

7.4. Discussion

BTHS is a rare X-linked genetic disease that affects young males and is caused by a mutation in the *taz* gene which results in heart and muscle weakness and, eventually, death [reviewed in (Kelley *et al.*, 1991b; Kelley, 2002; Barth *et al.*, 2004; Hauff *et al.*, 2006; Schlame *et al.*, 2006)]. The literature has anecdotally reported the occurrence of low serum cholesterol levels in BTHS boys for years, without further investigation. It is unknown what the specific role of hypocholesterolemia in BTHS is; nonetheless, low cholesterol can have a major impact on a child's health and development (Waterham, 2002). We characterized the biosynthesis of cholesterol in BTHS lymphoblasts in order to better understand the mechanism of hypocholesterolemia in relation to TAZ and CL remodeling, and to develop better approaches in the treatment of Barth Syndrome. The major findings of this study are 1. BTHS lymphoblasts from two patients with differing mutations have a reduced ability to up regulate CH *de novo* biosynthesis from [2-^{14}C]pyruvate or [1-^{14}C]acetate moieties in response to serum removal, 2. the reason for the reduced ability to up regulate CH *de novo* biosynthesis is a reduced ability of BTHS lymphoblasts to elevate HMGR enzyme activity in response to serum removal, and 3. Insig-1 mRNA expression is elevated in BTHS lymphoblasts upon serum removal indicating a direction toward enhanced HMGR degradation.

Serum CH levels in BTHS males were first reported as low in the early 90's (Kelley *et al.*, 1991b). More recently, Spencer *et al* reported a more thorough examination of the serum lipid profile in a cohort of BTHS males, compared to their unaffected siblings (Spencer *et al.*, 2006). The cohort was small and only 24% demonstrated a reduction in total serum CH below 2.84 mmol/L (110 mg/dL). However, upon further analysis they

found that 56% of the cohort demonstrated a reduced LDL profile 1.55 mmol/L (<60 mg/dL). In the current study, the CH content of BTHS lymphoblasts was unaltered in serum containing medium. However, serum removal resulted in a trend toward lowered CH in BTHS cells compared to control. In one case, ΔTAZ1, the reduction in cellular CH content upon serum removal was statistically significant. Unfortunately, these patient lymphoblasts samples were neither harvested at the same time, nor were the reported CH values measured at the same developmental age (**Table 7-2**). Hence, as CH demands change throughout different stages of development, it is not possible to directly compare the patients and assume that the lower CH values are indicative of a more severe CH impairment phenotype.

We observed a consistently lower induction of [2-^{14}C]pyruvate and [1-^{14}C]acetate incorporation into CH in BTHS lymphoblasts upon serum removal compared to control indicating a reduced ability of BTHS cells to up regulate *de novo* CH biosynthesis. Since the lowered induction of [2-^{14}C]pyruvate incorporation into CH in BTHS cells upon serum removal was similar to that of [1-^{14}C]acetate the reduction of CH synthesis was, the reduction is not likely due to a deficiency in mitochondrial uptake of pyruvate.

HMGR catalyzes the rate-limiting step of CH *de novo* biosynthesis in mammalian cells (Bucher *et al.*, 1960). In a previous study, it was noted that both radioactive acetate incorporation into CH and HMGR enzyme activity were unaltered in fibroblasts (Gibson *et al.*, 1991). In this study, all BTHS patient results were pooled, possibly leading to the large variation that resulted. Here we have shown variation between two BTHS samples, but an overall reduced induction of CH biosynthesis from [2-^{14}C]pyruvate and [1-^{14}C]acetate. The reason for the reduced induction of CH biosynthesis from [2-

^{14}C]pyruvate and [1-^{14}C]acetate precursors was likely the inability to induce HMGR activity in BTHS cells upon serum removal. Interestingly, the inability to induce HMGR activity upon serum removal was correlated with an increase in Insig-1 mRNA expression in both ΔTAZ1 and ΔTAZ2 cells. Insig-1 binding targets HMGR and facilitates accelerated degradation of the protein (Sever *et al.*, 2003a; Sever *et al.*, 2003b). Since Insig-1 mRNA expression was elevated in both ΔTAZ1 and ΔTAZ2 cells upon serum removal, it is possible that Insig-1 may play a role in the hypocholesterolemia phenotype of BTHS. In contrast, mRNA expression of HMGR and LDLR differed between ΔTAZ1 and ΔTAZ2 cells upon serum removal, indicating that heterogeneity in expression of these genes exists in BTHS patients with different mutations. This is an interesting observation since a correlation does not exist between the greater than 90 mutations described in BTHS and the severity of the disease.

The lymphoblast model we have used here is of major importance in aiding our understanding of the altered biochemistry and cellular functioning in BTHS. These cells provide a surrogate for BTHS patients in the absence of a viable mammalian model, and the harvesting of human lymphoblasts is far less invasive than many other cell lines. Unfortunately, not all cells perform the same functions, and therefore, not all biochemical alterations will be obvious in this cell line. For example, cholesterol *de novo* biosynthesis, although known to occur in all nucleated cells, predominantly occurs in hepatocytes (Pandak *et al.*, 2002). Since liver is the main source of VLDL and ultimately LDL, and more than half the BTHS males in the Spencer, *et.al.* cohort demonstrated a reduction in LDL levels (Spencer *et al.*, 2006), it is possible there are further defects in VLDL secretion that could not be observed in the lymphoblast model. In addition, only

certain tissues are steroidogenic, and express the enzymes and transporters responsible for converting CH into hormones and other sterol products in the mitochondria. It is known that tetralinoeoyl CL forms contact points for CH movement into the mitochondrial lumen (Gasnier *et al.*, 1998). Moreover, lymphoblasts do not produce any significant amount of CE for storage, as other cells do (Gottfried, 1967). As a result, any deficiencies in these pathways may not be observed in our cell model.

Our data suggests that, under normal culture conditions, BTHS lymphoblasts are able to maintain total intracellular CH levels similar to that of a control patient with no known pathology. This maintenance seems to be at the expense of an already maximal HMGR activity. As a result, during occasions of increased CH demand, such as serum removal, the BTHS lymphoblasts, being already taxed in their CH synthesis, are unable to further increase their production due to the inability to further increase HMGR activity. Our work provides the preeminent characterization for the hypocholesterolemia observed in two BTHS patients, and highlights the need for further work. The impact of hypocholesterolemia in BTHS needs to be investigated more thoroughly, particularly in response to times of increased cholesterol demand in developmental periods outside the "honeymoon period". We hypothesize that these are the periods when defects may become apparent, and potentially most detrimental to the patient.

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Chapter 8: Cardiolipin Synthesis is Required to Support Human Cholesterol Biosynthesis from Palmitate upon Serum Removal in HeLa cells

Chapter 8: Cardiolipin Synthesis is Required to Support Human Cholesterol Biosynthesis from Palmitate upon Serum Removal in HeLa cells

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Abbreviated Title: Cardiolipin regulates cholesterol synthesis in HeLa cells

Abstract

We examined whether cardiolipin (CL) synthesis was required to support cholesterol (CH) production from palmitate in HeLa cells. Knock down of human cardiolipin synthase-1 (hCLS1) in HeLa cells has been shown to reduce CL synthesis (Choi *et al.*, 2007). HeLa cells stably expressing shRNA for hCLS1 and mock control cells were incubated for 16 h with [^{14}C(U)]palmitate bound to albumin (1:1 molar ratio) in the absence or presence of serum. Knock down of hCLS1 in HeLa cells resulted in a reduction in [^{14}C(U)]palmitate incorporation into CL and CH. This reduction in [^{14}C(U)]palmitate incorporation into CH was most pronounced during incubation under serum-free conditions. The reduction in [^{14}C(U)]palmitate incorporation into CH was not due to alterations in total uptake of [^{14}C(U)]palmitate into cells or altered palmitate metabolism since [^{14}C(U)]palmitate incorporation into phosphatidylcholine the major [^{14}C(U)]palmitate containing lipid, and its immediate precursor 1,2-diacyl-*sn*-glycerol, was unaffected by hCLS1 siRNA. In addition, knock down of hCLS1 did not affect CH pool size indicating that CH catabolism was unaltered. Hydroxymethylglutaryl-Coenzyme A reductase enzyme activity and its mRNA expression were reduced by knock down of hCLS1 and this was most pronounced in HeLa cells cultured under serum-free conditions. These data indicate that CL synthesis is required to support human *de novo* CH biosynthesis under stress conditions.

8.1. Introduction

CL was the first polyglycerolphospholipid discovered and was first isolated from beef heart by Mary Pangborn [reviewed in (Hostetler, 1982)]. Cardiolipin (CL) is both synthesized and localized exclusively within mammalian mitochondria (Hostetler, 1982; Daum *et al.*, 1986). CL is localized to both inner and outer mitochondrial membranes and within contact sites in mammalian cells (Nicolay *et al.*, 1990). CL is required for the reconstituted activity of a number of key mitochondrial enzymes involved in cellular oxidative metabolism [reviewed in (Hoch, 1992; Hatch, 1998; Chicco *et al.*, 2007)]. CL anchors cytochrome *c* to the inner mitochondrial membrane (Tuominen *et al.*, 2002) and may play a key role in cytochrome *c* release and apoptosis (Ostrander *et al.*, 2001a; McMillin *et al.*, 2002; Ott *et al.*, 2002). CL also plays an essential role in mitochondrial biogenesis (Schlame *et al.*, 2000), and the assembly of respiratory enzyme supercomplexes (Pfeiffer *et al.*, 2003). Hence, CL may be the "glue" that holds the respiratory chain together (Zhang *et al.*, 2002).

CL is synthesized in mammalian cells by the CDP-DG pathway [reviewed in (Hatch, 2004)]. Phosphatidic acid is converted to cytidine-diphosphate-1,2-diacyl-*sn*-glycerol (CDP-DG) catalyzed by cytidinediphosphate-1,2-diacyl-*sn*-glycerol synthetase (CDS). There are two isoforms of this enzyme in mammalian tissues CDS-1 and CDS-2 (Halford *et al.*, 1998). CDP-DG condenses with glycerol-3-phosphate to form phosphatidylglycerol phosphate catalyzed by phosphatidylglycerol phosphate synthase (PGS). Phosphatidylglycerol phosphate does not accumulate in mammalian cells and is rapidly converted to phosphatidylglycerol by a phosphatidylglycerol phosphate phosphatase. The final step in the CL biosynthetic pathway involves the condensation of

phosphatidylglycerol with another molecule of CDP-DG to form CL catalyzed by cardiolipin synthase (CLS) (Hostetler *et al.*, 1972). The human CL gene (hCLS1) was recently cloned by four independent laboratories (Chen *et al.*, 2006; Houtkooper *et al.*, 2006; Lu *et al.*, 2006).

De novo cholesterol (CH) biosynthesis occurs in all nucleated cells and is up regulated in response to low intracellular sterol concentrations (Vance *et al.*, 2002). 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is the major rate limiting step in *de novo* cholesterol synthesis, (Brown *et al.*, 1999). HMG-CoA reductase activity in cells is regulated by sterols, primarily at the level of expression of HMGR mRNA (Goldstein *et al.*, 1990). It has been estimated that approximately two thirds of all cellular CH is from *de novo* biosynthesis (Endo *et al.*, 1989). Previous studies have shown that palmitate may be utilized for CH biosynthesis in mammalian cells and in the *de novo* biosynthesis of PLs (Vance *et al.*, 2002).

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

down of hCLS1 results in a reduced ability to synthesize CH *de novo* from [^{14}C(U)]palmitate upon serum removal in HeLa cells. These results suggest that CL synthesis is required to support CH biosynthesis under stress conditions.

8.2. Materials and Methods

8.2.1. Materials

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

8.2.2. Cell culture

HeLa cells were obtained from American Type Culture Collection. HeLa cells were transfected with plasmids containing shRNA to human CLS as previously described (Choi *et al.*, 2007). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 100 U Penicillin, 100 µg/ml Streptomycin. In addition, transfected cells had 10 µg/ml Blasticidin in their media as a selective reagent for propagation, but shared the same media as the untransfected cells (lacking in Blasticidin) during all experimental treatments. HeLa cells were incubated overnight with media containing or deficient in FBS and [^{14}C(U)]-Palmitic Acid (bound to albumin 1:1

molar ratio), and cells were incubated for 16 h. Cellular lipids were then isolated as described below.

8.2.3. Lipid Isolation and cholesterol determination

CH was isolated as described with modification (Folch *et al.*, 1957). Briefly, after the initial treatment of cells described above, the media was removed and cells washed with 2 ml PBS. Cells were scraped from the plates using a plastic scraper into 2 ml of methanol:H_2O (1:1, v/v). Cells were transferred with a Pasteur pipette to silanated glass tubes, and an aliquot was taken for total radioactivity and protein (25 µl, and 50 µl, respectively) determination. Protein assays were performed with a BioRad's Protein assay kit. Bovine serum albumin (BSA) was used as a standard. An additional 0.5 ml of H_2O and 2 ml of chloroform were added to each sample, followed by brief mixing using a vortex mixer. Samples were then centrifuged at 600 x *g* in a swinging bucket rotor for 10 min. The upper, aqueous layer was removed by suction, along with the protein interphase. Addition of 2 ml theoretical upper phase (methanol:0.9% NaCl:chloroform; 48:47:3, v/v) was followed by a second brief mixing as above and then centrifugation at 2,000 rpm for 5 min. Removal of the aqueous phase was followed by drying down of the organic phase with nitrogen gas. Samples were capped and stored at -20°C. A 50 µl portion of the total sample was spotted onto silica thin layer chromatography plates for one dimensional separation of CH. Plates were developed for approximately 1 h in Hexanes:Diethyl ether:glacial acetic acid (70:30:2, v/v). A portion of the total sample was spotted onto silica thin layer chromatography plates for separation of phosphatidylcholine (PC) and 1,2-diacyl-*sn*-glycerol (DG) as previously described

(Hatch & McClarty, 1996). Spots corresponding to lipids were visualized with iodine vapor and removed into plastic scintillation vials and radioactivity in the sample determined by liquid scintillation counting in a Beckman Model LS6500 Scintillation Counter. In some experiments, CH content of the cells was determined by colorimetric reaction of the Amplex Red CH assay kit from Invitrogen's Molecular Probes. After isolation of cellular lipids as described above, the lipid residue was reconstituted with 1% Triton X-100 in isopropanol, and CH assays were performed as per protocol. All isolates were measured immediately after drying down with nitrogen, as fresh samples seemed to yield the best results.

8.2.4. Real time-PCR

Cells were incubated as above and then RNA was isolated using the TRIzol method of phenol extraction and stored at -80°C. The isolated RNA was combined with Qiagen RT-PCR master mix and the appropriate primers to a total reaction volume of 25 µl. Reverse transcription (RT) was performed immediately prior to polymerase chain reaction (PCR) as part of the same cycler protocol. The cycler protocol for real time PCR on the Eppendorf Mastercycler ep Realplex 2, consisted of a 30 min RT step at 50°C, followed by a 15 min Taq activation step at 95°C followed by a 1 min separation at 95°C and melting curve that increased in temperature incrementally from 60°C to 95°C over the course of 20 min, taking fluorescence readings throughout to determine product quality. The primers used for CDS-1, CDS-2 and PGS in HeLa cells have been previously described (Hauff *et al.*, 2009). The primers used for HMGR and 18s RNA were as follows: HMGR (NCBI ID: NM_000859); Forward

,cggttGGAAGAGACAGGGATAAAC[FAM]G; Reverse, GGGTATCTGTTTCAGCCACTAAGG and 18s rRNA (X03205); Forward CTCGGGCCTGCTTTGAACAC, Reverse, cgggTGCTCTTAGCTGAGTGTCC[FAM]G. The PCR program for HMGR (400 nM) and housekeeping gene 18s rRNA (100 nM) primers ran 50 cycles of separation at 94°C for 15 seconds and annealing/elongation at 60°C for 45 seconds. Fluorescence readings were taken at the end of every elongation step. The changes in gene expression were analyzed on an Eppendorf Mastercycler ep Realplex, software version 1.5.474, and the data presented as mean fold change ($2^{-\Delta\Delta Ct}$) in mRNA expression (Livak *et al.*, 2001) relative to 18s rRNA, a gene not affected by short term incubation of cells under altered serum conditions (Schmittgen *et al.*, 2000).

