CHARACTERISATION OF MONOLYSOCARDIOLIPIN ACYLTRANSFERASE ACTIVITY

by

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ABSTRACT

Cardiolipin (CL) is a unique phospholipid synthesised exclusively in the mitochondria where it is also mainly localised. CL has been found to play an important role in the activation of several mitochondrial enzymes. Studies have indicated that the enzymes that synthesise CL may not be selective for the acyl groups present on CL's substrates. This implied that there must be specific de-acylation followed by re-acylation, or remodelling, of CL. Evidence is growing that not only the presence of CL, but a specific molecular species composition is important for the activation of mitochondrial enzymes.

Monolyso-CL acyltransferase is the enzyme responsible for re-acylating monolyso-CL to CL once a phospholipase A has removed one of the fatty acid tails. Because of CL's role in activating enzymes involved in energy metabolism, we investigated the effect of insulin on monolyso-CL acyltransferase activity and the composition of CL acyl groups. We hypothesised that some of the alterations in mitochondrial function may be due to the alteration in CL remodelling activity. Sprague-Dawley (SD) rats were treated with insulin (i.p. 12 U/rat) for 5 or 26 days. The hearts were then isolated and the mitochondrial fractions were collected. These mitochondria were assayed for monolyso-CL acyltransferase activity. In addition, streptozotocin-induced, diabetic SD rats were injected with saline for 5 days and the heart mitochondrial monolyso-CL acyltransferase activity was measured. The CL molecular species was examined using two-dimensional thin-layer chromatography and gas chromatography (GC). Both hypo- and hyperinsulinemic rats showed no significant change in monolyso-CL acyltransferase activity. GC analysis showed no significant change in the relative proportions of CL acyl molecular species in the rats with altered insulin levels.

The characteristics of monolyso-CL acyltransferase activity from rat heart mitochondria were studied. The effect of other non-labelled acyl-coenzyme A (CoA) species on the incorporation of either [1-^{14}C] linoleoyl-CoA or [1-^{14}C] oleoyl-CoA into CL was measured. All acyl-CoA's used in the study were able to interfere with radiolabel incorporation with one exception. Myristoyl-CoA did not affect the incorporation of [1-^{14}C] linoleoyl-CoA, but did affect the incorporation of [1-^{14}C] oleoyl-CoA. The effect of binding proteins on monolyso-CL acyltransferase activity was also measured. Fatty acid binding protein, lipid binding protein, and acyl-CoA binding protein isolated from various tissues were added to the monolyso-CL acyltransferase assays and were found to not affect monolyso-CL acyltransferase activity. The requirement of acyl-CoA as a substrate of monolyso-CL acyltransferase activity was examined. Mitochondrial samples were assayed with [1-^{14}C] linoleoyl-CoA, [1-^{14}C] oleoyl-CoA, [1-^{14}C] linoleic acid, or [1-^{14}C] oleic acid. Incorporation of radiolabel into CL only occurred when an acyl-CoA was present. Addition of ATP and CoA to [1-^{14}C] linoleic acid, or [1-^{14}C] oleic acid resulted in some incorporation.

In other experiments, the activity of monolyso-CL acyltransferase was localised using rat liver mitochondria. Since CL has been found to be synthesised exclusively in the mitochondrial inner membrane, we hypothesised the monolyso-CL acyltransferase activity would also be localised exclusively to the inner membrane. Mitochondria were isolated and the outer and inner membranes were separated using digitonin and differential centrifugation. Each fraction was then assayed for monolyso-CL acyltransferase activity. Intact mitochondria were also treated with trypsin to assess the amount of latency. Both the results of digitonin and trypsin treatment indicate that monolyso-CL acyltransferase activity can occur on the outer membrane which has some interesting implications.

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ABBREVIATIONS

$\times g_{max}$	maximal relative centrifugal force
ACBP	acyl-CoA binding protein
aLBP	adipocyte lipid binding protein
AT	acyltransferase
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CDP	cytidine 5'-diphosphate
CL	cardiolipin
CMP	cytidine 5'-monophosphate
CoA	coenzyme A
CPT-I	carnitine palmitoyl transferase I
CPT-II	carnitine palmitoyl transferase II
CTP	cytidine 5'-triphosphate
ddH_2O	ultra-filtered water
DG	diacylglycerol
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
FABP	fatty acid binding protein
g	gram
GC	gas chromatography
HDL	high density lipoprotein
HEPES	
hFABP	heart fatty acid binding protein
I.D.	internal diameter
kLBP	keratinocyte lipid binding protein
LBP	lipid binding protein
LDL	low density lipoprotein
mg	milligram
ml	millilitre
MLCL	monolysocardiolipin
mtDNA	mitochondrial deoxyribonucleic acid
nm	nanometer
NMR	nuclear magnetic resonance
O.D.	outer diameter
PA	phosphatidic acid
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PG	phosphatidyl glycerol
PGP	phosphatidyl glycerol phosphate
PLA_2	phospholipase A_2
PMSF	phenylmethylsulphonyl fluoride
PS	phosphatidyl serine

ROS	reactive oxygen species
SEM	standard error on the mean
Solvent A	chloroform:methanol (2:1 v/v)
T_3	triiodothyronine
T_4	tetraiodothyronine
TLC	thin-layer chromatography
TUP	theoretical upper phase ($CHCl_3$:CH_3OH:0.9% NaCl; 2:48:47 v/v/v)
VLDL	very low density lipoprotein

CHAPTER 1: INTRODUCTION

Lipids form a large group of molecules unified by the feature that they all contain long acyl tails linked to a hydrophilic backbone. This group includes triglycerides, glycerophospholipids, sphingolipids, and cholesterol esters. Triglycerides function as an energy storage vehicle in the form of fatty acids. Glycerophospholipids, sphingolipids, and cholesterol esters are important structural components of the cellular membrane.

As first predicted by Gorter and Grendel (1925), membrane lipids are arranged in a bilayer with the hydrophilic heads facing the exterior of the membrane and the hydrophobic acyl tails associating with each other on the interior of the membrane. Not only are biological membranes made of lipids, they also contain a plethora of imbedded proteins. This was proposed by Singer and Nicholson (1972) in their fluid mosaic model. This means that not only are lipids playing a role in the structure of cellular membranes, they serve as a medium to which membrane proteins may integrate. This presents a potential for interactions between membrane lipids and proteins.