8.2.5. Assay of in vitro HMG-CoA reductase

Cells were incubated as above and removed from the plates and suspended on ice in 2 ml homogenizing buffer (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose), followed by homogenization with 2 x 20 strokes of a Dounce A homogenizer. The homogenate was centrifuged at 4°C, 1,000 x g for 5 min and the supernatant centrifuged at 4°C, 10,000 x g for 15 min. The resulting supernatants were then further centrifuged for 1.5 h at 4°C, 100,000 x g, and the resulting microsomal pellets were resuspended in homogenizing buffer and kept on ice while protein content was determined. Protein assays were performed with BioRad's Protein assay. BSA was used as a standard. *In vitro* HMG-CoA reduction assays were performed as described (Ohashi *et al.*, 2003). Reaction buffer containing, at final concentration, 5 mM β-nicotinamide adenine dinucleotide phosphate

reduced, tetrasodium salt (NADPH - made fresh), 10 mM Ethylenediamine-tetraacetic acid (EDTA), 10 mM dithiothreitol (DTT), 100 mM Tris-HCl (pH 7.4), was added to 50 µg microsomal protein per sample. The reactions were initiated by the addition of DL-3-[glutaryl-3-^{14}C] hyroxy-3-methylglutaryl coenzyme A (4.5 µCi/µmol), cold HMG-CoA to 110 µM, and incubation at 37°C for 30 min. Addition of HCl to 1N, and incubation for a further 30 min at 37°C was required to lactonize the mevalonate formed. Samples were then stored in the freezer overnight and then spotted onto dried silica thin layer chromatography plates. Plates were developed for 2 h in thin layer chromatography tanks containing acetone-benzene (1:1 v/v). Subsequently, the plates were exposed to iodine vapor and spots corresponding to R_f = 0.6-0.9 were removed into scintillation vials and radioactivity determined, i.e. [^{14}C]mevalonate formation.

8.2.6. *Statistics*

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

8.3. Results and Discussion

We previously demonstrated that knock-down of hCLS1 reduced hCLS1 mRNA expression and CL synthesis in HeLa cells (Choi *et al.*, 2007). In this study, we began by examining the expression of genes of the CDP-DG pathway of CL biosynthesis in serum-supplemented and serum-depleted HeLa cells in which hCLS1 was knocked down. HeLa cells stably expressing shRNA to hCLS1 (hCLS) or mock controls (Mock) were incubated for 16 h with medium in the absence or presence of serum. Total RNA was prepared and mRNA expression of CDS-1, CDS-2, PGS and hCLS1 determined. Knock down of hCLS1 did not affect expression of CDS-1, CDS-2 or PGS in HeLa cells incubated in serum-containing or serum-free medium (**Figure 8-1A-C**). As previously demonstrated, (Choi *et al.*, 2007) knock down of hCLS1 reduced hCLS1 mRNA expression approximately 20-40% (data not shown). Thus, knock down of hCLS1 using shRNA reduces only hCLS1 mRNA expression but not other enzymes of the CDP-DG pathway of CL synthesis. We next examined CL synthesis from [^{14}C(U)]palmitate. HeLa cells stably expressing shRNA to hCLS1 (hCLS) or mock controls (Mock) were incubated for 16 h in medium containing [^{14}C(U)]palmitate in the absence or presence of serum. The cells were then harvested and radioactivity incorporated into CL determined. We used 0.1 mM palmitate bound to albumin (1:1 molar ratio) to achieve the labeling, since this is representative of circulating plasma palmitate levels and higher palmitate:albumin ratios result in apoptosis (Ostrander *et al.*, 2001a). Total uptake of [^{14}C(U)]palmitate into growing cells was unaltered between mock ($0.68 \pm 0.02 \times 10^7$ dpm/mg) and hCLS ($0.70 \pm 0.04 \times 10^7$ dpm/mg) cells. In addition, total uptake of [^{14}C(U)]palmitate into growth-arrested (serum-free) cells was unaltered between mock

($0.69 \pm 0.05 \times 10^7$ dpm/mg) and hCLS ($0.67 \pm 0.03 \times 10^7$ dpm/mg) cells. Incorporation of [^{14}C(U)]palmitate into CL was reduced 42% ($p<0.05$) from 0.07 ± 0.01 % of total dpm to 0.04 ± 0.01 % of total dpm by knock down of hCLS1 in serum-supplemented HeLa cells and was reduced 50% ($p<0.05$) from 0.08 ± 0.01 % of total dpm to 0.04 ± 0.01 % of total dpm by knock down of hCLS1 in HeLa cells incubated in serum-free conditions. Thus, knock down of hCLS1 reduced CL biosynthesis from [^{14}C(U)]palmitate in HeLa cells grown in serum-containing or serum-free medium.

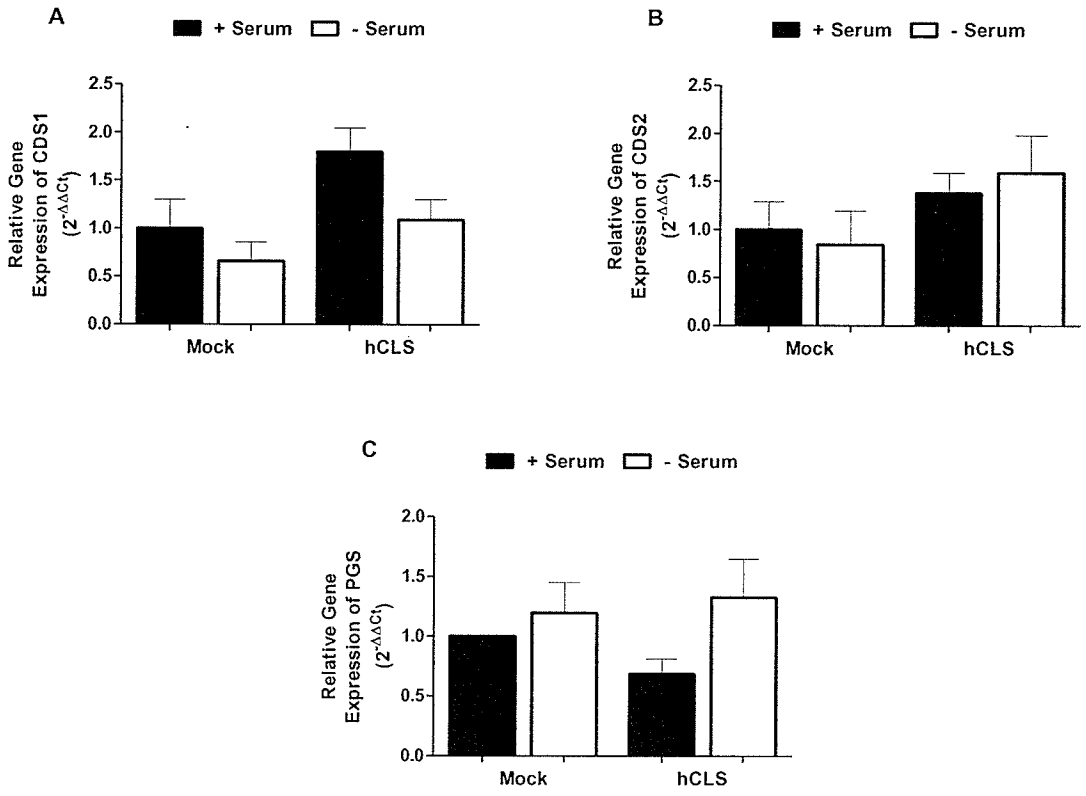

Figure 8-1: Expression of enzymes of the CDP-DG pathway of CL biosynthesis in HeLa cells.

HeLa cells stably expressing shRNA to hCLS1 (hCLS) or mock controls (Mock) were incubated for 16 h with medium in the absence or presence of serum. Total RNA was prepared and mRNA expression of CDS-1 (**A**), CDS-2 (**B**) and PGS (**C**) determined. Data represent the mean $\pm$ SEM (n=3).

To examine if CL synthesis is required to support CH synthesis from palmitate, HeLa cells stably expressing shRNA to hCLS1 (hCLS) or mock controls (Mock) were incubated for 16 h in medium containing [^{14}C(U)]palmitate in the absence or presence of serum and the radioactivity incorporated into CH determined. Serum removal would be expected to upregulate CH biosynthesis (Fogelman *et al.*, 1977). Serum removal resulted in elevated CH synthesis from [^{14}C(U)]palmitate in mock transfected HeLa cells (**Figure 8-2A**). Thus, palmitate can be used as a carbon source for CH synthesis in HeLa cells. However, knock down of hCLS1 resulted in a 20% decrease ($p<0.05$) and 40% decrease ($p<0.05$) in [^{14}C(U)]palmitate incorporation into CH in HeLa cells grown in serum-containing or serum-free medium, respectively. HeLa cell growth is attenuated under serum-free conditions (Hauff *et al.*, 2009). Since total uptake of [^{14}C(U)]palmitate into cells was unaltered and reduction in [^{14}C(U)]palmitate incorporation into CH was observed for cells incubated in either the absence or presence of serum, the decrease in [^{14}C(U)]palmitate incorporation into CH was not simply due to a potential growth defect caused by reduced CL synthesis. Thus, knock down of hCLS1 reduces [^{14}C(U)]palmitate utilization for CH biosynthesis in HeLa cells grown in serum-containing or serum-free medium. However, the effect was most significant when the cells were grown in the absence of serum, a condition where CH demand is highly up regulated (Fogelman *et al.*, 1977).

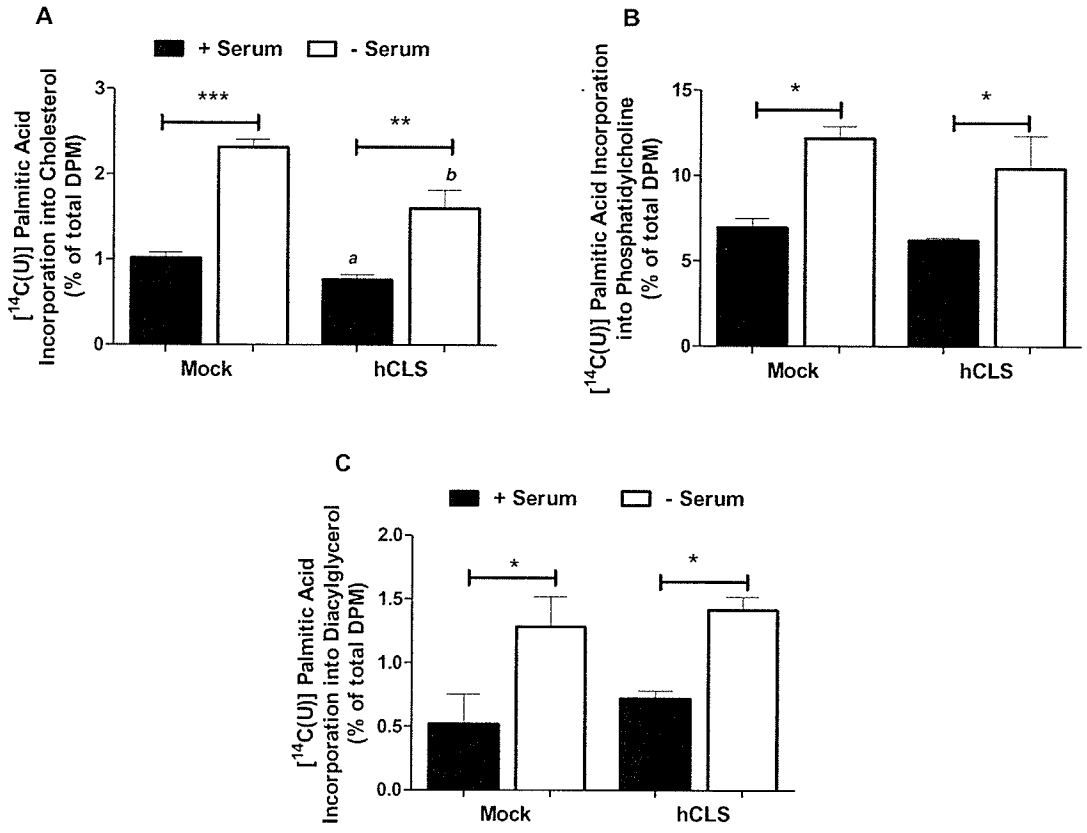

Figure 8-2: Synthesis of lipids from [^{14}C(U)]palmitate in HeLa cells.

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

Palmitate is a saturated fatty acid which will enter into most PLs via *de novo* biosynthesis (Vance *et al.*, 2002). It could be argued that general [^{14}C(U)]palmitate metabolism was disrupted by knock down of hCLS1. To address this, HeLa cells stably expressing shRNA to hCLS1 (hCLS) or mock controls were incubated 16 h with medium containing [^{14}C(U)]palmitate in the absence or presence of serum and radioactivity incorporated into the major palmitate containing lipid PC and its immediate precursor DG, determined. Serum removal resulted in an elevation in [^{14}C(U)]palmitate incorporation into PC and DG compared to serum supplemented cells, respectively (**Figure 8-2B-C**). Knock down of hCLS1 did not affect incorporation of [^{14}C(U)]palmitate into PC or DG in HeLa cells grown in serum-containing or serum-free medium. Thus, the reduction in [^{14}C(U)]palmitate incorporation into CH with knock down of hCLS1 was not due to changes in metabolism of the major palmitate containing lipid or its immediate precursor in HeLa cells.

The reason for the reduction in [^{14}C(U)]palmitate incorporation into CH with knock down of hCLS1 was examined. HeLa cells stably expressing shRNA to hCLS1 (hCLS) or mock controls were incubated for 16 h with medium in the absence or presence of serum, microsomal fractions were prepared and HMGR enzyme activity determined. Serum removal resulted in an increase in HMGR activity in both mock and hCLS cells (**Figure 8-3A**). Knock down of hCLS1 in HeLa cells reduced HMGR *in vitro* activity 46% ($p<0.05$) in the absence of serum compared to control. The effect was only significant when cells were grown in the absence of serum. Finally, we examined expression of HMGR mRNA in these cells using real time-PCR. HeLa cells stably expressing shRNA to hCLS1 (hCLS) or mock controls were incubated in the absence or presence of serum

for 16 h total RNA prepared and HMGR mRNA expression determined. Knock down of hCLS1 in HeLa cells reduced HMGR mRNA expression 28% ($p<0.05$) in the absence of serum compared to control (**Figure 8-3B**). The effect was significant when cells were grown in the absence of serum, and paralleled the reduced HMGR enzyme activity. These data indicate that the reduction in [^{14}C(U)]palmitate incorporation into CH might be due in part to reduced activity and mRNA expression of the key rate-limiting enzyme of CH biosynthesis in HeLa cells grown under serum-free conditions.

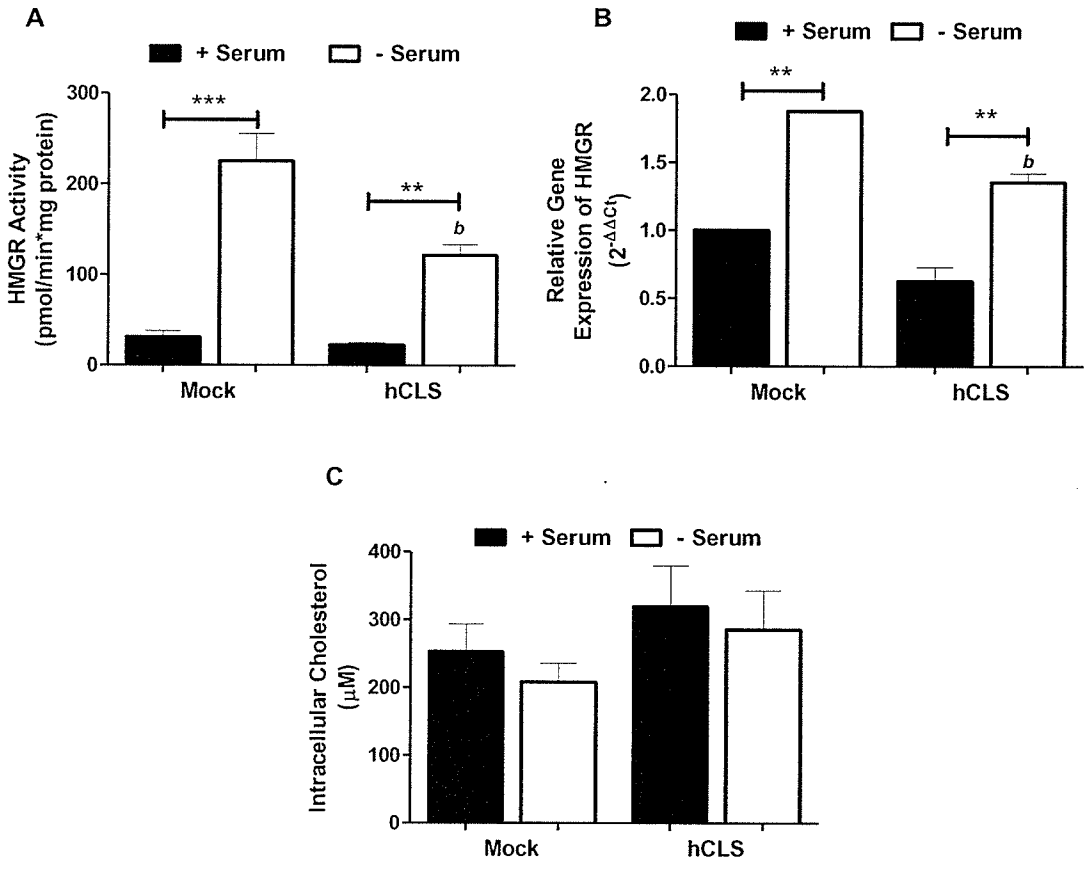

Figure 8-3: HMGR activity and mRNA expression and CH pool size in HeLa cells.

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

To examine if knock down of hCLS1 altered CH degradation, HeLa cells stably expressing shRNA to hCLS1 (hCLS) or mock controls were incubated for 16 in the absence or presence of serum and the pool size of CH determined. Knock down of hCLS1 did not affect the CH pool size when cells were incubated in either in the absence or presence of serum (**Figure 8-3C**). Thus, knock down of hCLS1 did not alter CH degradation.

8.4. Conclusion

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

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Chapter 9: Other Lipids Affected by BTHS or hCLS1 Inhibition

Chapter 9: Other Lipids Affected by BTHS or hCLS1 Inhibition

9.1. Introduction

In an attempt to characterize the lipid biochemistry in BTHS, the incorporation of various radiolabeled precursors into each of the readily separated PLs (PC, PE, PA, PG, and CL), the neutral lipids (DG, and TG), CH and CE were investigated. In addition, expression levels of various enzymes, transporters and transcription factors were examined. By considering these responses in the presence and absence of serum, the biochemistry of BTHS lymphoblasts can be evaluated at basal conditions and under conditions of cell stress, whereby CH biosynthesis is induced (Fogelman *et al.*, 1977). To further the investigation, a HeLa cell line stably transfected with shRNA to hCLS1, or its mock control, were also analyzed. The addition of this cell line to the examination allowed us to determine whether the changes observed in the BTHS lymphoblasts were the result of the tafazzin mutation, or the lack of mature CL.

Acetate is a substrate that can freely penetrate cell membranes, and thus, is least likely to be affected by import proteins that may be altered in BTHS (Sabine, 1977; Murray *et al.*, 1996). As previously mentioned (***Mitochondrial Biochemistry***), the mitochondrion is an important intermediate in cellular metabolism (Murray *et al.*, 1996), and modifies the acetate or pyruvate moiety to acetyl-CoA for further processing. Once made, in order for the cell to utilize this acetyl-CoA for biosynthesis, it must be exported via the citrate carrier (Murray *et al.*, 1996), which may still affect our results, as CL has been shown to influence the activity of the citrate carrier (Palmieri *et al.*, 1993; Giudetti *et al.*, 2002).

It was noted that the BTHS cells consistently exhibited more acidic media when cultured, even over a 16 h period, as determined by phenolphthalein red. As a result, lymphoblasts were cultured in media containing *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid* (HEPES), a buffering agent capable of maintaining physiological pH despite changes in carbon dioxide. However, the differences in CH incorporation of [1-^{14}C]acetate were absent in experiments completed in BTHS lymphoblasts grown in media containing HEPES. Therefore, a series of experiments were designed to examine this effect further. Acidosis has been shown to increase the expression of the ER chaperone protein GRP-78, indicative of ER stress (Aoyama *et al.*, 2005). A decrease in phosphorylation leads to an increase in activation of GRP78, a member of the heat shock 70 family of proteins (Sugawara *et al.*, 1993; Hendershot *et al.*, 1994). Furthermore, ER stress has been shown to be involved in the development of heart failure (Gaspar *et al.*, 2008).

In addition to glucose, glycerol can be readily incorporated into the PL backbone (Wylie *et al.*, 1997; Van *et al.*, 2007). Since CL is known to modulate the activity of the mitochondrial enzyme, glycerol-3-phosphate dehydrogenase, [reviewed in (Hatch, 1998)], and CL was shown to preferentially utilize glycerol under serum depleted conditions, see previously (Hauff *et al.*, 2009), incorporation of the precursor, [1,3-^{3}H]glycerol, into CH was also examined.

Studies have established that altered β-oxidation can cause a severe pathology, and has been demonstrated to occur in some cardiomyopathies (Saudubray *et al.*, 1999; Cheng *et al.*, 2004); as such, we wanted to find out if β-oxidation was altered in BTHS, or our hCLS1 knockdown cells. Palmitate is a major source of lipid for β-oxidation. CPT transports palmitate into the mitochondria, where it undergoes sequential removal of

acetate (Vance *et al.*, 2002). The resulting acetyl-CoA moieties can then be used as substrates for CH. On the other hand, palmitate can be incorporated into the phospho- and neutral lipids directly. By comparing these results in our BTHS cell lines, and our HeLa cells stably transfected with shRNA to hCLS1, we were able to determine if any alterations in β-oxidation exist in BTHS, and whether it is a result of CL a general deficiency or the mutation in tafazzin.

Glucose is taken up by glucose transporters, such as the GLUT1 transporter (Young *et al.*, 1995; MacIver *et al.*, 2008). Once in the cytosol, glucose undergoes glycolysis to produce pyruvate (Mayes, 1996). Since glucose is so broadly utilized, we used a more distal substrate, 2-[^{14}C]pyruvate, to corroborate that any findings were the result of mitochondrial deficiencies rather than additional glycolytic alterations. Pyruvate can enter the cellular membrane via a number of monocarboxylate transporters, which are not expected to require CL [reviewed in (Meredith *et al.*, 2008)]. Once formed, pyruvate is imported into the mitochondria for further processing to acetyl-CoA. Active uptake of pyruvate into the mitochondria requires the pyruvate carrier, which is known to require CL for its full functionality (Paradies *et al.*, 1997b), but has not been sequenced to date. As an alternative to this protein, we looked at the expression of the mitochondrial enzyme complex responsible for the conversion of pyruvate to acetyl-CoA, PDC. The PDC is inhibited by phosphorylation on the serine residues of E1 by the PDKs, of which there are 4 known isoforms (Roche *et al.*, 2001; Sugden *et al.*, 2003; Sugden *et al.*, 2006). Each isoform of PDK responds by an increase in expression during starvation, but each is subtly responsive to a different set of stimuli. PDK2 exhibits a greater sensitivity to the energy status of the cell and is highly sensitive to the absence of pyruvate, while PDK4 is

more responsive to acute alterations in lipid availability which has earned it the term lipid-status responsive isoform (Roche *et al.*, 2001). Under conditions of starvation, the inhibition of the PDC prevents muscle and other tissues from catabolizing glucose and gluconeogenesis precursors, such as pyruvate, in an attempt to save these resources for the brain, which requires glucose. In this case, metabolism shifts toward fat utilization via β-oxidation (Majer *et al.*, 1998). It is important to note that, although the cells in our studies underwent serum starvation, this is not the same as a state of glucose-sparing starvation, in which one is deprived of glucose, as glucose was still present in the medium at a concentration of 2g/L. However, some of the enzymes responsive to glucose starvation exhibit this decrease in the absence of serum as well, one such enzyme is the PDC (Tan *et al.*, 1998).

In the reverse direction, pyruvate can be sequestered from anabolism of lipids by the reverse reaction, PEPCK. Increase in the activity of this enzyme would favour gluconeogenesis in the cell (Chakravarty *et al.*, 2005), rather than the utilization of glucose, and therefore pyruvate, as a carbon or energy source. Phosphorylation of this enzyme indicates an inactive state (Inoue *et al.*, 2006), thus we examined the expression and phosphorylation of PEPCK.

De novo CH biosynthesis can occur in all nucleated cells (Vance *et al.*, 2002) by cleavage of the SREBP-2 in response to low intracellular sterol concentrations. The cleaved SREBP-2 promotes transcription of a number of genes whose products are involved in CH biosynthesis, as well as the LDLR, to increase CH uptake (Goldstein *et al.*, 2002). Under conditions of high ER sterol concentration, the SREBP cleavage-activating protein/SREBP-2 complex undergoes a conformational change that allows it to bind to

Insig-1, thereby preventing exit from the ER and further transcription of the genes involved in increasing intracellular CH concentration [reviewed in (Goldstein *et al.*, 2006; Espenshade *et al.*, 2007)]. As a result, SREBP-2 and Insig-1 are proteins which oppose each other in the regulation of intracellular CH levels.

ABCA1 is a CH exporter that is increased by the action of oxysterols on the liver X receptor (LXR), [reviewed in (Santamarina-Fojo *et al.*, 2001)]. The activity of ABCA1 is important in exporting CH from cells peripheral to the liver. The ABCA1 transporter serves to package CH and PLs in HDL particles for transport to the liver (Fielding *et al.*, 1995; Vance *et al.*, 2002). In Tangier's disease, a deficiency in the functioning of this CH exporter leads to CH accumulation in peripheral cells, a reduction in circulating HDL, and an increase in the level of CL in fibroblasts (Fobker *et al.*, 2001). Thus, a reduction in the expression of ABCA1 could lead to a reduction in plasma CH concentrations in BTHS.

In order to further investigate the CH biosynthetic pathway, HMGR activity, explored previously (***BTHS lymphoblasts fail to exhibit increased HMGR activity upon serum removal***), was inhibited and followed by supplementation of the cells with its product mevalonate. By inhibiting HMGR, the major rate-limiting enzyme in CH biosynthesis, and providing the cells with exogenous, radiolabeled RS-[2-^{14}C]mevalonate, whether residual CH synthesis is altered in BTHS was determined.

This chapter examines the effect of decreased CL on lipid biochemistry and the genes that control CH synthesis.

9.2. Results

9.2.1. Expression of CL Synthetic Enzymes are altered in BTHS

Since CL levels are altered in BTHS (Schlame *et al.*, 2003), we investigated the mRNA expression of the major CL biosynthetic enzymes using real time RT-PCR. Cells were incubated for 16h in the presence or absence of serum, and then harvested for total RNA and the relative expression of CL biosynthetic genes were determined. No significant changes in CDS2 or PGS mRNA expression occurred in any of the BTHS cells (**Figure 9-1B, C**). However, ΔTAZ 2 exhibited a significant increase in the level of CDS1 mRNA expression, in both the absence and presence of serum (**Figure 9-1A**). Downstream, the control cells exhibited a 48.5% reduction in the levels of hCLS1 mRNA expressed in response to serum starvation (**Figure 9-1D**). None of the BTHS cells exhibited any decrease in hCLS1. This maintenance of hCLS1 expression in BTHS lymphoblasts, while the controls displayed a reduction, may be an attempt to maintain the levels of CL in the BTHS lymphoblasts. These results suggest that the CL synthetic pathway is, at least partially, altered in BTHS. These alterations are likely the result of a compensating mechanism in an attempt to maintain appropriate levels of CL within the cell. It is interesting that CDS1 and hCLS1 exhibited alterations, but not PGS. This may be a result of the final reaction, which requires the condensation of an extra CDP-DG moiety to PG, effectively doubling the amount of product required from CDS (Chang *et al.*, 1998; Chen *et al.*, 2006; Lu *et al.*, 2006). It is also remarkable that the three BTHS cell lines did not exhibit uniform changes in the expression of CL synthetic enzymes.

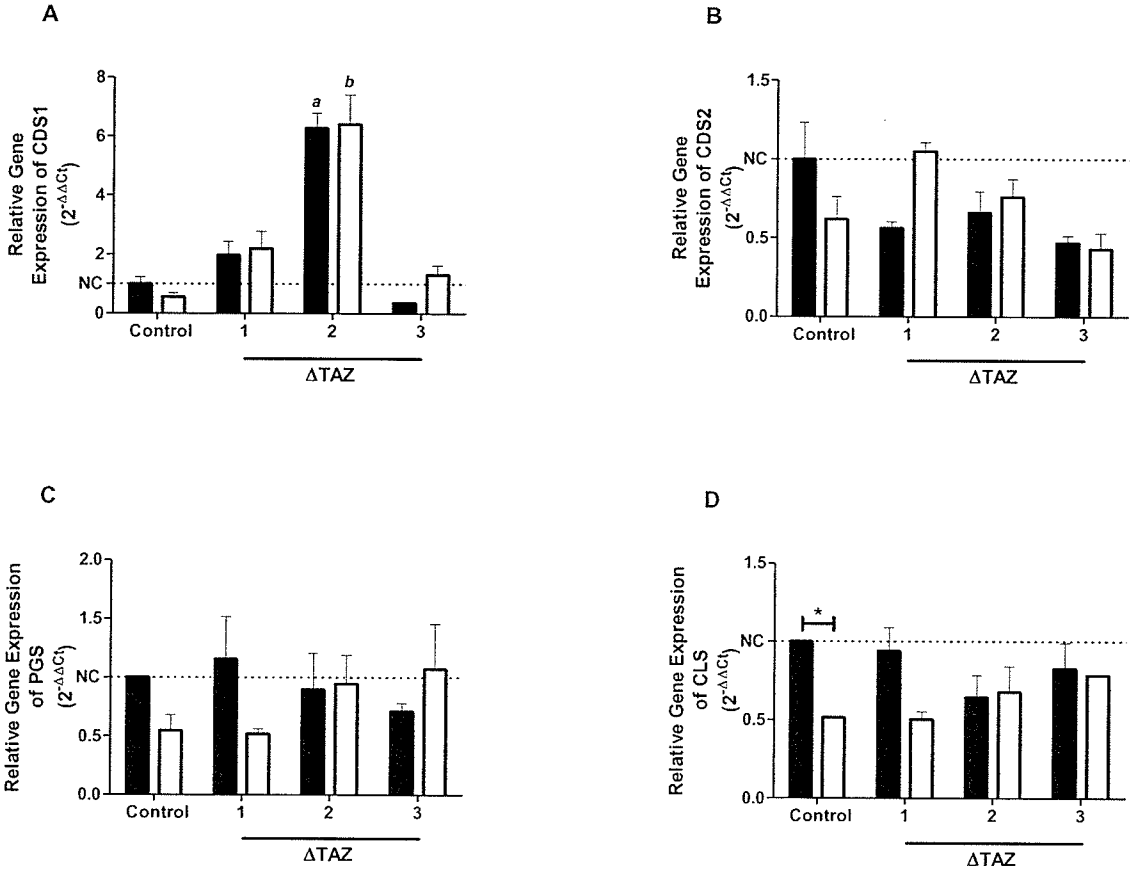

Figure 9-1: Expression of enzymes of the CDP-DG pathway of CL biosynthesis in BTHS cells.

Lymphoblasts from BTHS patients, ΔTAZ, or controls were incubated for 16 h with medium in the absence (open bars) or presence (closed bars) of serum. Total RNA was prepared and mRNA expression of CDS-1 (**A**), CDS-2 (**B**) PGS (**C**) and CLS (**D**) determined. Data represent the mean ± SEM (n=3), *$p<0.05$; $^{a}p<0.05$ compared to control +serum; $^{b}p<0.05$ compared to control -serum.

9.2.2. [^{14}C(U)]Palmitate Incorporation into Neutral and Phospholipids of BTHS and hCLS HeLa cells

The total uptake of [^{14}C(U)]palmitate was unaltered in all of the BTHS and hCLS cells incubated in the absence or presence of serum, as compared to controls (**Table 9-1**). However, the ΔTAZ 3 cells revealed a 2.9 fold increase in total uptake of [^{14}C(U)]palmitate in the absence of serum, compared to their counterparts incubated in the presence of serum. Even though CL levels were reduced in BTHS, we did not observe any significant changes in the level of [^{14}C(U)]palmitate incorporated into this lipid (**Table 9-1**). In addition, we didn't observe any significant changes in the incorporation of [^{14}C(U)]palmitate into any of the PLs. However, it is known that lymphoblasts predominantly use glucose and glutamine as their fuel source (Bental *et al.*, 1993). Thus it wasn't surprising that the incorporation of [^{14}C(U)]palmitate into all of the lipids were lower in these cells. Interestingly, ΔTAZ 1 cells incubated in the absence of serum display a significant increase in the incorporation of [^{14}C(U)]palmitic acid into DG, TG and CEs (**Table 9-1**). It is interesting to note that the variation associated with the incorporation of [^{14}C(U)]palmitate into the neutral lipids and CH was greater in the ΔTAZ patients than in the controls, under both serum deplete and serum replete conditions. This may relate to the acidic environment that is hypothesized to be an increase in the ER stress response that is observed in these cells (***[1-14C]Acetate Incorporation into Phospho- and Neutral Lipids***), as a previous study demonstrated that both CH and TG synthesis were upregulated in response to the UPR (Werstuck *et al.*, 2001).

Table 9-1: [^{14}C(U)]Palmitate incorporation into cholesterol, neutral and phospholipids in BTHS lymphoblasts compared to control.