There are many examples of membrane lipids that do more than simply act as inert substances forming a selective barrier between two volumes. Lipids are active in signal transduction pathways. Phosphatidyl inositol-4,5-bisphosphate (PIP_2) can be cleaved by phospholipase C to form inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) that act in cell signalling pathways. The arachidonyl tails of lipids serve as a source for synthesis of prostaglandins, thromboxanes, and other eicosanoids. Sphingolipids act as sites for biological recognition such as those that determine blood type.

Diversity of glycerophospholipids

Biological membranes are made up of a diverse group of lipids. The glycerophospholipids alone include phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl choline (PC), phosphatidyl inositol (PI), phosphatidyl glycerol (PG), and cardiolipin (CL). The generation of diversity requires a wide range of enzymes. The biosynthesis of glycerophospholipids involves multi-step reactions. These synthetic pathways were initially worked out by Kennedy and Kanfer (Kanfer and Kennedy, 1963; Kanfer and Kennedy, 1964) and are summarised in Figure 1.1.

Each glycerophospholipid can be categorised according to various properties. For example, the phospholipids can be distinguished by charge. Anionic phospholipids include PS, PI, PG, and CL. Zwitterionic phospholipids include PE and PC. Phospholipids are not only chemically diverse, they also distribute within each cell in a diverse manner. For example, in rat liver, the most abundant lipid in all membranes is PC. It makes up 44% of the total phospholipids in mitochondria, 60% in endoplasmic reticulum, 48% in lysosomes, 51% in Golgi, and 40% in plasma membrane (Daum and Vance, 1997). Then next most abundant phospholipid is PE, which makes up 34% of the total lipids in mitochondria, 23% in endoplasmic reticulum, 17% in lysosomes, 21% in Golgi, and 24% in plasma membrane. PI ranges from making up 5% of the total lipids in mitochondria to 12% in Golgi. Cardiolipin makes up about 14% of the total lipids in mitochondria.

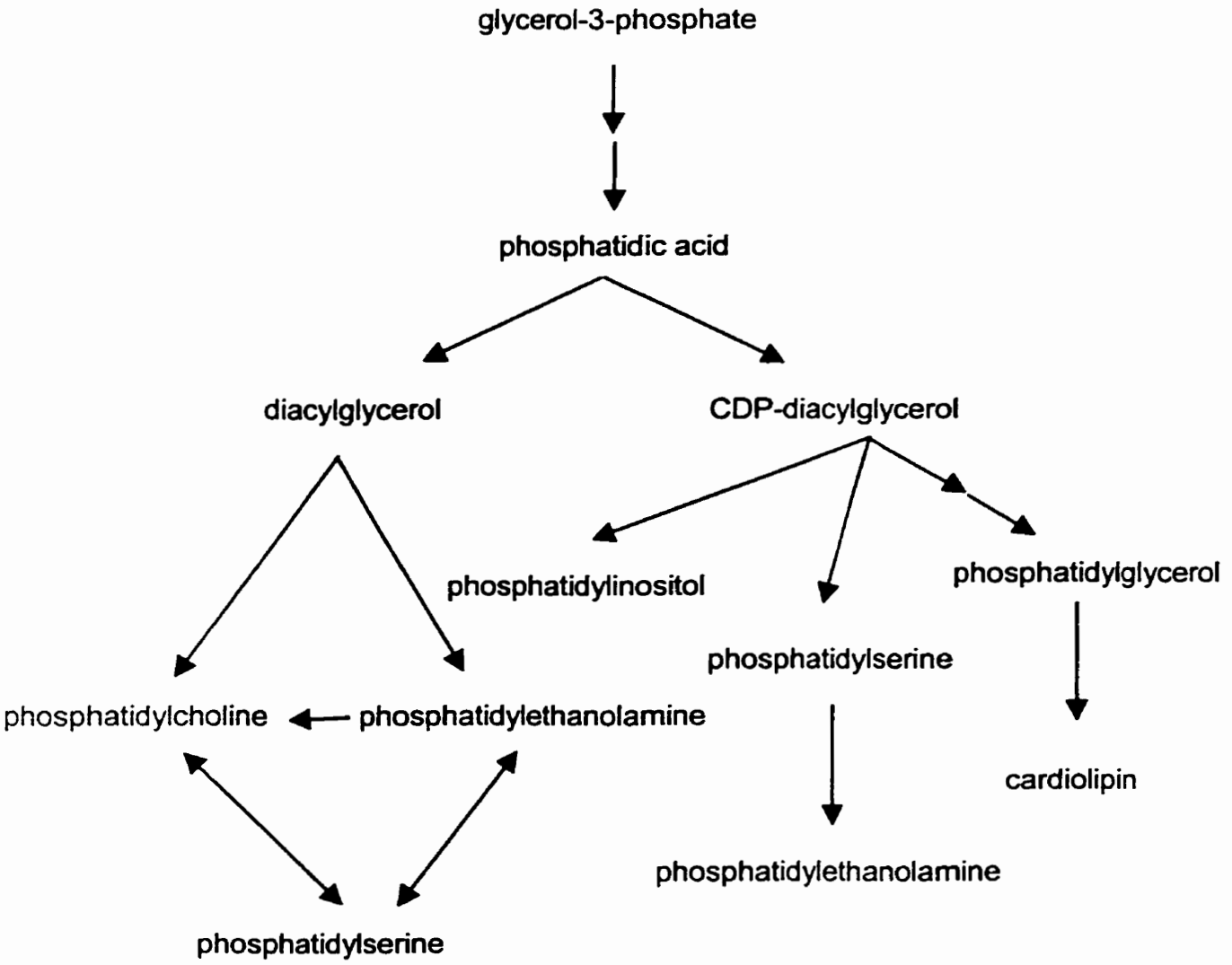

Figure 1.1: Summary of Kennedy pathways in phospholipid biosynthesis.

Lipids by no means distribute homogenously. Phospholipids also show diversity in their transverse distribution in the lipid bilayer. This transbilayer lipid asymmetry is present in most membranes (Cullis *et al.*, 1996). In human erythrocyte plasma membranes approximately 80% of the PC is on the outer monolayer while only 20% is on the inner monolayer. Approximately 18% of the PE is on the outer monolayer while 82% is on the inner monolayer. PS is almost exclusively distributed on the inner monolayer (Cullis *et al.*, 1996).