Lipid	Palmitate Incorporation (% total DPM ±SEM)							
	Control		ΔTAZ1		ΔTAZ2		ΔTAZ3	
Serum	+	-	+	-	+	-	+	-
Cholesterol	1.46 ±0.28	1.38 ±0.07	3.92 ±1.05	3.18 ±0.53	2.37 ±0.81	2.38 ±0.82	1.76 ±0.46	2.12 ±0.62
Cholesterol Esters	0.05 ±0.00	0.11 ±0.00	0.06 ±0.01	0.21 ±0.04*†	0.07 ±0.00‡	0.09 ±0.01	0.05 ±0.01	0.10 ±0.02
Diacylglycerol	0.65 ±0.19	0.90 ±0.16	1.94 ±0.73	2.50 ±0.79†	0.74 ±0.23	0.95 ±0.21	0.97 ±0.40	1.10 ±0.24
Triacylglycerol	2.11 ±0.76	1.30 ±0.39	7.01 ±3.23	9.34 ±3.93†	3.91 ±1.71	3.00 ±1.31	3.25 ±1.36	2.25 ±0.71
Phosphatidyl-choline	9.99 ±1.09	16.92 ±3.98	7.46 ±1.46	10.56 ±2.35	7.27 ±0.75	9.96 ±1.85	7.67 ±0.93	11.71 ±1.90
Phosphatidyl-ethanolamine	1.61 ±0.23	2.67 ±0.71	1.93 ±0.83	1.31 ±0.38	1.11 ±0.26	2.20 ±0.66	1.16 ±0.30	2.38 ±0.72
Phosphatidic acid	0.09 ±0.02	0.13 ±0.03	0.11 ±0.04	0.14 ±0.04	0.13 ±0.05	0.17 ±0.04	0.13 ±0.04	0.16 ±0.04
Phosphatidyl-glycerol	0.19 ±0.02	0.34 ±0.08	0.28 ±0.06	0.35 ±0.08	0.30 ±0.05	0.47 ±0.14	0.32 ±0.04	0.63 ±0.23
Cardiolipin	0.21 ±0.03	0.42 ±0.15	0.22 ±0.05	0.25 ±0.07	0.37 ±0.10	0.34 ±0.10	0.55 ±0.28	0.35 ±0.14
Total *(DPM/mg protein *10^{7})*	0.82 ±0.05	1.31 ±0.24	0.89 ±0.06	1.59 ±0.30	1.15 ±0.19	1.40 ±0.37	0.87 ±0.11	2.52 ±0.92*
Percent Basal	**156%**		**201%**		**172%**		**346%**	

*-compared to +Serum; †-compared to Control –Serum; ‡-compared to Control +Serum

In HeLa cells with knock down hCLS1, CL synthesis from [^{14}C(U)]palmitate was decreased by approximately 43%, compared to that of the control, incubated in the presence of serum (**Table 9-2**). In addition, the inhibition of CL synthesis seems to have had an effect on the production of precursors in CL synthetic pathway. The major [^{14}C(U)]palmitate accumulation occurred at the level of PA, as a 66% increase in PA was observed in the presence of serum, as compared to the mock control (**Table 9-2**). [^{14}C(U)]Palmitate uptake by the HeLa cells cultured with or without serum was unaltered by the inhibition of hCLS1, thus, these effects are not a result of altered [^{14}C(U)]palmitate uptake.

Table 9-2: [^{14}C(U)]Palmitate incorporation into neutral and phospholipids of HeLa cells stably transfected with shRNA to hCLS compared to mock controls.

Lipid	Palmitate Incorporation (10^{-2} % total DPM ±SEM)			
	Mock		hCLS	
Serum	+	-	+	-
Cholesterol	1.02 ±0.07	2.31 ±0.10*	0.76 ±0.06‡	1.60 ±0.21*†
Diacylglycerol	0.52 ±0.23	1.28 ±0.23*	0.72 ±0.06	1.42 ±0.10*
Triacylglycerol	1.01 ±0.25	1.29 ±0.08	0.61 ±0.02	1.11 ±0.03
Phosphatidylcholine	6.94 ±0.55	12.18 ±0.72*	6.20 ±0.15	10.42 ±1.92*
Phosphatidylethanolamine	1.50 ±0.14	2.89 ±0.36*	1.20 ±0.24	1.82 ±0.31
Phosphatidic acid	0.19 ±0.03	0.68 ±0.06*	0.56 ±0.03‡	0.82 ±0.16
Phosphatidylglycerol	0.41 ±0.08	0.36 ±0.02	0.19 ±0.02	0.36 ±0.15
Cardiolipin	0.07 ±0.00	0.08 ±0.01	0.04 ±0.00‡	0.04 ±0.00
Total *(DPM/mg protein *10^7)*	0.68 ±0.02	0.69 ±0.05	0.70 ±0.04	0.67 ±0.03
Percent Basal	101%		97%	

*-compared to +Serum; †-compared to Mock –Serum; ‡-compared to Mock +Serum

9.2.3. Glycolysis is altered in BTHS cells but not in HeLa cells with a knock down in hCLS1

Overall incorporation of D-[^{14}C(U)]glucose into lipids was quite low. The total D-[^{14}C(U)]glucose uptake was significantly increased in the ΔTAZ2 and 3 cells compared to controls under serum supplemented and serum free conditions (**Table 9-3**). This difference in uptake may have had an impact on our results, as the only significant differences in D-[^{14}C(U)]glucose incorporation were in the ΔTAZ2 and 3 cells. Incorporation of D-[^{14}C(U)]glucose into both PG and CL was significantly increased in ΔTAZ2 cells in the absence of serum, compared to the presence of serum (**Table 9-3**). In contrast, the ΔTAZ3 cells exhibited a significant increase in D-[^{14}C(U)]glucose incorporation into CH, CEs, DG and TG in the presence of serum, compared to control lymphoblasts (**Table 9-3**).

Table 9-3: D-[^{14}C(U)]Glucose incorporation into cholesterol, neutral and phospholipids of BTHS lymphoblasts compared to controls.

Lipid	Glucose Incorporation (% total DPM ±SEM)							
	Control		ΔTAZ1		ΔTAZ2		ΔTAZ3	
Serum	+	-	+	-	+	-	+	-
Cholesterol	0.03 ±0.003	0.03 ±0.004	0.03 ±0.01	0.05 ±0.003	0.01 ±0.002	0.03 ±0.01	0.05 ±0.005‡	0.06 ±0.01
Cholesterol Esters	0.009 ±0.001	0.015 ±0.001	0.010 ±0.001	0.015 ±0.003	0.005 ±0.001	0.008 ±0.001	0.031 ±0.003‡	0.016 ±0.007
Diacylglycerol	0.006 ±0.001	0.012 ±0.001	0.008 ±0.002	0.014 ±0.001	0.005 ±0.001	0.015 ±0.003	0.023 ±0.002‡	0.018 ±0.007
Triacylglycerol	0.024 ±0.003	0.020 ±0.004	0.045 ±0.011	0.039 ±0.003	0.021 ±0.002	0.018 ±0.004	0.064 ±0.004‡	0.036 ±0.012
Phosphatidyl-choline	0.007 ±0.000	0.007 ±0.000	0.007 ±0.000	0.008 ±0.000	0.003 ±0.001	0.006 ±0.003	0.003 ±0.000	0.005 ±0.001
Phosphatidyl-ethanolamine	0.008 ±0.001	0.011 ±0.002	0.008 ±0.002	0.018 ±0.003	0.005 ±0.001	0.015 ±0.002	0.011 ±0.001	0.019 ±0.006
Phosphatidic acid	0.015 ±0.010	0.006 ±0.001	0.005 ±0.000	0.007 ±0.001	0.004 ±0.001	0.009 ±0.001	0.003 ±0.000	0.006 ±0.001
Phosphatidyl-glycerol	0.006 ±0.000	0.009 ±0.002	0.007 ±0.002	0.012 ±0.002	0.005 ±0.000	0.012 ±0.001*	0.005 ±0.001	0.010 ±0.002
Cardiolipin	0.007 ±0.001	0.009 ±0.002	0.009 ±0.002	0.010 ±0.001	0.004 ±0.001	0.012 ±0.002*	0.007 ±0.001	0.010 ±0.001
Total *(DPM/mg protein*10^{7})*	0.30 ±0.01	2.07 ±0.23*	0.36 ±0.02	2.00 ±0.06*	0.38 ±0.02‡	4.67 ±0.98*†	0.40 ±0.01‡	2.40 ±0.17*
Percent Basal	**691%**		**560%**		**1224%**†		**601%**	

*-compared to +Serum; †-compared to Control –Serum; ‡-compared to Control +Serum

HeLa cells expressing the hCLS knock down and the mock controls both exhibited a significant increase in D-[^{14}C(U)]glucose incorporation into CH and DG in the absence of serum, compared to the presence of serum (**Table 9-4**). However, in the presence of serum, HeLa cells with reduced hCLS demonstrated a significant increase in D-[^{14}C(U)]glucose incorporation into both CE and PA (**Table 9-4**). There were no significant differences in the total uptake of D-[^{14}C(U)]glucose in the HeLa cells stably transfected with shRNA to hCLS.

Table 9-4: D-[^{14}C(U)]Glucose incorporation into cholesterol, neutral and phospholipids into HeLa cells stably transfected with shRNA to hCLS1 compared to mock control cells.

Lipid	Glucose Incorporation (% total DPM ±SEM)			
	Mock		hCLS	
Serum	+	-	+	-
Cholesterol	0.11 ±0.01	0.22 ±0.03*	0.13 ±0.01	0.25 ±0.03*
Cholesterol Esters	0.03 ±0.004	0.03 ±0.01	0.06 ±0.01‡	0.03 ±0.01
Diacylglycerol	0.09 ±0.01	0.15 ±0.01*	0.10 ±0.01	0.18 ±0.03*
Triacylglycerol	0.17 ±0.04	0.21 ±0.07	0.16 ±0.03	0.29 ±0.12
Phosphatidylcholine	0.25 ±0.03	0.42 ±0.07	0.33 ±0.03	0.52 ±0.13
Phosphatidylethanolamine	0.06 ±0.01	0.06 ±0.01	0.09 ±0.02	0.09 ±0.01
Phosphatidic acid	0.01 ±0.001	0.01 ±0.001	0.03 ±0.006‡	0.02 ±0.004
Phosphatidylglycerol	0.02 ±0.01	0.01 ±0.002	0.02 ±0.002	0.01 ±0.003
Cardiolipin	0.006 ±0.001	0.007 ±0.001	0.010 ±0.002	0.013 ±0.005
Total ***(DPM/mg protein *10^7)***	1.92 ±0.09	2.04 ±0.20	1.77 ±0.28	2.29 ±0.08
Percent Basal	**108%**		**128%**	

*-compared to +Serum; †-compared to Mock –Serum; ‡-compared to Mock +Serum

Total 2-[^{14}C]pyruvate uptake in control and BTHS lymphoblasts was increased in the absence of serum (**Table 9-5**). The incorporation of radioactivity into PG and PA, precursors in the CL biosynthetic pathway, were elevated in the ΔTAZ3 cells, as compared to controls in the absence of serum (**Table 9-5**). No significant differences were observed in the incorporation of 2-[^{14}C]pyruvate into CL in the presence or absence of serum (**Table 9-5**).

Incorporation of 2-[^{14}C]pyruvate into PE in control lymphoblasts was significantly greater in the absence than in the presence of serum (**Table 9-5**). This was not true for the BTHS lymphoblasts, as they were unable to incorporate 2-[^{14}C]pyruvate into PE in the absence of serum as effectively as controls. However, this decrease in incorporation into PE under serum free conditions, as compared to control lymphoblasts, was not significant. A similar effect was observed in the incorporation of 2-[^{14}C]pyruvate into PC. Unlike the control lymphoblasts, the ΔTAZ2 and 3 cells did not exhibit a significant increase in 2-[^{14}C]pyruvate incorporation into PC in the absence of serum, as compared to the presence of serum (**Table 9-5**). In fact, the ΔTAZ2 cells exhibited a significant decrease in 2-[^{14}C]pyruvate incorporation into PC in the absence of serum, as compared to control cells. Upstream, in DG incorporation, trends observed were the same. The reduction in 2-[^{14}C]pyruvate incorporation in the absence of serum was even more pronounced in DG, as all of the BTHS demonstrated a significant decrease in incorporation, compared to controls (**Table 9-5**). In contrast, there was a significant increase in the level of 2-[^{14}C]pyruvate incorporated into PA in the absence of serum in ΔTAZ3 lymphoblasts, compared to control cells (**Table 9-5**). This data suggests that a defect in lipid metabolism may occur somewhere between the production of PA and DG

in these BTHS lymphoblasts. In contrast, synthesis of TG, from 2-[^{14}C]pyruvate, was significantly increased in BTHS lymphoblasts in the presence of serum, but not significantly different in the absence of serum (**Table 9-5**). Thus, 2-[^{14}C]pyruvate was able to progress through to TG synthesis in the presence of serum, however it was sequestered prior to incorporation into DG in the absence of serum, compared to control cells.

Table 9-5: 2-[^{14}C]Pyruvate incorporation into neutral and phospholipids of BTHS lymphoblasts compared to control cells.

Lipid	Pyruvate Incorporation (% total DPM ±SEM)							
	Control		ΔTAZ1		ΔTAZ2		ΔTAZ3	
Serum	+	-	+	-	+	-	+	-
Cholesterol	1.44 ±0.11	2.74 ±0.34*	1.78 ±0.10	1.93 ±0.30	1.10 ±0.03	1.71 ±0.20	0.80 ±0.12‡	1.36 ±0.15†
Cholesterol Esters	0.04 ±0.004	0.02 ±0.003	0.06 ±0.01	0.02 ±0.003*	0.06 ±0.01	0.03 ±0.003	0.07 ±0.01	0.02 ±0.003*
Diacylglycerol	0.06 ±0.01	0.26 ±0.02*	0.07 ±0.02	0.10 ±0.02†	0.04 ±0.01	0.12 ±0.01*†	0.04 ±0.00	0.15 ±0.03*†
Triacylglycerol	0.28 ±0.02	0.32 ±0.03	0.59 ±0.08‡	0.28 ±0.04*	0.53 ±0.01‡	0.33 ±0.02	0.56 ±0.09‡	0.31 ±0.03*
Phosphatidyl-choline	0.68 ±0.05	3.54 ±0.23*	0.33 ±0.09	2.10 ±0.89*	0.35 ±0.17	0.66 ±0.41†	0.44 ±0.13	1.81 ±0.32
Phosphatidyl-ethanolamine	0.17 ±0.02	0.53 ±0.02*	0.20 ±0.01	0.32 ±0.16	0.16 ±0.02	0.32 ±0.09	0.15 ±0.01	0.12 ±0.09
Phosphatidic acid	0.03 ±0.004	0.11 ±0.02	0.05 ±0.01	0.13 ±0.02	0.05 ±0.01	0.11 ±0.02	0.06 ±0.01	0.24 ±0.04*†
Phosphatidyl-glycerol	0.03 ±0.001	0.06 ±0.01	0.05 ±0.01	0.12 ±0.07	0.03 ±0.004	0.13 ±0.02	0.08 ±0.02	0.30 ±0.06*†
Cardiolipin	0.02 ±0.01	0.03 ±0.005	0.02 ±0.000	0.03 ±0.01	0.03 ±0.01	0.04 ±0.01	0.02 ±0.01	0.04 ±0.01
Total *(DPM/mg protein *10^{7})*	0.11 ±0.004	1.10 ±0.12*	0.16 ±0.003	1.20 ±0.16*	0.17 ±0.01	0.98 ±0.15*	0.23 ±0.06	1.09 ±0.22*
Percent Basal	**988%**		**749%**		**567%**†		**482%**†	

*-compared to +Serum; †-compared to Control –Serum; ‡-compared to Control +Serum

Since the uptake and incorporation of D-[^{14}C(U)]glucose (**Table 9-3**) and 2-[^{14}C]pyruvate (**Table 9-5**) was altered in BTHS cells, we investigated the expression pattern of the GLUT1 glucose import protein as well as a few key enzymes involved in pyruvate processing. Import of glucose occurs mainly through the GLUT1 transporter in lymphoblasts [reviewed in (MacIver *et al.*, 2008)], therefore, we investigated its expression pattern, as a representation of glucose uptake. In the presence of serum, GLUT1 mRNA expression was reduced in ΔTAZ2 cells, compared to controls. In the absence of serum, GLUT1 mRNA expression was significantly decreased in control and ΔTAZ1 cells, compared to the presence of serum, but not in ΔTAZ2 cells (**Figure 9-2A**). Interestingly, GLUT1 mRNA expression in ΔTAZ1 cells was reduced, compared to control cells in the absence of serum. This implies that glucose uptake may be altered in BTHS, but underscores the variability observed between the different patients. Overall, this indicates a complexity in BTHS metabolism that is likely one of the reasons that a correlation between genotype and phenotype in this disease has not been identified.

There were no significant differences in the expression of PDC in BTHS cells compared to control cells (**Figure 9-2B**). However, in the absence of serum, PDC expression was reduced in control and ΔTAZ2 cells, compared to incubation in the presence of serum. Furthermore, lipid responsive inhibitor of PDC, PDK4, was upregulated in the presence of serum, in both ΔTAZ1 and 3 cells (**Figure 9-2C**). This data suggests there is an inhibition of PDC activity in ΔTAZ1 and 3 cells, but not in ΔTAZ2 or control cells in the presence of serum. In contrast, there was a significant decrease in the energy responsive PDC inhibitor, PDK2 (**Figure 9-2D**). This would suggest that the activity of PDC is upregulated, though no significant decrease in PDK2 phosphorylation was detected in the

ΔTAZ1 cells (**Figure 9-2F**). Taken together, it appears that no significant deficiency in energy is activating PDK2, however a deficiency in lipids are acting to upregulate the inhibitory activity of PDK4 in the ΔTAZ1 and 3 cells. Further studies to elucidate the exact nature of this interaction will be required.

PEPCK was reduced in the ΔTAZ3 cells in the presence of serum, compared to control cells, (**Figure 9-2F**). While all of the BTHS cells were significantly reduced in the absence of serum, as compared to controls. In addition, the ΔTAZ1 cells, incubated in the presence of serum, exhibited a decrease in phosphorylation of PEPCK (**Figure 9-2G**). Thus, even when PEPCK mRNA expression was unaltered, as was the case for the ΔTAZ1 cells in the presence of serum, phosphorylation of PEPCK may still inhibit its activity. This data indicates that gluconeogenesis is decreased in BTHS cells, compared to control lymphoblasts. This may be the result of an increased requirement for the glucose in the anaplerotic or lipid biosynthetic reactions (Murray *et al.*, 1996; Owen *et al.*, 2002). Taken together, the expression of various glycolysis and gluconeogenesis enzymes suggests that BTHS lymphoblasts favour the action of glycolysis over gluconeogenesis to a greater extent than control lymphoblasts. In addition, there may be a greater activity of PDC, leading to the increased production of acetyl-CoA. This, however, could still be the result of a deficiency in pyruvate import into the mitochondria causing a deficiency in substrate availability, a function we were unable to characterize.

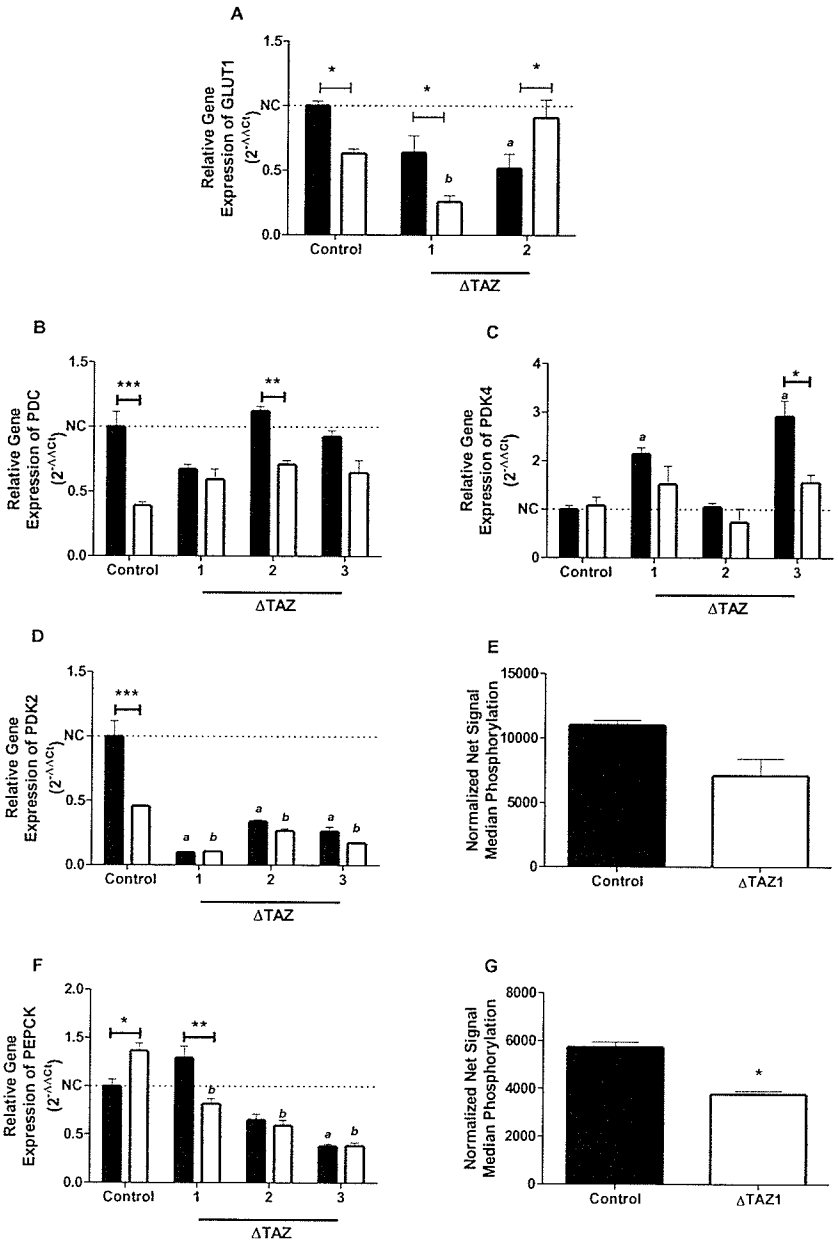

Figure 9-2: Expression of enzymes of the glycolytic pathway, and phosphorylation patterns in BTHS cells.

Lymphoblasts from BTHS patients, ΔTAZ, or controls were incubated for 16 h with medium in the absence (open bars) or presence (closed bars) of serum. Total RNA was prepared and mRNA expression of GLUT1 (**A**), PDC (**B**), PDK4 (**C**), PDK2 (**D**), and PEPCK (**F**) determined. Phosphorylation of PDK2 (**E**) and PEPCK (**G**) in ΔTAZ1 compared to control in the presence of serum was determined by Kinex™ Antibody Microarray. Data represent the mean ± SEM (n=3), *p<0.05; **p<0.01; ***p<0.001; ^{a}p<0.05 compared to control +serum; ^{b}p<0.05 compared to control -serum.

Unlike the BTHS lymphoblasts, the HeLa cells with knock down of hCLS exhibited no difference in the levels of GLUT1 mRNA expression in the presence or absence of serum (**Figure 9-3A**). This is not entirely surprising, as 13 GLUT family members have been identified, and in most cells, the GLUT1 isoform is ubiquitously expressed [reviewed in (Zhao *et al.*, 2007)]. In addition, none of the other glycolytic enzymes examined (PDC, PDK2, PDK4, PEPCK) exhibited any differences in mRNA expression in the presence or absence of serum (**Figure 9-3B-E**). This is in contrast to the BTHS cells, which demonstrated many changes in the levels of PDC, its kinases, PDK2 and 4, and PEPCK (**Figure 9-2B-G**). These results are surprising, as studies by Paradies *et al* established that the pyruvate transporter required CL for efficient functioning (Paradies *et al.*, 1991), yet the data here suggests that the mutation in Tafazzin is the reason for the alterations in 2-[^{14}C]pyruvate and D-[^{14}C(U)]glucose metabolism. Alternatively, the alterations observed in the BTHS lymphoblasts may be the result of the preference for glucose as a metabolite in lymphoblasts (Bental *et al.*, 1993). Or this may simply be the result of a dose-dependent response to CL, indicating that the differences we see may be the result of variations in the CL levels in each of the cell lines.

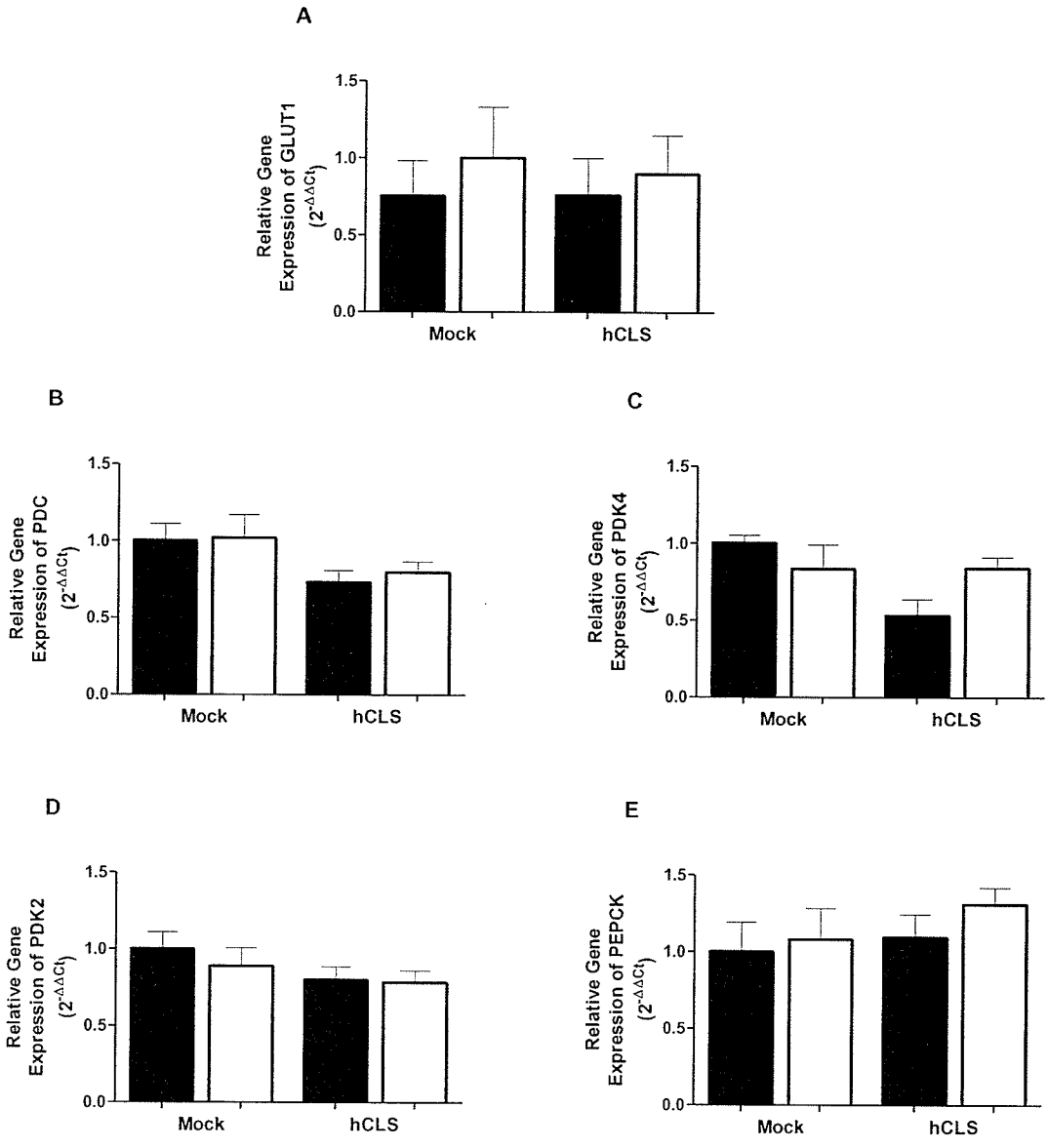

Figure 9-3: Expression of enzymes of the glycolytic pathway in HeLa cells.

HeLa cells stably expressing shRNA to hCLS1 (hCLS) or mock controls (Mock) were incubated for 16 h with medium in the absence (open bars) or presence (closed bars) of serum. Total RNA was prepared and mRNA expression of GLUT1 (**A**), PDC (**B**), PDK4 (**C**), PDK2 (**D**) and PEPCK (**E**) determined. Data represent the mean $\pm$ SEM (n=3).

9.2.4. [1-^{14}C]Acetate Incorporation into Phospho- and Neutral Lipids

None of the PLs exhibited any significant changes in incorporation of [1-^{14}C]acetate under either serum replete or serum deplete conditions (**Table 9-6**). In addition, there were no alterations in total [1-^{14}C]acetate uptake. Interestingly, there were no significant changes in the incorporation of [1-^{14}C]acetate into CL in any of the three BTHS cell lines (**Table 9-6**). Though this is noteworthy, it is not surprising, as previous studies have noted that the *de novo* synthesis of CL was unaltered, since the defect in CL lies in the remodeling process (Neuwald, 1997; Schlame *et al.*, 1997; Xu *et al.*, 2003b).

BTHS cells incorporated 2-4x more [1-^{14}C]acetate into TG than the controls (**Table 9-6**). This effect was decreased in the presence of serum, though, [1-^{14}C]acetate incorporation into TG remained significantly increased in the ΔTAZ1 and 3 cells when incubated in the absence of serum. ΔTAZ 2 exhibited a smaller increase in incorporation into TG under serum replete conditions but this was not significant in serum deprived BTHS cells. Interestingly, the control cells exhibited no change in the level of incorporation into TG whether they were incubated in the presence or absence of serum (**Table 9-6**). Though an increase in TG production in the presence of serum was observed in BTHS cells, the fact that it could not be maintained under serum depletion is intriguing. Strangely, although [1-^{14}C]acetate incorporation into TG was higher than controls under all conditions, the only change in DG incorporation was a decrease in the incorporation into DG in the ΔTAZ 3 patient, in the presence of serum (**Table 9-6**). This decrease was not evident when the ΔTAZ 3 cells were incubated in the absence of serum.

Though CE are not a major form of storage in the lymphoblast (Gottfried, 1967), there was some incorporation of [1-^{14}C]acetate detected in the CEs (**Table 9-6**). Incorporation

of [1-^{14}C]acetate into CE was not significantly altered with serum removal in the control cells. In contrast, both the ΔTAZ 2 and 3 patients exhibited a 50% reduction in [1-^{14}C]acetate incorporation into CE when they were incubated in the absence of serum. Conversely, the cells from the ΔTAZ 1 individual exhibited a 44% increase in [1-^{14}C]acetate incorporation into CE in the absence of serum, compared to incubation in the presence of serum. The differing effects observed in the CE synthesis of each patient may reflect variations in the metabolism within each of these patients. This, once again, attests to the complexity of the BTHS disease state and implies there is much more we need to learn before we will be able to understand what modifies the phenotype in each of these patients. Even when cell culture conditions are carefully controlled, the responses between each patient are not uniform.

Table 9-6: [1-^{14}C]Acetate incorporation into neutral and phospholipids in BTHS lymphoblasts versus controls

Lipid	Acetate Incorporation (% total DPM ±SEM)							
	Control		ΔTAZ1		ΔTAZ2		ΔTAZ3	
Serum	+	-	+	-	+	-	+	-
Cholesterol	1.84 ±0.26	3.25 ±0.28*	3.93 ±0.09‡	4.95 ±0.32†	2.03 ±0.31	2.67 ±0.14	3.21 ±0.46‡	3.74 ±0.44
Cholesterol Esters	0.07 ±0.00	0.11 ±0.02	0.09 ±0.01	0.16 ±0.02*	0.08 ±0.01	0.04 ±0.01†	0.14 ±0.02‡	0.07 ±0.01*
Diacylglycerol	0.90 ±0.18	1.42 ±0.05	0.73 ±0.13	1.65 ±0.25	0.80 ±0.17	0.77 ±0.28	0.20 ±0.04‡	1.31 ±0.59
Triacylglycerol	1.09 ±0.12	1.01 ±0.13	4.11 ±0.07‡	3.02 ± 0.11*†	2.38 ±0.18‡	1.24 ±0.10*	3.20 ±0.25‡	2.03 ± 0.13*†
Phosphatidyl-choline	3.70 ±0.14	5.14 ±1.57	4.66 ±0.71	7.98 ±2.54	4.12 ±0.37	5.19 ±1.10	3.72 ±0.33	8.36 ±2.16
Phosphatidyl-ethanolamine	0.72 ±0.06	0.58 ±0.14	0.94 ±0.21	0.71 ±0.18	0.76 ±0.10	0.67 ±0.12	1.06 ±0.09	1.23 ±0.26
Phosphatidic acid	0.12 ±0.05	0.31 ±0.14	0.15 ±0.06	0.31 ±0.14	0.17 ±0.08	0.29 ±0.12	0.21 ±0.08	0.27 ±0.11
Phosphatidyl-glycerol	0.05 ±0.02	0.07 ±0.01	0.13 ±0.04	0.30 ±0.09	0.11 ±0.02	0.19 ±0.05	0.12 ±0.02	0.29 ±0.11
Cardiolipin	0.06 ±0.02	0.16 ±0.06	0.09 ±0.03	0.13 ±0.02	0.05 ±0.01	0.08 ±0.01	0.05 ±0.01	0.08 ±0.02
Total *(DPM/mg protein *10^{7})*	1.76 ±0.49	3.95 ±1.83	2.74 ±1.20	3.28 ±1.74	2.90 ±0.62	3.69 ±1.56	2.65 ±0.61	3.30 ±1.35
Percent Basal	**178%**		**88%**		**112%**		**108%**	

*-compared to +Serum; †-compared to Control –Serum; ‡-compared to Control +Serum

There were no significant differences in the incorporation of [1-^{14}C]acetate into most of the lipids investigated in the HeLa cells stably transfected with shRNA to CLS. Nor was there a difference in the total uptake of [1-^{14}C]acetate in these cells. Surprisingly, [1-^{14}C]acetate incorporation into CL was not altered (**Table 9-7**). Unlike the BTHS cells, however, there were some alterations of [1-^{14}C]acetate incorporation into the precursors of CL biosynthesis, PA and PG. Under full serum conditions, there was a significant buildup of [1-^{14}C]acetate in PA. However, upon serum withdrawal, the buildup of [1-^{14}C]acetate appeared to occur in the immediate precursor to CL, PG (**Table 9-7**). This suggested that, although CL exhibited no significant change in the levels of [1-^{14}C]acetate incorporation, there was an alteration in the synthetic pathway upon serum removal.