This diversity in phospholipids with respect to chemical structure, tissue distribution, and transmembrane distribution suggests that each phospholipid plays specific functions or confers specifc properties to biological membranes. The differences in distributions of phospholipids suggest that the biosynthetic rates are regulated. It also suggests there must be mechanisms that determine the amount of a specific lipid in a particular membrane.

The following discussion will mainly focus on cardiolipin (CL), a unique phospholipid localised almost exclusively in the mitochondria. The biosynthesis and remodelling will be discussed as well as possible roles of CL in the cell.

Localisation of cardiolipin

CL was first discovered by Pangborn (1942) from isolated lipid extracts of beef heart. In eukaryotes, CL has been almost exclusively found in mitochondria. Therefore, tissues with high concentrations of mitochondria such as heart, liver, and skeletal muscle would be expected to contain higher amounts of CL. In rat heart, CL has been found to make up about 15% of total lipid mass (Poorthius *et al.*, 1976).

CL has been putatively thought to be localised exclusively to the inner mitochondrial membrane (Cullis *et al.*, 1996). Like other membrane lipids, CL has displayed an asymmetrical distribution between the inner and outer leaflet of the inner mitochondrial membrane. Studies in rat liver, bovine heart, and pig heart showed that approximately 75-90% of CL is localised on the inner leaflet of the inner mitochondrial membrane (Harb *et al.*, 1981; Krebs *et al.*, 1979; Nilsson and Dallner, 1977). However, a more recent study showed that only 43% of the total CL was localised to the inner leaflet of the mitochondrial inner membranes of rat liver, quiescent L1210 cells, and respiring yeast (Petit *et al.*, 1994). Studies of yeast mitochondria showed that CL distribution across the inner membrane changed depending on whether the growth was dependent on fermentation or on gluconeogenesis (Gallet *et al.*, 1997). When yeast were exposed to a growth condition more dependent on oxidative phosphorylation, the distribution of CL on the mitochondrial inner membrane was altered from 80% on the outer leaflet to only 30%. This suggested that the transverse distribution of CL on the mitochondrial inner membrane was specifically altered depending on the reliance on mitochondria for ATP generation.

CL is tightly associated with many inner mitochondrial membrane proteins such as cytochrome *c* oxidase (Vik *et al.*, 1981). This tight association with proteins would be expected to determine the distribution of CL along the mitochondrial inner membrane. In fact, the distribution of CL along the plane of the mitochondrial membrane has been shown to display heterogeneity (Brdiczka *et al.*, 1974). Subfractionation of mitochondrial inner membranes using density gradient centrifugation showed that CL is concentrated in subfractions which contain relatively more cytochrome aa_3 than any other fraction. This

demonstrated that CL is more concentrated in cristae membrane rather than the inner boundary membrane.

The thought that CL was exclusively localised on the mitochondrial inner membrane has been challenged by Hovius and co-workers (1990). Using digitonin to separate the mitochondrial inner and outer membranes they concluded that 23% of total mitochondrial CL is on the outer membrane. The fact that CL was not exclusively localised on the inner membrane is further supported by studies that suggested mitochondrial contact sites between the inner and outer membranes were enriched in CL (Ardail *et al.*, 1990; Simbeni *et al.*, 1991). These studies showed that 27% of the lipids in the contact sites are CL. It is believed that CL and PE which constitute about 45% of the lipids in the contact sites can form a nonbilayer arrangement known as hexagonal (H_{II}) phase as proposed by Cullis and co-workers (1978). These H_{II} phase lipids could serve as channels that could be involved in import of proteins and phospholipids into the mitochondria.

Further studies into the asymmetry on the mitochondrial outer membrane using PLA_2 digestion suggested that 100% of the outer membrane CL was on the outer leaflet (Hovius *et al.*, 1993). This conclusion was based on the assumption that CL could not quickly transverse between the two monolayers. However, studies of yeast mitochondria suggested that newly synthesised CL, at least in the inner membrane, could rapidly be translocated to the outer leaflet (Gallet *et al.*, 1997). This places doubt to the conclusion that CL on the mitochondrial outer membrane was exclusively localised to the outer monolayer.

CL is only synthesised in the mitochondria. The mechanism by which CL is transported between lipid monolayers and membranes is unknown. The studies cited above indicate that there must be a specific mechanism controlling the localisation of CL. CL has recently been

found to be a normal component in human plasma lipoproteins (Deguchi *et al.*, 2000). Very low density lipoprotein (VLDL), high density lipoprotein (HDL), and low density lipoprotein (LDL) were all found to contain CL representing 0.42%, 0.40%, and 0.77% of the relative lipid content in each lipoprotein, respectively. This implied that CL could be exported from the mitochondria.

Cardiolipin synthesis

CL is one of only a few lipids that are synthesised in the mitochondria (Daum and Vance, 1997). Most of the other lipids in the mitochondria are imported from the cytosol. The CL biosynthetic pathway was first proposed by Kennedy and co-workers (Kiyasu *et al.*, 1963) and is diagrammed in Figure 1.2. The first step involves the conversion of CTP and phosphatidic acid (PA) to CDP-diacylglycerol (CDP-DG) by CTP:PA cytidylyltransferase. The second step involves the condensation of glycerol-3-phosphate and CDP-DG to phosphatidyl glycerol phosphate (PGP) by PGP synthase. This is followed by a rapid dephosphorylation to phosphatidyl glycerol by PGP phosphatase (Greenberg and Lopes, 1996). Finally, in eukaryotes only, CL synthase catalyses the synthesis of CL from CDP-DG and PG (Hostetler *et al.*, 1971). In prokaryotes, this last step differs in that CL synthase does not use CDP-DG as a substrate, but instead catalyses the formation of CL from two molecules of PG (Hirschberg and Kennedy, 1972).

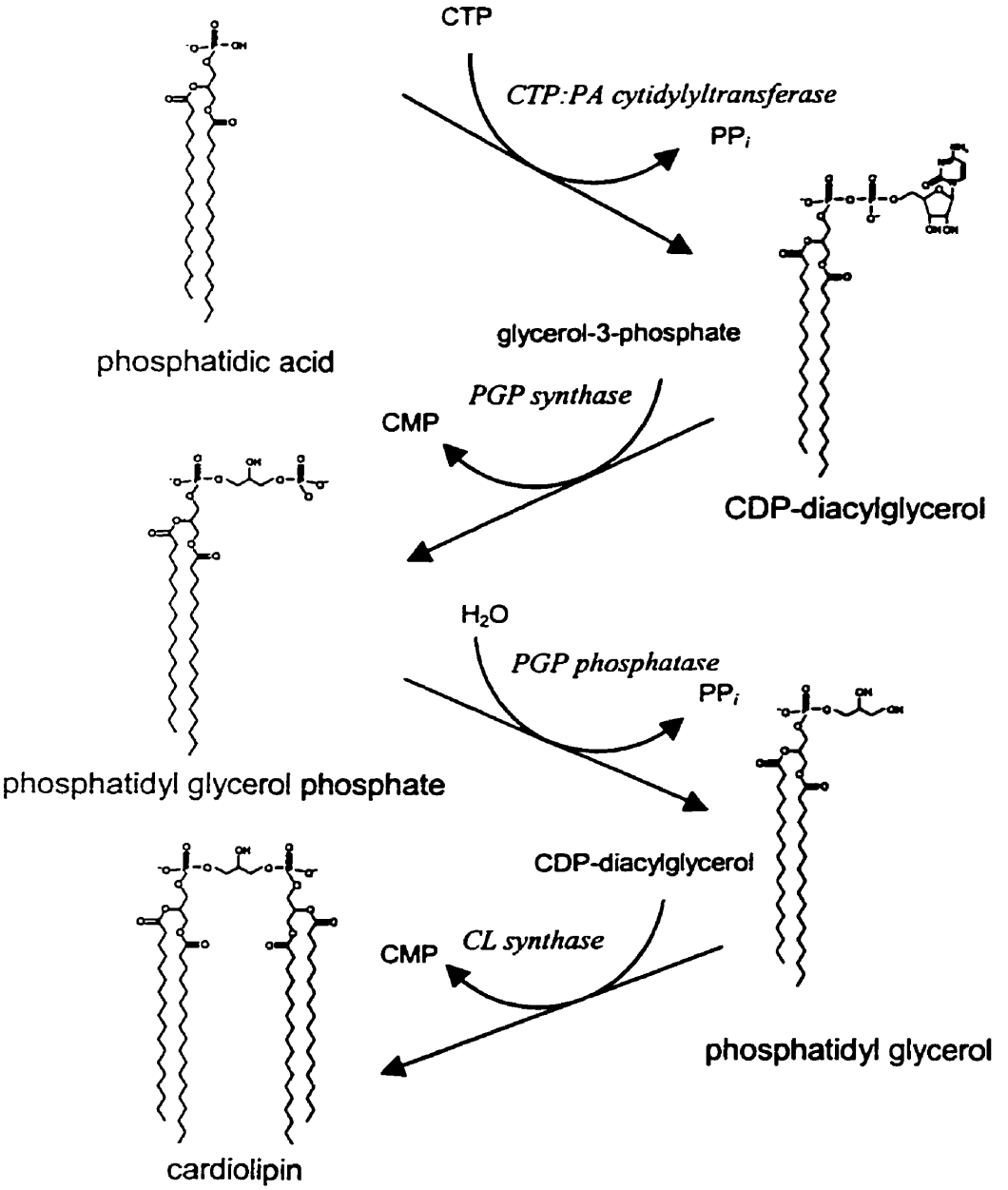

Figure 1.2: Biosynthetic pathway of cardiolipin in eukaryotes. The enzyme that catalyses each synthetic step is italicised.

In the synthesis of CL the conversion of PA to CDP-DG appeared to be the rate limiting step (Hatch, 1994). In fact, the availability of CTP for this reaction may be what regulates the amount of CL synthesis (Hatch and McClarty, 1996). The expression of CL synthase also appears to be regulated by mitochondrial developmental factors with the greatest expression in yeast during the stationary phase (Jiang *et al.*, 1999).

Role of cardiolipin

The presence of CL appears to be a common feature to aerobic organisms. In eukaryotes, it is present in all mitochondria. In prokaryotes, CL has been commonly found in aerobes. The presence of mitochondria in eukaryotes is believed to have arisen from an engulfment of an aerobic prokaryote early in evolution which brought about a symbiotic relationship. Therefore the presence of CL in aerobic prokaryotes may have developed due to an evolutionary advantage for oxidative phosphorylation.

CL appears to play an important role in the stability and function of integral proteins of energy-transducing membranes (McAuley *et al.*, 1999). It has been found to play a role in the activity of several mitochondrial enzymes such as cytochrome *c* oxidase (Sedlák and Robinson, 1999; Vik *et al.*, 1981), carnitine palmitoyltransferase (Fiol and Bieber, 1984), creatine phosphokinase (Muller *et al.*, 1985), pyruvate translocator (Hutson *et al.*, 1990), tricarboxylate transporter (Kaplan *et al.*, 1990), NADH dehydrogenase (Awasthi, *et al.*, 1969), ATP synthase (Eble *et al.*, 1990), cytochrome bc_1 complex (Gomez and Robinson, 1999), adenine nucleotide carrier (Beyer and Klingenberg, 1985), and glycerol-3-phosphate dehydrogenase (Belezani and Janesik, 1989).

For at least some of the above enzymes, the removal of tightly associated CL molecules reversibly inactivated the enzyme. Although not proven, this therefore suggested that CL plays an important role in energy production. Perhaps the most well characterised interaction of the above enzymes is the interaction of CL with cytochrome *c* oxidase. CL is believed to affect the catalytic activity principally through electrostatic and conformational interactions. Cytochrome *c* oxidase was found to have four to five CL molecules that are more tightly bound to the enzyme than all other lipids surrounding it (Sedlák and Robinson, 1999). When cytochrome *c* oxidase was completely delipidated, enzyme activity is lost (Vik *et al.*, 1981). This activity could be maximally restored with lysolipid and CL but not PC or PE. Activity could not be restored to the level prior to delipidation, which suggested that CL might play a role in the structural integrity of the enzyme as well as in its functionality.