Incorporation of [1-^{14}C]acetate into CH in both HeLa cell mock control and hCLS cells was significantly increased in response to serum withdrawal, while CEs were decreased (**Table 9-7**). This response would be expected as a healthy response to serum lipid deprivation. Given the major changes in [1-^{14}C]acetate incorporation into TG, CH and CEs observed in BTHS cells, the defect may be due to either a lack of CL greater than the knock down we achieved in the hCLS cells, or some additional factor.

Table 9-7: [1-^{14}C]Acetate incorporation into cholesterol, neutral and phospholipids of HeLa cells with shRNA to hCLS1 versus a Mock control.

Lipid	Acetate Incorporation (% total DPM ±SEM)			
	Mock		hCLS	
Serum	+	-	+	-
Cholesterol	0.48 ±0.07	1.01±0.15*	0.53 ±0.08	1.07 ±0.13*
Cholesterol Esters	0.07 ±0.01	0.02 ±0.01*	0.09 ±0.01	0.03 ±0.003*
Diacylglycerol	0.32 ±0.05	0.48 ±0.07	0.36 ±0.01	0.54 ±0.09
Triacylglycerol	0.48 ±0.09	0.36 ±0.07	0.63 ±0.12	0.40 ±0.06
Phosphatidylcholine	2.03 ±0.29	3.02 ±0.56	2.33 ±0.78	3.49 ±0.34
Phosphatidylethanolamine	0.31 ±0.05	0.38 ±0.09	0.49 ±0.04	0.51 ±0.09
Phosphatidic acid	0.01 ±0.00	0.03 ±0.01	0.03 ±0.01‡	0.05 ±0.01
Phosphatidylglycerol	0.05 ±0.02	0.04 ±0.01	0.07 ±0.01	0.09 ±0.00†
Cardiolipin	0.05 ±0.01	0.08 ±0.02	0.08 ±0.01	0.10 ±0.01
Total *(DPM/mg protein *10^7)*	2.29 ±0.08	1.16 ±0.04*	2.26 ±0.06	1.20 ±0.03*
Percent Basal	51%		53%	

*-compared to +Serum; †-compared to Mock –Serum; ‡-compared to Mock +Serum

The ΔTAZ1 lymphoblasts, and its maternal control, were cultured in media containing HEPES, a common buffer in cell media, or in the normal growth medium, lacking HEPES buffer. The ΔTAZ1 cells cultured in normal growth media incorporated significantly more [1-^{14}C]acetate into CH than the maternal control, as previously demonstrated (**Table 9-6**). However, these alterations in [1-^{14}C]acetate incorporation into CH could be recovered by the addition of the pH buffer, HEPES (**Figure 9-4A**). Taken together with the increase in [1-^{14}C]acetate into TG, this suggests that an ER stress response may occur in BTHS cells. Thus, the phosphorylation of a major UPR protein, GRP78, was investigated. In comparison to control lymphoblasts, ΔTAZ1 cells exhibited a reduction in phosphorylation, implicating an increase in GRP78 activity (**Figure 9-4B**). This data lends credence to the idea that the UPR is active in BTHS, and may play a role in the BTHS disease state. In a recent study, it was suggested that TG synthesis can be used as a 'metabolic sink', during ER secretory stress, to remove excess free fatty acids (Gaspar *et al.*, 2008), which accumulate in response to an acidic environment (Spector *et al.*, 1980). Furthermore, increased pH, as observed in the BTHS lymphoblasts (data not shown), has been demonstrated to induce the ER stress response in the form of UPR (Aoyama *et al.*, 2005). In support of this theory, culturing the BTHS lymphoblasts in the pH buffer, HEPES, was able to recover the level of [1-^{14}C]acetate incorporation into CH in ΔTAZ1 cells. Conversely, the stably transfected HeLa cells, exhibiting a reduction in CL synthesis exhibited no alterations in either DG or TG metabolism, thus, this effect may be unique to BTHS cells.

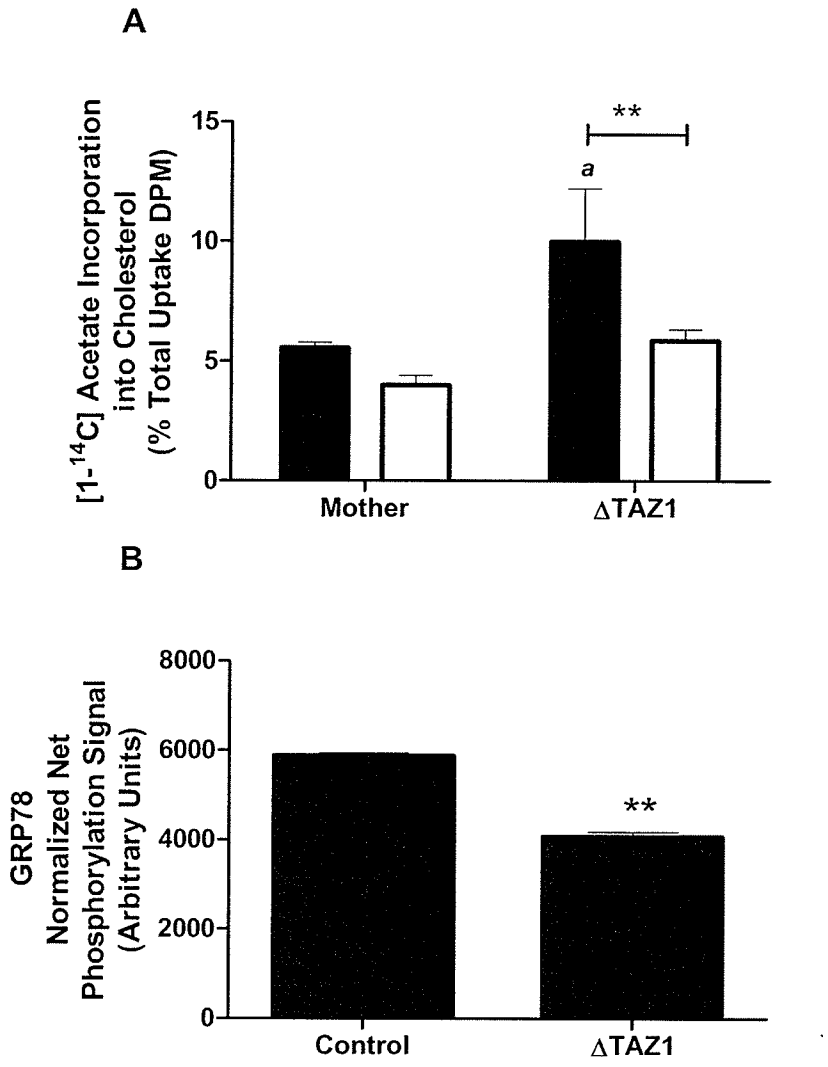

Figure 9-4: Synthesis of CH from [1-^{14}C]acetic acid in BTHS cells cultured in media containing the pH buffer, HEPES.

(**A**) ΔTAZ1 and lymphoblasts from the corresponding mother were incubated for 16h in media containing [1-^{14}C]acetate and the buffer, HEPES (open bars), or the regular culture media described in the materials and methods (closed bars). Cells were harvested and radioactivity incorporated into CH determined. (**B**) Phosphorylation of GRP78 in ΔTAZ1 compared to control in the presence of serum was determined by Kinex™ Antibody Microarray. Data represent the mean ±SEM (n=3), ** $p<0.01$; ^{a} $p<0.05$ compared to Mother –HEPES.

9.2.5. CH Biosynthesis in Cells Exhibiting Reduced CL Levels

Glycerol is another major precursor for lipid synthesis.We investigated the incorporation of [1,3-^{3}H]glycerol into CH of ΔTAZ2 and control cells in the presence or absence of serum (**Figure 9-5**) and found no alterations. Although CL exhibited a preference for [1,3-^{3}H]glycerol under serum free conditions in HeLa cells (Hauff *et al.*, 2009), neither the presence nor absence of serum resulted in a change from control levels of [1,3-^{3}H]glycerol incorporation into CH in BTHS cells.

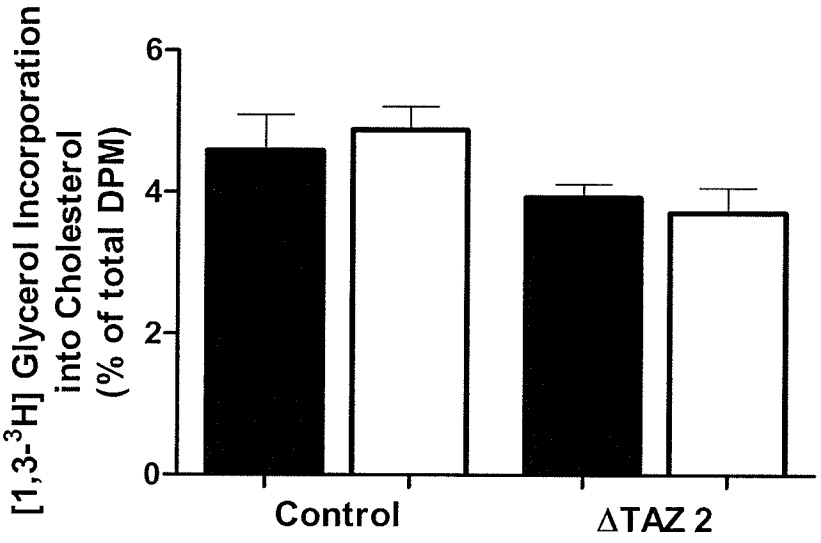

Figure 9-5: Synthesis of CH from [1,3-^{3}H]Glycerol in BTHS lymphoblasts.

Lymphoblasts from control and ΔTAZ2 were incubated for 16 h in medium containing [1,3-^{3}H]glycerol in the absence (open bars) or presence (closed bars) of serum. Cells were harvested and radioactivity incorporated into CH determined. Data represent the mean ± SEM (n=3).

In order to examine the effect of decreased CL levels on the CH biosynthetic pathway downstream of HMGR, control and BTHS lymphoblasts were incubated for 6 h in medium containing mevastatin, a drug known to inhibit the activity of HMGR (Roweïl *et al.*, 1997). The cells were then supplemented with RS-[2-^{14}C]mevalonic acid for 16 h in the presence of mevastatin. The incorporation of mevalonate into CH was unchanged in ΔTAZ1 lymphoblasts, compared to control cells (**Figure 9-6**), indicating that CH synthesis downstream of HMGR was unaffected by the BTHS phenotype.

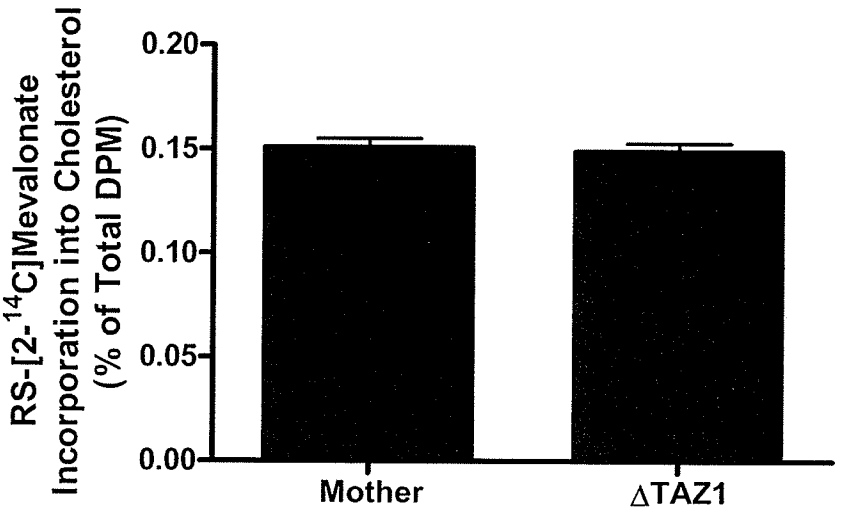

Figure 9-6: Synthesis of CH from RS-[2-^{14}C]mevalonic acid in BTHS cells.

Lymphoblasts from a BTHS patient, ΔTAZ1, or carrier control, mother, were incubated for 6 h in the inhibitor of CH synthesis, mevastatin, then in16 h in medium containing RS-[2-^{14}C]mevalonate and mevastatin presence of serum. Cells were harvested and radioactivity incorporated into CH determined. Data represent the mean ± SEM (n=3).

We examined whether SREBP-2 mRNA expression was altered in BTHS lymphoblasts. No significant change in SREBP-2 mRNA expression was observed in BTHS cells compared to control (**Figure 9-7A**). The reason for this may be the ubiquitous expression of SREBP-2, and the regulation of this transcription factor is predominantly at the level of post translational processing, as described previously (***Regulation of Cholesterol synthesis***).

Next we determined whether mRNA expression of the CH export protein, ABCA1, was altered in BTHS lymphblasts. In the absence of serum the ΔTAZ1 and 2 cells exhibited 61% and 46% reduction in ABCA1 mRNA expression, respectively, as compared to controls (**Figure 9-7B**). This would indicate that, under conditions of increased CH demand in BTHS, CH export is reduced. This is likely a consequence of the inability of the BTHS cells to respond to increased demands for CH at the level of biosynthesis, see previously (***CH levels are reduced in BTHS lymphoblasts upon serum removal***). Interestingly, the patient that exhibited the greatest incorporation of [1-^{14}C]acetate into CH in the presence of serum (**Table 9-6**), ΔTAZ1, was also the patient that exhibited the greatest levels of ABCA1 mRNA expression in the presence of serum (**Figure 9-7B**). The ABCA1 mRNA expression levels were similar to control in the presence of serum. As CH is only exported by ABCA1 when the cell has an excess of CH, this suggests that the ΔTAZ1 cells may have the least difficulty maintaining CH under basal conditions and implicates a role for ABCA1 in the hypocholesterolemia reported in BTHS.

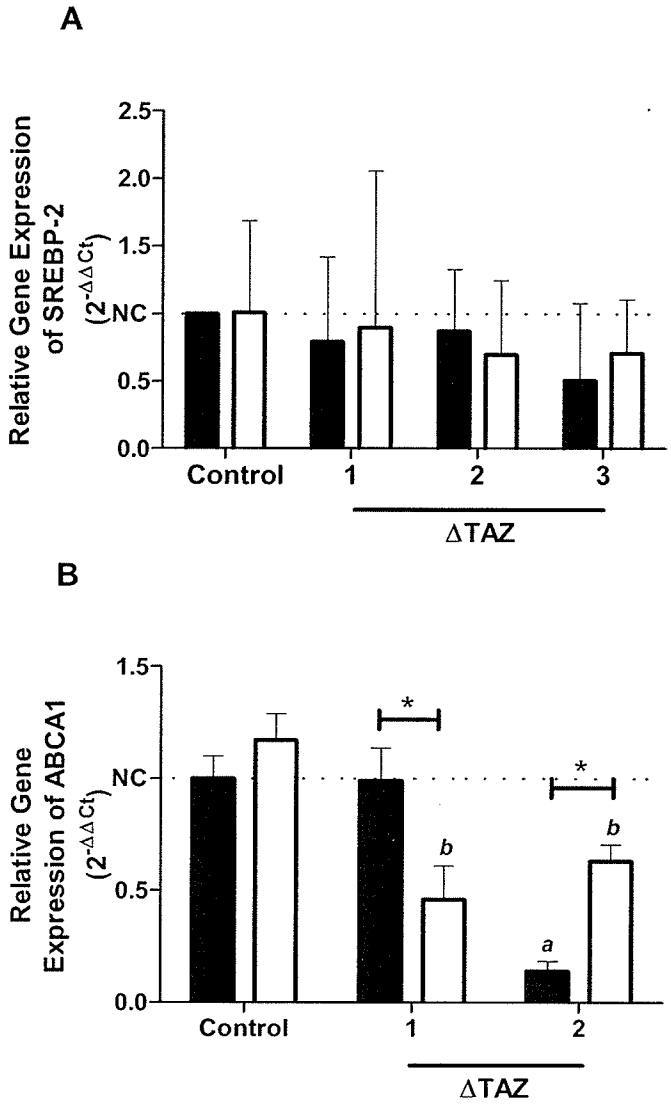

Figure 9-7: Expression of CH responsive genes in BTHS cells.