Is this requirement for CL specific or does this interaction exist simply because CL is one of the more abundant lipids on the mitochondrial membrane? One study examined the specificity of mitochondrial ATP synthase (Eble *et al.*, 1990). Through ^{31}P nuclear magnetic resonance (NMR) spectroscopy, it was found that only CL was tightly bound to the enzyme and neither PC, PS, or PG could substitute.

In some cases, CL may serve as a target to localise an integral protein to the mitochondria. Phospholipase A hydrolysis of CL, but not PC or PE solubilised the NADH dehydrogenase on the mitochondrial membrane (Awasthi, *et al.*, 1969). This suggested that CL specifically integrated the enzyme to the mitochondrial membrane. CL has been crystallised as part of the enzyme complex of the photoreaction centre in the photosynthetic bacteria *Rhodobacter sphaeroides* (McAuley *et al.*, 1999). This showed that there was a combination of ionic and van der Waals interactions between CL and the enzyme and that

binding of CL occurred at residues that were conserved among species. As CL is localised mainly in the mitochondria, perhaps proteins that are specific for the mitochondrial membrane have evolved to specifically recognise CL. Studies into why CL is localised only to the mitochondrial membrane are incomplete at this time.

CL is also believed to be important in protein import into the mitochondria (Jiang *et al.*, 2000; Leenhouts *et al.*, 1996; Eilers *et al.*, 1989). This is supported by the finding that CL is highly enriched in the mitochondrial contact sites (Ardail *et al.*, 1990). These contact sites are the putative sites for protein and phospholipid import into the mitochondria (Schleyer and Neupert, 1985; Simbeni *et al.*, 1990).

In order to better define the role of CL, CL synthase was purified and cloned from *Sacchromyces cerevisiae* (Jiang *et al.*, 1997; Tuller *et al.*, 1998; Chang *et al.*, 1998b). Null mutants were generated which were deficient in CL. Based on previous studies, the absence of CL may have been expected to seriously affect functioning of the mitochondria. Surprisingly, the absence of CL appeared to have no appreciable effect on the viability of the cells. Perhaps no significant effects were seen because the elevated PG in these cells was a suitable substitute for CL in this case. A PGP synthase null mutant that lowers the levels of both PG and CL was found to be non-lethal. However, the yeast could not grow on glycerol-containing plates which was consistent with a defect in mitochondrial oxidative phosphorylation (Chang *et al.*, 1998a). The results of these findings suggested that CL may not be essential for life. However, CL appeared to affect the optimal activity of mitochondrial enzymes. Further studies into the CL synthase null mutants showed the impaired ability of the yeast to grow under elevated temperatures (40°C) (Jiang *et al.*, 1999; Koshkin and Greenberg, 2000). This impairment correlated with an impairment in oxidative

phosphorylation (Koshkin and Greenberg, 2000). This was characterised by a complete uncoupling between respiration and phosphorylation. The relationship between the absence of CL and the uncoupling of oxidative phosphorylation was hypothesised to be through the interaction between CL and membrane enzymes. Specifically, the absence of CL was thought to have modified the adenine nucleotide carrier such that the intermembrane space became leaky and thus had a decreased membrane potential (Koshkin and Greenberg, 2000). CL was also hypothesised to play a direct role in maintaining the membrane potential by both stabilising the membrane bilayer (Shibata *et al.*, 1994) and decreasing membrane fluidity (Yamauchi *et al.*, 1981). Whereas an increase in temperature in the absence of CL would have destabilised the mitochondrial membrane, resulting in the loss of the proton gradient.

Cardiolipin molecular species

Cardiolipin is unique in that it has four acyl tails instead of the two like all other glycerophospholipids. Molecular species refers to the specific acyl composition of a phospholipid. It is generally accepted that phospholipids have a saturated fatty acid at the *sn*-1 position and an unsaturated fatty acid at the *sn*-2 position (Schlame and Rüstow, 1990). CL is different in that 60-80% of the acyl tails tends to be linoleic acid (18:2). In other words, the *sn*-1 position(s) tends to have an unsaturated fatty acid. The proportions of molecular species of CL in rat liver mitochondria were found to be as follows: 57% were (18:2-18:2)-(18:2-18:2); 35% were (18:1-18:2)-(18:2-18:2); and 8% were (18:1-18:2)-(18:1-18:2) (Schlame *et al.*, 1993). A similar trend of high amounts of linoleic acid were found in many other tissues such as the hearts of humans, rats, and cattle; skeletal muscle of rats and

humans (Hoch, 1992). However not all tissues showed a high level of linoleic acid. For example, rat lung CL was found to contain 44% stearic acid (18:0) and only 15.2 % linoleic acid. Rat brain CL contained 38% oleic acid (18:1) and only 13.1 % linoleic acid (Hoch, 1992).

The molecular species of CL is also different in yeast. CL of *Saccharomyces cerevisiae* was found to contain 48% oleic acid, 48% palmitoleic acid (16:1), and 4% palmitic acid (16:0). The reason for near homogeneity of acyl tails in some mammalian tissues and the variability in molecular species between species is unknown.

Cardiolipin remodelling

The characterisation of the role of CL by the null mutants has thus far been done using yeast only. As discussed in the previous section, mammalian CL molecular species differs from yeast. Is there a specific reason for this difference? Perhaps the specific molecular species of CL seen in mammals plays an important role. The heart, liver, and skeletal muscles have been shown to have CL with high linoleoyl composition. Although not all tissues exhibit high amount of linoleic acid in CL, the ones that do are tissues that have high energy demands. Perhaps the preferred molecular species for CL is important for oxidative phosphorylation.