Lymphoblasts from BTHS patients, ΔTAZ1-3, or controls were incubated for 16 h with medium in the absence (open bars) or presence (closed bars) of serum. Total RNA was prepared and mRNA expression of SREBP-2 (**A**) and ABCA1 (**B**) determined. Data represent the mean ± SEM (n=3), *$p<0.05$; $^{a}p<0.05$ compared to control +serum; $^{b}p<0.05$ compared to control -serum.

In contrast to lymphoblasts, SREBP-2 was significantly reduced in hCLS HeLa cells incubated with serum, compared to mock controls (**Figure 9-8A**). This corresponded to a minor increase in Insig-1 expression in these hCLS HeLa cells in the presence of serum (**Figure 9-8B**). However, in the absence of serum, when CH synthesis is expected to increase (Fogelman *et al.*, 1977) no changes were observed in Insig-1 mRNA expression in hCLS1 cells, compared to controls (**Figure 9-8B**). In addition, no significant changes were observed in the expression of the CH importer, LDLR (**Figure 9-8C**), nor the CH exporter, ABCA1 (**Figure 9-8D**), in the cells stably transfected with shRNA to hCLS1 (hCLS) incubated in the presence or absence of serum. This was different from BTHS cells, suggesting that the effects are not due to changes in the level of CL, but are the result of either the TAZ mutation or altered CL remodeling.

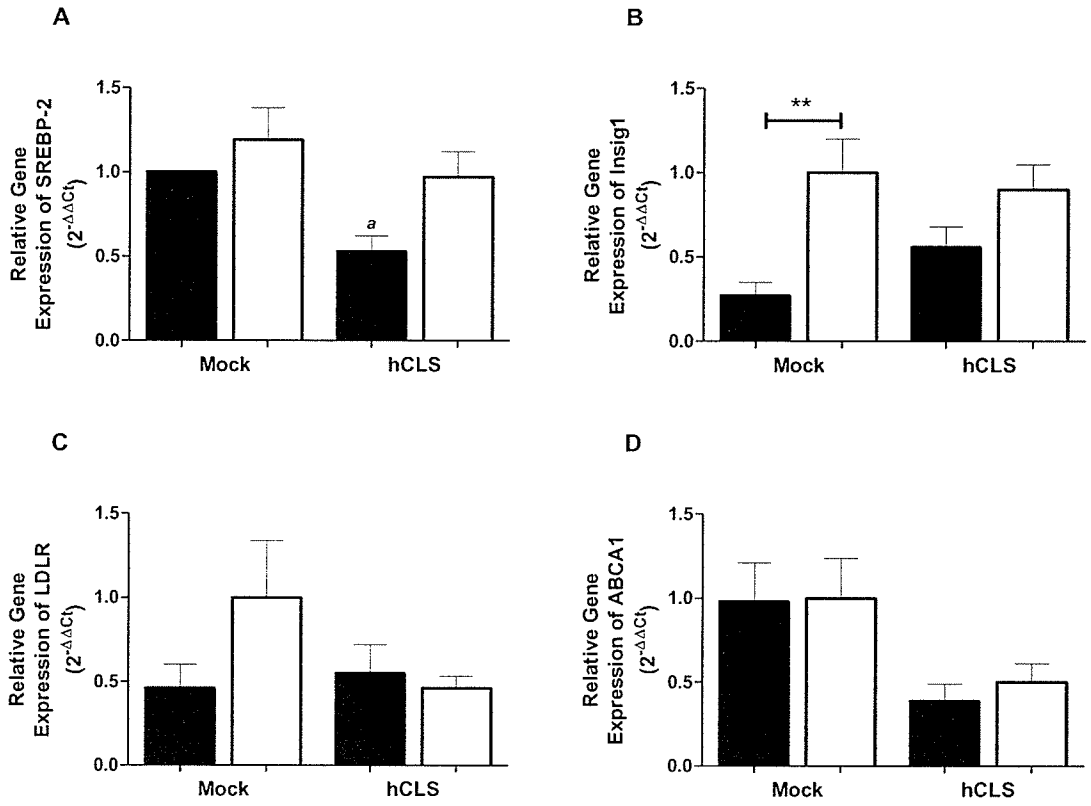

Figure 9-8: Expression of CH responsive genes in HeLa cells.

HeLa cells stably expressing shRNA to hCLS1 (hCLS) or mock controls (Mock) were incubated for 16 h with medium in the absence (open bars) or presence (closed bars) of serum. Total RNA was prepared and mRNA expression of SREBP-2 (**A**), Insig1 (**B**), LDLR (**C**) and ABCA1 (**D**) determined. Data represent the mean $\pm$ SEM (n=3), **$p<0.01$; $^{a}p<0.05$ compared to control +serum.

9.3. Discussion

In general, biosynthesis of PC and PE from any of the precursors tested exhibited no significant differences in BTHS or hCLS cells. In addition, the levels of PC and PE synthesis were the same regardless of whether or not serum was present in the medium. The hCLS HeLa cells, however, exhibited changes in the levels of radiolabeled substrate incorporation into PA, PG, and CL, indicative of the inhibition of CL synthesis. Synthesis of CL from [^{14}C(U)]palmitic acid was reduced in the presence of serum. No significant differences were observed in the production of CL from D-[^{14}C(U)]glucose or [1-^{14}C]acetate, but this may reflect the fact that palmitate is the predominant carbon source in HeLa cells (Stanisz *et al.*, 1983). Conversely, in the presence of serum, incorporation of all three precursors into PA was increased. In the absence of serum [1-^{14}C]acetic acid incorporation into PG was also increased.

The BTHS lymphoblasts exhibited no significant differences in incorporation of labeled precursors into PA or CL. In contrast, incorporation of [1-^{14}C]acetate into PG was increased. This may be the result of the cells' attempt to compensate for the lack of mature CL species, as there are some cases where PG can partially recover some CL-dependent function in cells lacking CL [reviewed in (Houtkooper *et al.*, 2008)]. In addition, it may be the result of an increased flux through the CL synthetic pathway, continuing through to an increase in MLCL. In support of this was the observed increase in the mRNA expression of CDS1 and hCLS1 in BTHS lymphoblasts.

Though there are some subtle differences in the responses of HeLa cells and lymphoblasts to different precursors and serum status, the message is clear. Global metabolism from palmitate, acetate and glycolysis is not challenged in response to the

BTHS mutation in tafazzin, or the inhibition of CL synthesis, as PLs are largely unaffected. The alterations observed in these cells are likely the result of an attempt to compensate for their respective defects.

Chapter 10: Conclusions and General Discussion

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Barth syndrome is a multifaceted disease, one where a single mutation can cause multiple deficiencies and changes in metabolism. More than 90 different mutations have been identified in BTHS to date (Gonzalez, 2008), resulting in dilated cardiomyopathy, cyclic neutropenia, type II 3-MGA, moderate hypocholesterolemia, and failure to thrive (Barth *et al.*, 1999; Mazzocco *et al.*, 2001) [reviewed in (Gonzalez, 2005; Hauff *et al.*, 2006)]. Serum CH levels in BTHS males were first reported as low in the early 90's (Kelley *et al.*, 1991b), but until recently, there has been no serious attempt to investigate the validity, impact or cause of this hypocholesterolemia. It is unknown what the specific role of hypocholesterolemia in BTHS is; nonetheless, low CH can have a major impact on a child's health and development (Waterham, 2002). We have hypothesized four main mechanisms for serum CH to be reduced; (1) is a reduction in CH synthesis, either as a result of a global defect in carbon processing, or a defect more specific to CH. (2) Alternatively, CH degradation could be enhanced, resulting in an overall decrease in the total amount of CH. The remaining potential mechanisms involve the transport of CH. (3) A decrease in the export of CH from the cells, as in Tangier's Disease (Francis *et al.*, 1995; Fobker *et al.*, 2001) or (4) an increase in CH uptake by the cells, as occurs in response to polyphenols, would both result in lower serum CH levels. We have attempted to broadly characterize as many of these sources of serum CH as we were able to with our resources. However, as was discussed (***Cellular Model***), there are some aspects which our cell models were insufficient to explore, and will need to be the topic of future investigations.

We characterized the biosynthesis of CH in BTHS lymphoblasts in order to better understand the mechanism of hypocholesterolemia in relation to TAZ and CL remodeling, and to develop better approaches in the treatment of BTHS. In addition, we examined the biosynthesis of CH in HeLa cells in which hCLS was knocked down to determine if CL plays a role in CH biosynthesis. Although the literature suggests circulating CH levels are reduced in BTHS patients, our data exhibited an unanticipated trend towards increased CH production. This increase in CH production needn't result in an increase in plasma CH; however, it does make the cause of hypocholesterolemia in BTHS a more difficult case to solve.

The major findings of this study are broad, and start to piece together the deficiencies in BTHS and CH synthesis; however, much investigation into the exact mechanism of hypocholesterolemia in BTHS is still required. We found that BTHS lymphoblasts from three patients with differing mutations have a reduced ability to up regulate CH *de novo* biosynthesis from [2-^{14}C]pyruvate or [1-^{14}C]acetate precursors in response to serum removal (**Figure 10-1**). The reason for the reduced ability to up regulate CH *de novo* biosynthesis is a reduction in the ability of BTHS lymphoblasts to elevate HMGR enzyme activity in response to serum removal. Insig-1 mRNA expression is elevated in BTHS lymphoblasts upon serum removal, indicating a direction toward enhanced HMGR degradation.

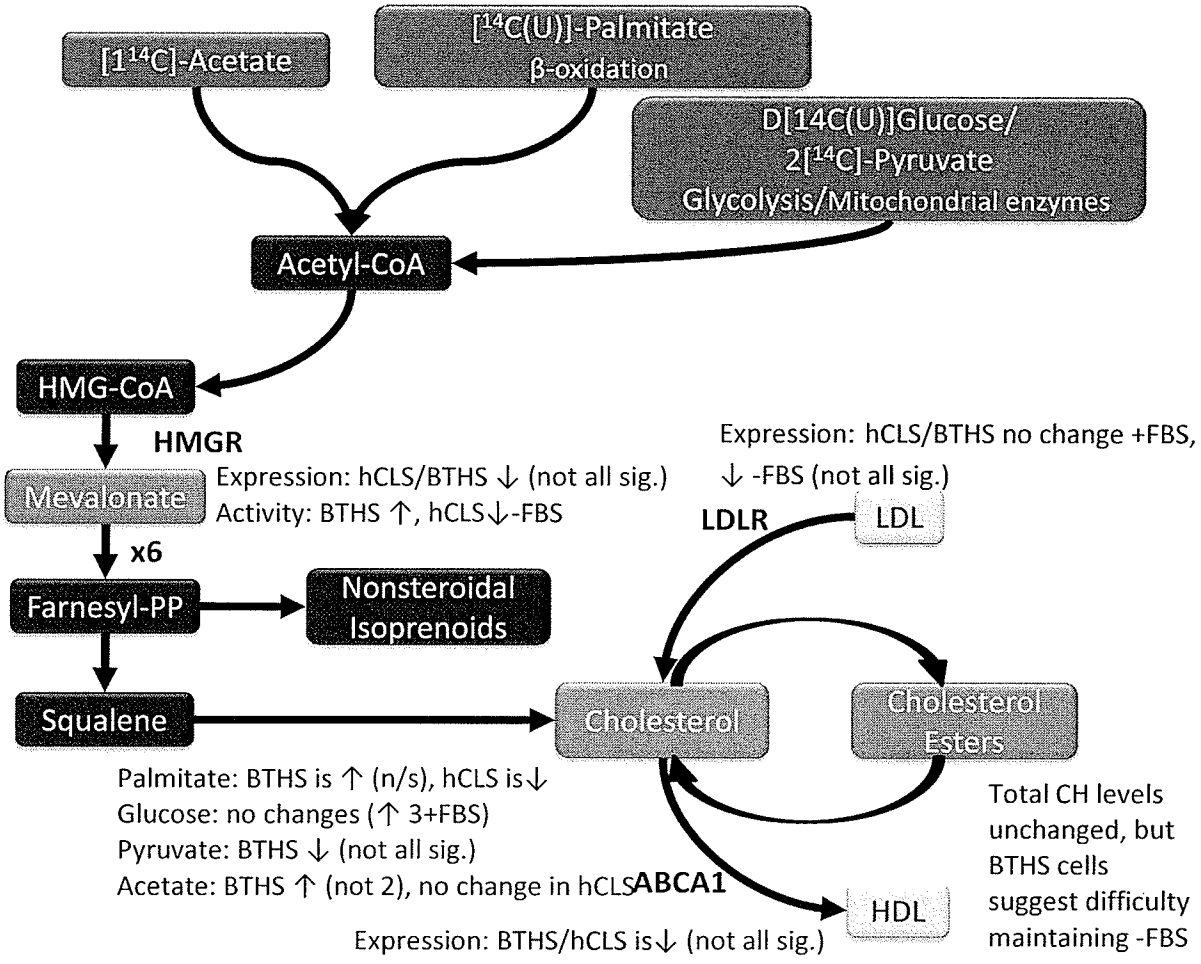

Figure 10-1: Summary of CH metabolism in BTHS lymphoblasts and hCLS knock down in HeLa cells

Three different sources of radiolabeled carbon were used to investigate the role of mitochondrial metabolism on CH biosynthesis. The biosynthesis of CH was further studied by looking at the expression and activity of HMGR, the rate limiting step in CH synthesis, as well as the expression of the major CH transporters for uptake, LDLR, and export, ABCA1.

Knock down of hCLS1 and hence CL synthesis reduced CH synthesis from [^{14}C(U)]palmitate. This effect was most pronounced under the condition in which CH synthesis needs to be up regulated, during growth in serum free medium (Fogelman *et al.*, 1977). (**Figure 10-1**). Thus, CL synthesis is required to support human CH biosynthesis from [^{14}C(U)]palmitate under stress conditions in HeLa cells. No significant changes were observed in the total CH levels of either the BTHS lymphoblasts or the hCLS1 knockdown cells. However, in the BTHS cells a subtle difficulty in maintaining CH levels upon removal of serum was observed. A greater difference in total CH levels may have been obtained if we had depleted the cells' internal CH stores before incubating them in the experimental conditions, as internal stores were maintained at control levels prior to serum deprivation.

CH transport in the BTHS cells seems to be affected. Although the LDLR exhibited a decrease in the level of mRNA expression in TAZ1 cells in the absence of serum, this evidence does not suggest a cause for hypocholesterolemia. However, there was a decrease in ABCA1 mRNA expression, a result that could be responsible for the decreased serum CH in BTHS. Further study into the function of CH export in BTHS cells is warranted, as this observation was not supported by the hCLS cells, which exhibited a trend toward decreased ABCA1, but no statistically significant difference. This may indicate that a greater decrease in CL is required to significantly alter CH export.

The only PLs altered in hCLS HeLa cells, appeared to be the result of alterations in CL biosynthesis. The hCLS cells exhibited more changes in substrate incorporation into PA, PG and CL, in association with altered transcription of the corresponding enzymes

(**Figure 10-2**). Though an increase in CL was associated with cell cycle progression (Bergeron *et al.*, 1970), less than 20% of normal CL levels (Valianpour *et al.*, 2002a) may support DNA replication (Hauff *et al.*, 2009), suggesting that their ability to progress through the cell cycle is unaltered.