Studies have indicated that the enzymes that synthesise CL may not be selective for the acyl groups present on CL's substrates (Rüstow *et al.*, 1989). When the acyl groups of CL were examined, they differed from those of its precursors. In rat liver mitochondria, approximately 84% of the acyl tails of CL were found to be linoleic acid (18:2) with the next largest acyl group being oleic acid (18:1) at about 10% (Hostetler, 1982). In rat heart,

approximately 88.2% of CL acyl tails were found to be linoleic acid and about 7.7% were oleic acid (Ma *et al*, 1999). In the human atria, linoleic acid was found to make up about 67.6% of CL acyl tails and oleic acid was found to make up about 14.7% (Gloster and Harris, 1969). When compared to PG, one of the precursors in CL synthesis, it was found that in rat liver mitochondrial PG, only 20% of the tails were linoleic acid and about 21% were oleic acid (Hostetler, 1982). To get from a precursor that is relatively diverse in its acyl tails to an almost homogenous molecular species suggested that there must be a specific cycle of deacylation followed by reacylation of CL. This cycle is referred to as remodelling. Remodelling of glycerophospholipids was first proposed by Lands (1960). The remodelling pathway that is of interest to our studies was first hypothesised by Schlame and Rüstow (1990). This is diagrammed in Figure 1.3. This pathway involves first deacylation of CL by phospholipase A_2 followed by reacylation involving monolyso-CL acyltransferase and an acyl-CoA. Endogenous CL was found to not be deacylated by phospholipase A_2 (PLA_2) (de Winter *et al.*, 1987). Thus, the cycle was proposed to only occur with newly synthesised CL since exogenously added CL was shown to undergo remodelling (Schlame and Rüstow, 1990).

An alternative mechanism of remodelling could occur by transacylation. In this mechanism, rather than using acyl-CoA as a substrate, the reacylation could occur by direct transfer of the fatty acid from one phosphate to another. However, incubating [^{3}H]monolyso-CL with crude mitochondrial extract in the absence of acyl-CoA resulted in insignificant [^{3}H]CL formation (Ma *et al.*, 1999). Thus, this indicates that transacylation is an unlikely mechanism.

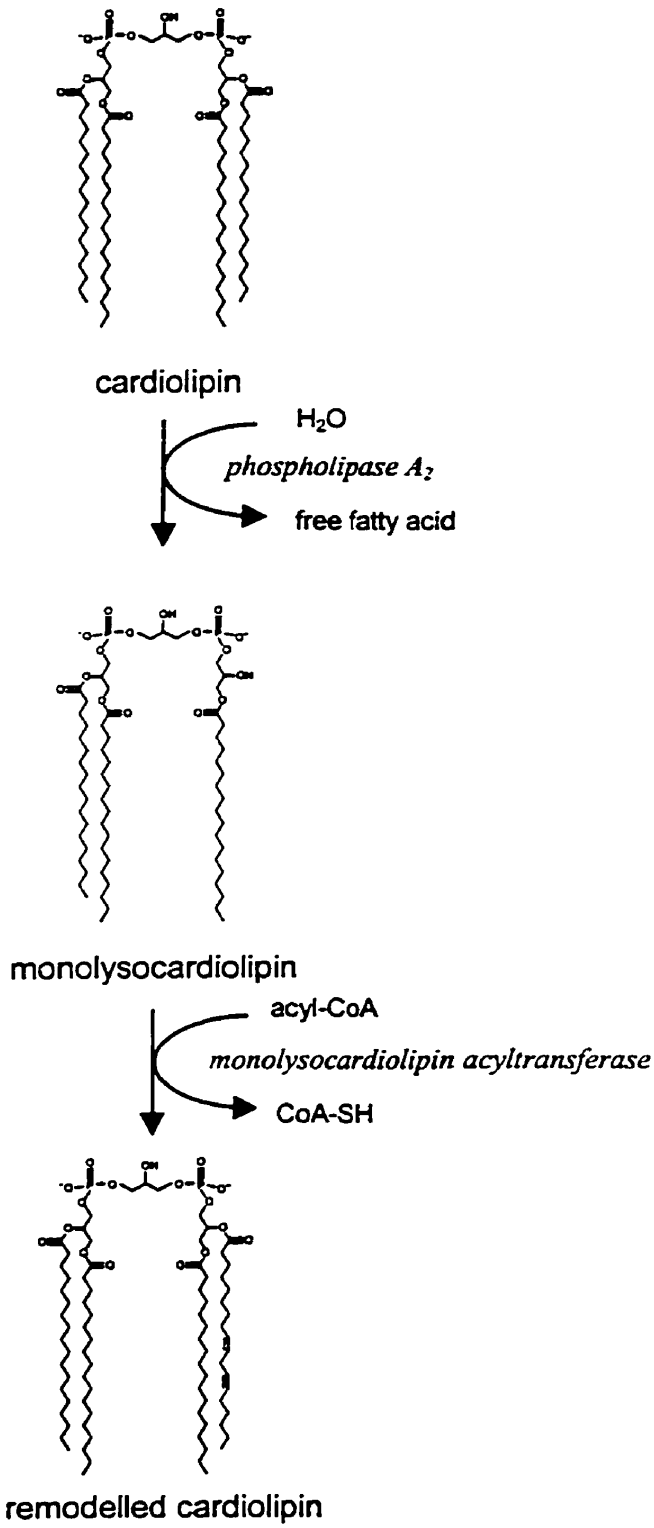

Figure 1.3: Proposed remodelling pathway for cardiolipin. Cardiolipin is first deacylated to monolyso-CL and then reacylated using the appropriate acyl CoA species as substrate. The enzyme that catalyses each synthetic step is italicised.

Evidence is growing that not only the presence of CL, but a specific molecular species composition is important for the activity of mitochondrial enzymes. When rats were fed a diet deficient in linoleic acid, the amount of linoleic acid acyl tails on cardiolipin decreased (Yamaoka *et al.*, 1990). These lipids were isolated from the rat and used *in vitro* to reconstitute cytochrome *c* oxidase. The linoleoyl-deficient CL was unable to activate the enzyme as well as CL with high linoleic acid content (Yamaoka-Koseki *et al.*, 1991). In a chinese hamster lung fibroblast cell line deficient in oxidative energy production, there was an alteration in CL molecular composition (Rusnak *et al.*, 1997). The levels of unsaturated fatty acids such as palmitolate, oleate, and linoleate were altered in these cells. However, contrary to what would have been expected based on the studies by Yamaoka-Koseki and co-workers (1991), the increase in linoleic acid in the CL of the deficient cells apparently did not help oxidative phosphorylation. There are likely other mechanisms to explain the deficiencies and perhaps one could speculate that the cell is increasing the level of unsaturated fatty acyl groups on CL in an attempt to improve functionality. Nonetheless, the remodelling activity in these cells was altered, thus again suggesting that specific CL molecular composition may play a role in bioenergetics.