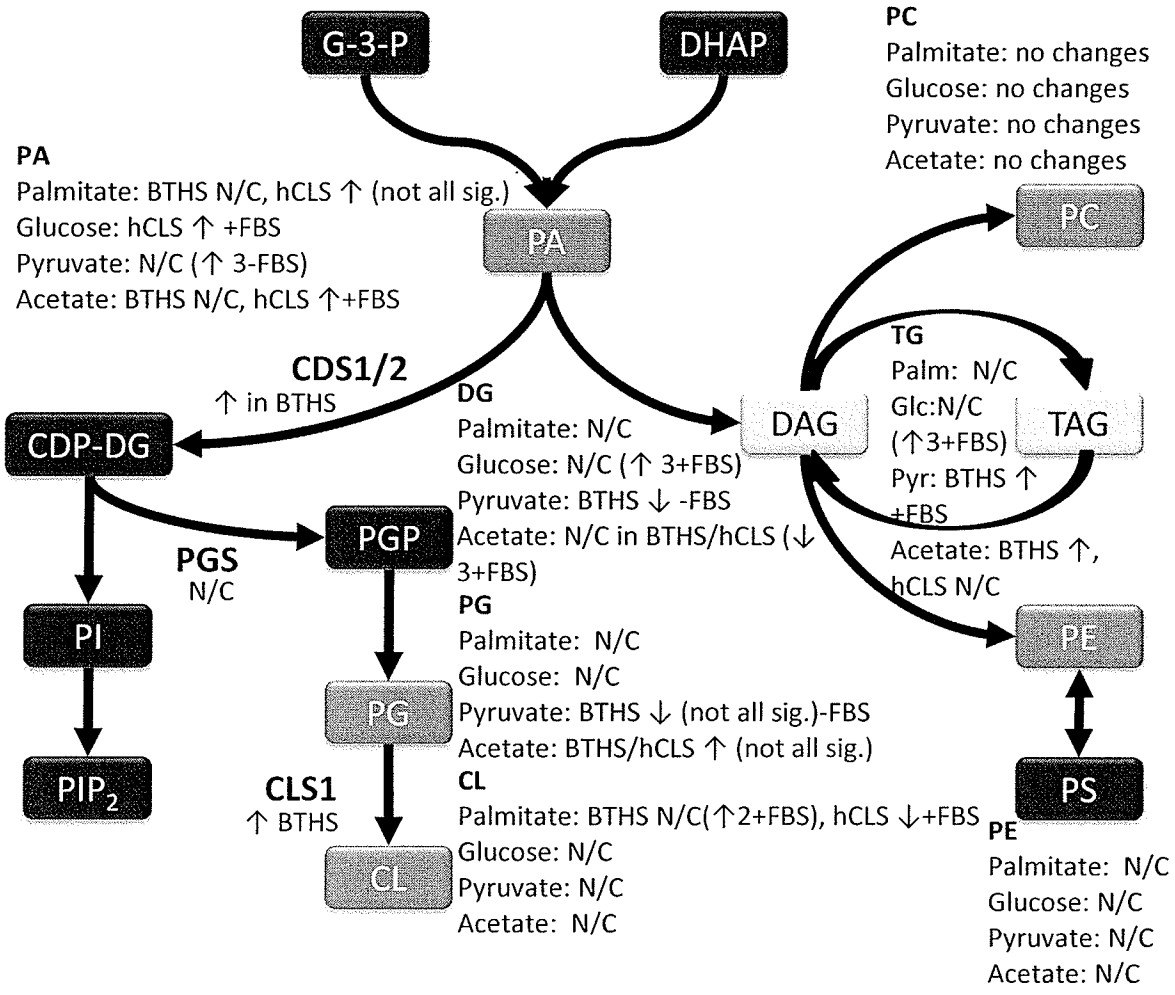

Figure 10-2: Summary of the synthesis of neutral and phospholipids in BTHS lymphoblasts and hCLS knock down HeLa cells

Four radiolabeled carbon sources were used to investigate the role of mitochondrial metabolism on PL synthesis. The major findings are summarized here. No changes were found in either PC or PE. Both TG and PA were generally increased. The ΔTAZ3 patients exhibited some changes in DG, while all BTHS cells exhibited a decrease in 2-[^{14}C]pyruvate incorporation into DG in the absence of serum. PG was increased with [1-^{14}C]acetate, but decreased with 2-[^{14}C]pyruvate, while CL was significantly decreased with [^{14}C(U)]palmitate utilization. (N/C) no change.

An interesting finding was that TG synthesis was increased in BTHS cells. This increased TG synthesis was associated with a likely increase in GRP78 activity, as determined by a reduction in GRP78 phosphorylation, and suggests that an ER stress response may be active in BTHS cells. Culturing BTHS cells in HEPES, a pH buffer, returned CH synthesis from [1-^{14}C]acetate to control levels (**Figure 10-2**), suggesting that an imbalance in pH was altering the metabolism of BTHS lymphoblasts. Much heterogeneity exists in BTHS patients with different mutationsand a correlation does not exist between the greater than 90 mutations described in BTHS and the severity of the disease.

Furthermore, some, but not all of the alterations identified in the BTHS lymphoblasts are likely due to the lack of mature CL, as evidenced by similarities in the hCLS knock down model. The absence of differences in the level of PG or PE production in either cell line (**Figure 10-2**) suggests that a global defect in lipid metabolism is not responsible for the hypocholesterolemia of BTHS.

Our work provides the preeminent characterization for the hypocholesterolemia observed in three BTHS patients and a model of CL inhibition, and highlights the need for further work. The impact of hypocholesterolemia in BTHS needs to be investigated more thoroughly, particularly in response to times of increased CH demand in developmental periods outside the "honeymoon period". We hypothesize that these are the periods when defects may become apparent, and potentially most detrimental to the patient.

10.1. Future Directions

Though we were able to characterize many of the biochemical alterations associated with the synthesis and transport of CH, and lipids, we were unable to identify the cause of these modifications. Thus, much work is still required before we fully understand the complex mechanism of hypocholesterolemia in BTHS.

Due to the close association between immunity and CH [reviewed in (Ravnskov, 2003)], it seems likely that the neutropenia frequently observed in BTHS, is either precipitated by the hypocholesterolemia, or may be further impaired by the presence of hypocholesterolemia. It may be particularly important, given our results, which revealed a difficulty in BTHS cells to respond to increased CH demand. Further investigation into the cause and effect of this relationship is highly warranted.

As mentioned, it is interesting that CH synthesis in BTHS appeared to be increased under basal conditions, rather than decreased, as one might expect. A global defect in mitochondrial processing of carbon intermediates was not identified; however our results indicated that further investigation into the functioning of the pyruvate transporter and PDC is defensible. In the absence of a mitochondrial defect in processing, our results also suggest a possible defect in trafficking of CH. Within this study, we have made a preliminary effort to identify alterations in the uptake and export of CH. While LDLR results were inconclusive, ABCA1 appeared decreased in BTHS cells, suggesting that a deficiency in CH export occured in BTHS. It is, therefore, important to investigate the function of these transporters in BTHS cells to further identify their role in the hypocholesterolemia. Furthermore, the lack of CH export anticipated by the work within may be a response to other alterations in CH metabolism. It is possible that the CH

produced, along with the excess TG, is being sequestered in lipid droplets, causing the regulatory machinery to respond as though a hypocholesterolemic state exists in these cells. However, this effect would likely be better studied in a cell line more conducive to CH storage, as lymphoblasts don't produce significant amounts of CE (Gottfried, 1967). Fluorescent staining with the lipid stain, Oil Red O or lipid analogue, BODIPY, would allow us to identify any qualitative or large quantitative changes in lipid droplet formation. Alternatively, processing of the LDL receptor-CH complex may be altered in BTHS, affecting the ability of CH to enter the ER and negatively regulate CH synthesis. This process is known to require an acidic environment in the lysosome (Sugii *et al.*, 2003; Soccio *et al.*, 2004; Radhakrishnan *et al.*, 2008). If the cytosol is more acidic than normal conditions allow, as our Hepes experiments suggest, then the appropriate functioning of the lysosome may be hindered. In this way, CH synthesis would be increased, though LDL uptake is maintained or increased, causing a low serum cholesterol effect (Davalos *et al.*, 2006). Thus, trafficking in the BTHS cells may be altered; fluorescent labeling of LDL particles would allow the progress of CH to be followed through the cell. However, one study demonstrated that late endosomal lysophosphatidic acid, required for appropriate CH trafficking, is unaltered in BTHS (Hullin-Matsuda *et al.*, 2007). Alternatively, we have described a decrease in the mRNA expression of ABCA1. However, that is not a direct indication of decreased CH export in BTHS. An inhibition in protein degradation could make up for the lack of *de novo* expression of ABCA1. Similar transport studies could be examined for CH export in BTHS cells.

Accumulation of TG in BTHS cells, combined with the response to pH buffer, HEPES, may suggest that BTHS activates the UPR. If this were the case, it would suggest a stress related alteration in the cellular metabolism. Further investigation of this pathway in the BTHS lymphoblasts may lead to further insights into BTHS.

As the isoprenoid pathway is closely linked to CH biosynthesis, it is possible that this pathway is also affected in BTHS [reviewed in (Houten *et al.*, 2003)]. Further investigation into the synthesis and the functional activity of the isoprenoid pathway in BTHS may be warranted. It is unlikely that the increased activity we see in HMGR results in an excess of farnesol and/or geranylgeraniol, as excesses exhibit an inhibition of both PC synthesis and cell cycle progression [reviewed in (Houten *et al.*, 2003)]. In this study, we established that neither PC synthesis, nor cell cycle progression were inhibited in BTHS. Other alterations in the isoprenoid processing may, however, result in the increased utilization of CH synthetic intermediates for isoprenoid synthesis. If this were the case, fewer resources would be available for CH synthesis, and an increase in HMGR activity would not result in an increase in CH production. We observed that the BTHS cells often had higher baseline activity in CH processing, but never exhibited an increase in the total CH, therefore potential for this mechanism exists. However, many of the radiolabeled precursors exhibited an increase in CH production. This suggests that there is less sequestering of substrates for other products and more turn over in the CH product. One aspect we were unable to fully examine in this cell model was the potential for an increased turn over in CH. A series of studies looking at the effects of polyunsaturated fatty acids (PUFA) suggest that an increase in LA species in PC, as was observed in one BTHS study (Schlame *et al.*, 2003), can lead to the ability of HDL

particles to accomodate more CE due to a reduction in stearic inhibition. In addition, intake of PUFAs increased the rate of bile synthesis and excretion [reviwed in (Paul *et al.*, 1980)]. These two conditions would lead to a decrease in circulating CH that could be exacerbated by the dietary supplementation of BTHS with LA, which is sometimes prescribed to increase LA_4-CL levels (Valianpour *et al.*, 2003).

There are many potential deficiencies in our cellular model, not the least of which is fact that lymphoblasts are neither the major source of CH in the body, nor are they a steroidogenic tissue. Clinical studies by Spencer *et al.* are helping to better characterize the clinical responses to tafazzin mutations. One study identified LDL particles as the predominant deficiency, suggesting that the major defect in CH would be better studied in a hepatocyte model (Spencer *et al.*, 2006).

This work has raised many interesting questions regarding the processing of CH in the BTHS cell and thus, much work remains to be explored. As the first systematic characterization of lipid biochemistry in BTHS and the only attempt to determine the cause of hypocholesterolemia reported in BTHS, this work represents an important contribution to understanding BTHS and has broad implications for how CH and CL are related.

Appendix

Appendix I

TLC plates were scraped according to the following schematics that were obtained by running standards on separate TLC plates, then aligning the origins to determine the relative mobility on the plates.

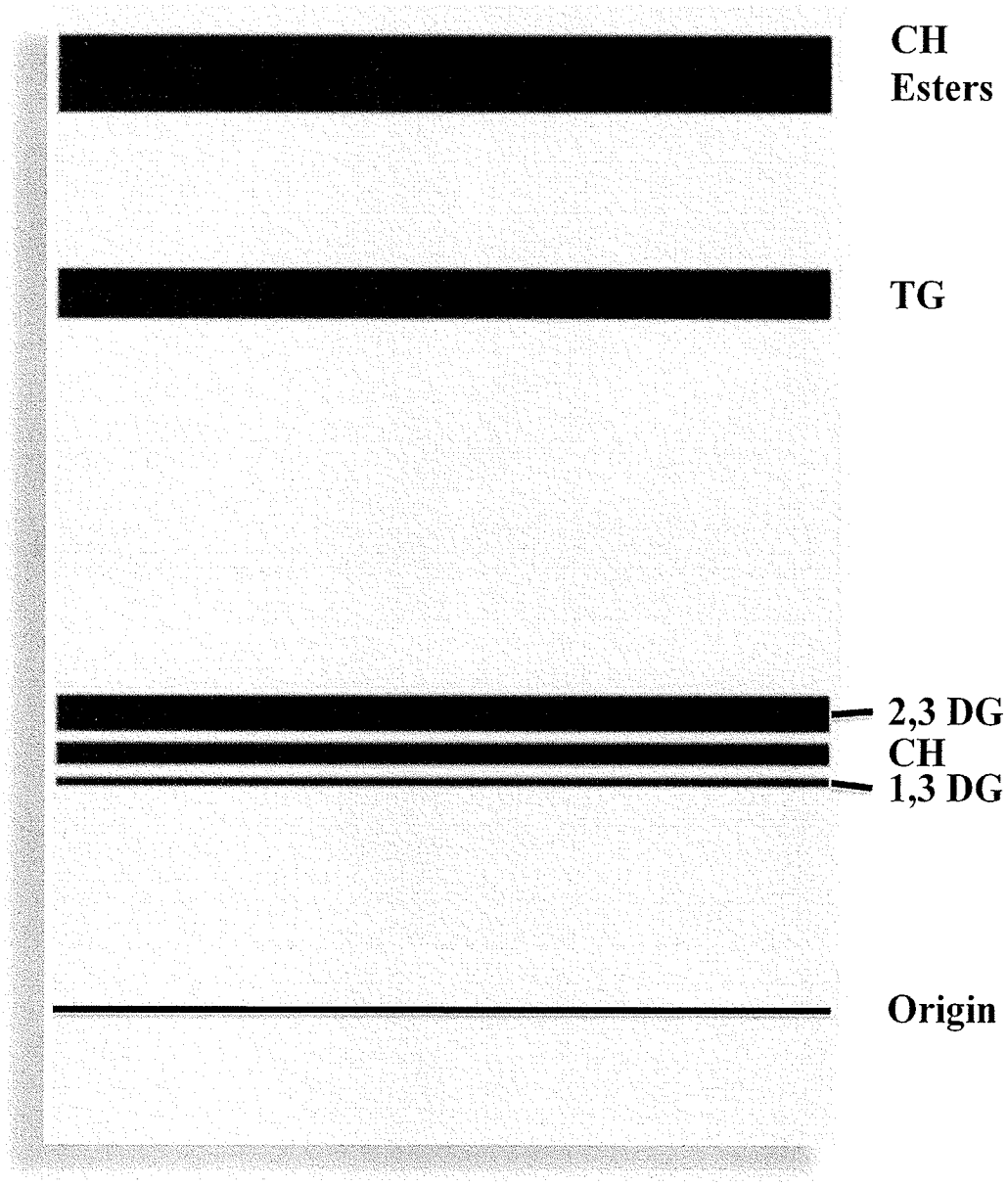

Figure AI- 1: TLC for Neutral lipids

One dimensional TLC plates were spotted in neighbouring lanes with standards identified, CH, DG, TG and CEs and run in Hexanes:Diethyl ether:glacial acetic acid (70:30:2, v/v) for approximately an hour. The standards were visualized with iodine and the resulting schema identified the relative mobility of each lipid. The close proximity of DG with CH mandated the addition of standard to every sample run.

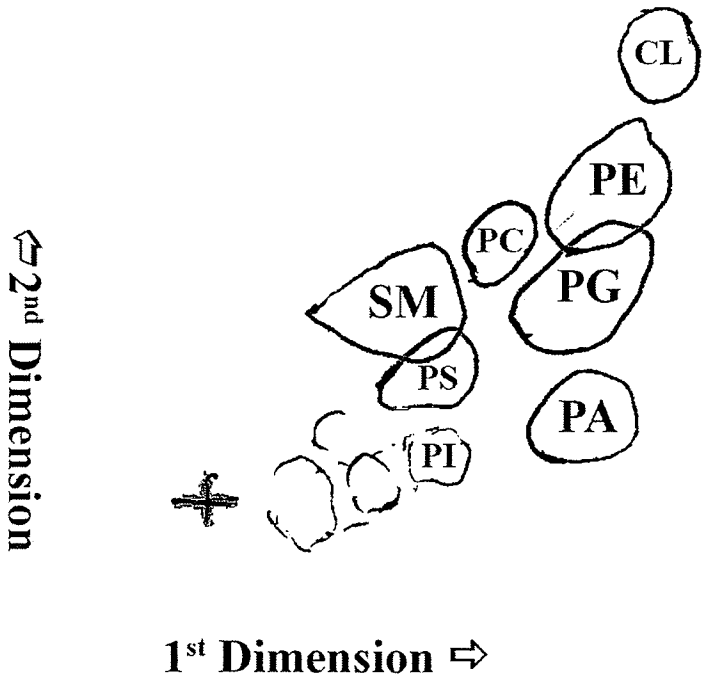

Figure AI- 2: 2D TLC of polar lipids.

$10cm^2$ plates were spotted with individual lipids identified, CL (cardiolipin), PE (phosphatidylethanolamine), PG (phosphatidylglycerol), PA (phosphatidic acid), PC (phosphatidylcholine), SM (sphingomyelin), PS (phosphatidylserine), and PI (phosphatidylinositol) and run in chloroform:methanol:NH_4OH:H_2O (70:30:2:3, v/v) for up to an hour. The plates were left to dry for up to 3 h at room temperature, rotated 90° counterclockwise, and developed for 15-20 min in the second dimension, chloroform:methanol:water (65:35:5, v/v). After exposure to iodine to visualize the lipids, the origins of each plate were aligned, and the relative mobility of each mapped. The close proximity of the lipids to each other warranted the addition of standard to each sample.

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