Possible role of monolyso-CL acyltransferase in ageing

The cause of cellular decline in the ageing process is thought to be caused by mitochondrial decay (Hagen *et al.*, 1998). The reasons for a decline in mitochondrial function are not fully known. However, mitochondrial diseases such as mitochondrial myopathy have already been shown to correlate with mutations in mitochondrial DNA (mtDNA) (Holt *et al.*, 1988). Thus, it was postulated that the process of ageing is a

mitochondrial disease and is due to a lifelong, progressive accumulation of mutations in mtDNA (Linnane *et al.*, 1998). These mutations lead to a gradual decline in bioenergy capacity.

The cause of the mtDNA damage was thought to be attributed to reactive oxygen species (ROS) (Hagen *et al.*, 1998). Mitochondrial DNA is more exposed to oxidative damage than nuclear DNA. However, genetic factors may not be the only cause of mitochondrial dysfunction. ROS may also cause damage to other mitochondrial components such as the lipid membranes.

It is generally accepted that lipids are subject to oxidation during ageing. As discussed above, CL and particularly CL containing high amounts of linoleic acid are able to most effectively enhance cytochrome *c* oxidase activity (Yamaoka-Koseki *et al.*, 1991). Unsaturated fatty acid tails of lipids such as CL are highly susceptible to peroxidative attack by ROS. Exposure of submitochondrial particles to ROS lead to a loss of cytochrome *c* oxidase activity as well as a loss of CL (Paradies *et al.*, 2000). Replacement of the lost CL with peroxidised CL showed no restoration of activity. There is no doubt that loss of CL will affect cytochrome *c* oxidase activity, but this study showed that oxidative damage to CL acyl groups will also result in loss of activity.

It is known that levels of CL decrease with age (Paradies and Ruggiero, 1990). This is known to result in a decrease in cytochrome *c* oxidase activity. The lost activity could be attributed to lower levels of the enzyme. However, it has been shown that cytochrome *c* oxidase levels in mitochondria are similar between young and old rats (Paradies *et al.*, 1993b). During ageing, the fatty acid composition of mitochondrial membrane lipids has been shown to increase in polyunsaturated fatty acids up to 12 months of age (Castelluccio *et*

al., 1994). This is followed by a decrease. The question of whether the molecular species of CL follows this trend was studied (Paradies *et al.*, 1993b). Rats from two time points were studied: young rats (4-5 months old) and old rats (27-28 months old). The study of the fatty acid composition of CL showed no difference between the ages. This would indicate that the molecular species of CL does not change during ageing. However, the peak in polyunsaturated fatty acids as reported by Castelluccio *et al.* (1994) was at 12 months while an extrapolated comparison between 4 months and 27 months would have been expected to show similar levels of unsaturated fatty acids. In addition, it has been shown that the fatty acid composition of CL in rat livers and human skeletal muscles do increase in linoleic acid from fetus to adult (Hoch, 1992). Thus, the question of whether the molecular species of CL changes during ageing is still debatable. The significance of changing CL molecular species during ageing contributing to mitochondrial dysfunction is currently unknown. As CL is believed to have a fairly slow turnover rate (Hostetler, 1982), an accumulation of CL with oxidised acyl tail could conceivable occur during ageing. Whether or not the molecular species of CL remains constant or changes as we age, it is of interest to study the CL remodelling pathway to access how the mitochondria maintains (or fails to maintain) a particular fatty acid composition. As already discussed, molecular species of CL with linoleoyl tails rapidly increase from fetus to adult, whether this is due to an increase in monolyso-CL acyltransferase activity or some other factor is unknown at this time.

Characteristics of monolyso-CL acyltransferase

The following three chapters describe some characteristics of monolyso-CL acyltransferase which include the effects of insulin and fatty acid binding protein and differing substrates on activity as well as submitochondrial localisation of activity.

These characterisations will hopefully give more insight into answering the following questions: Why does CL tend to have such a high proportion of linoleic acyl tails in certain tissues? Does the particular molecular species of CL found in liver, skeletal muscle, and heart play an important role in the mitochondria? If so, what effect does ageing have on CL molecular species? How would this affect functioning of mitochondria? If we inhibit the activity of this enzyme, will rats age faster? How is monolyso-CL acyltransferase activity controlled?

These studies are important to this field because CL remodelling may play a key role in the maintenance or regulation of oxidative phosphorylation. A better understanding of the regulation and acitivity of the monolyso-CL acyltransferase is crucial in determining this relationship.

CHAPTER 2: EFFECT OF INSULIN ON CARDIOLIPIN MOLECULAR REMODELLING

Introduction

The discussions in Chapter 1 suggested that both the presence and molecular species of CL play an important role in mitochondria. The importance of maintaining a balance of CL levels relative to other lipids as well as the proper molecular species led to speculation that this process must be regulated. Thus in this chapter, the possible regulation of CL remodelling in cardiac tissue by hormones was explored.

Homones and CL metabolism

Hormones maintain homeostasis in the face of changing conditions. The involvement of hormones in lipid metabolism is not novel. Insulin promotes the synthesis of triglycerides (Sherwood, 1997). Glucagon, growth hormone, and catecholamines stimulates release of fatty acids from adipose tissue (Saudek and Eder, 1979). The above hormones produce these effects by either stimulating or inhibiting the activities of hormone-sensitive lipase (Bernlohr and Simpson, 1996). Thus, through the actions of these hormones, the availability of fuel for energy production is regulated.

CL is widely believed to be important for the activity of several mitochondrial membrane enzymes such as cytochrome *c* oxidase (Vik *et al.*, 1981) and ATP synthase (Eble *et al.*, 1990). A decline in CL levels in rat heart mitochondria during ageing was correlated

with a decline in activity of mitochondrial enzymes (Paradies *et al.*, 1992; Paradies and Ruggiero, 1990). Thus it would appear that the maintenance of proper CL levels is important and likely regulated.

Role of thyroid hormone

Thyroxin has been found to have an effect on CL synthesis (Hostetler, 1991; Cao *et al.*, 1995). Thyroid homones are derived from tyrosine and exist in two forms, thyroxin (tetraiodothyronine, T_4) and triiodothyronine (T_3). They are synthesised in the thyroid gland and the levels circulating in the body are controlled by the hypothalamus-pituitary-thyroid axis (Sherwood, 1997). The effects of thyroid hormone in the body are widespread. These include effects on overall metabolic rate, growth rate, development, and functioning of the nervous system.

Thyroid hormone increases the basal metabolic rate particularly increasing the consumption rate of O_2 and energy (Sherwood, 1997). In a related effect, thyroid hormone increases heat production. Thyroid hormone influences both the synthesis and breakdown of carbohydrates, fat, and protein depending on the level of hormone. Normal growth requires thyroid hormone to stimulate growth hormone release as well as promote the effects of growth hormone (Sherwood, 1997).

In the heart, it has been known for over 200 years that thyroid hormone causes hypertrophy (Dillmann, 1990). The heart is a major target organ for this hormone. Thyroid hormone increases the heart's responsiveness to catecholamines, thus increasing cardiac output (Sherwood, 1997).

At the molecular level, there are at least two mechanisms by which thryoid hormone mediates its effects. The first is by being transported into the nucleus where it binds to specific nuclear receptors which in turn promotes transcription (Apriletti *et al.*, 1998). The second mechanism falls under the category of extranuclear effects and does not require protein synthesis. This mechanism has been shown to be responsible for producing effects such as stimulation of amino acid and sugar transport (Segal *et al.*, 1977) and increase in calcium efflux from myocytes (Mylotte *et al.*, 1985). There is also thought to be a large macromolecular complex on the inner mitochondrial membrane that can bind thyroid hormone known as the mitochondrial thyroid hormone receptor (Sterling *et al.*, 1978). Thyroid hormone has also been shown to increase the amount of protein import into the mitochondria (Craig *et al.*, 1998).

Thyroid hormone does have an effect on CL metabolism. Hypothyroidism was found to decrease both CL levels and cytochrome *c* oxidase activity (Paradies *et al.*, 1993a). Thyroid hormone was found to increase the activity of PGP synthase by 250% (Cao *et al.*, 1995) and cardiolipin synthase in rat liver by 52% (Hostetler, 1991). This leads to the speculation that some of the alterations in metabolism by thyroid hormone could be mediated through CL. Control of mitochondrial respiration by thyroid hormones has been suggested to be at least partially exerted at the level of CL synthase (Hostetler, 1991).

Hormonal regulation of remodelling

It was previously shown that the molecular species of CL is important for mitochondrial energy production (Yamaoka-Koseki *et al.*, 1991; Rusnak *et al.*, 1997). Therefore, regulation of the CL remodelling activity is likely. Indeed, administration of thyroxin was

found to increase monolyso-CL acyltransferase activity in rat heart mitochondria 1.6 times over control (Mutter *et al.*, 2000). The studies thus far do not explain how activity is being increased by thyroxin. Possibilities include specific increase in monolyso-CL acyltransferase levels by thyroxin or enhancement of monolyso-CL acyltransferase activity of existing enzymes. The question of whether monolyso-CL acyltransferase activity could be modulated by other hormones was of interest. Thus in this study, the effects of insulin on monolyso-CL acyltransferase activity was examined.

Function of insulin

Like thyroid hormone, insulin is another hormone involved in cellular metabolism. It is synthesised by the β-cells of the pancreas and is secreted in response to increased blood glucose levels. Insulin plays a role in anabolism. Its primary function is to promote cellular uptake of glucose, fatty acids, and amino acids from the blood (Sherwood, 1997). It also promotes the conversion of these substances into glycogen, triglycerides, and protein, respectively. Insulin exerts its function by binding to extracellular receptors that in turn transducts the signal to the intracellular side of the receptor by activation of tyrosine kinase. This then activates a cascade of protein kinases that result in inhibition of some enzymes and activation of others. These events also activate the glucose transporter as well as promote synthesis of proteins.

The lack of insulin results in diabetes mellitus. Chronic diabetes mellitus is associated with cardiomyopathy (Fein *et al.*, 1981; Penpargkul *et al.*, 1980; Regan *et al.*, 1981). Although the cause of this cardiomyopathy is likely to be multifaceted, one of the effects seen is a 30% loss of CL in sarcolemmal membranes (Makino *et al.*, 1987). It is also known

that streptozotocin-induced diabetic rat cardiocytes have decreased PG synthesis but this does not affect CL biosynthesis (Hatch *et al.*, 1995). In addition, streptozotocin treatment of neonatal rats was also found to increase the lipid proportion of CL in the whole body by 22% (Rabinowitz and Craig, 1989). These two findings suggest that the transfer of CL from the mitochondria to sarcolemma is impaired. The function of CL in membranes other than mitochondria has never been characterised, but potential involvement of CL with the activity of membrane proteins such as Ca^{2+}-stimulated ATPase, which is important for cardiomyocyte contractility, can be extrapolated.

Insulin is also known to play a role in the synthesis of unsaturated fatty acids. This relationship is unclear but diabetic rats have been found to have depressed Δ9-desaturase activity which is restored upon addition of insulin (Cook, 1996). It is unknown how this impairment of desaturase activity would affect the balance of unsaturated fatty acids in the cell. Nor is it known whether this will affect the molecular species of CL.

The effect of insulin on CL remodelling was studied. Insulin plays an obvious role in cellular energetics. It would seem plausible that insulin would have an effect on CL remodelling, since the CL molecular species may activate enzymes involved in energy metabolism. Here we investigated the effect of insulin on monolyso-CL acyltransferase activity and CL molecular species.

Materials and Methods

Materials

Bovine monolyso-CL (a mixture of 1'(1-acyl-*sn*-glycerol-3-phosphoryl)-3'(1'',2''-diacyl-*sn*-glycerol-3-phosphoryl)glycerol and of 1'(1,2-diacyl-*sn*-glycerol-3-phosphoryl)-3'(1''-acyl-*sn*-glycerol-3-phosphoryl)glycerol) was obtained from Avanti Polar Lipids (Alabaster, AL). [1-^{14}C] oleoyl-coenzyme A was obtained from DuPont NEN (Boston, MA). [1-^{14}C] linoleoyl-coenzyme A was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Bradford reagent was purchased from Bio-Rad Laboratories (Hercules, CA). TLC plates were Whatman K6 silica gel 60 Å, 0.25 mm thickness, 20 x 20-cm plates (Clifton, NJ). Streptozotocin was obtained from Sigma Chemical Company (St. Louis, MO). The insulin used was Humulin® N from Eli Lilly (Toronto, ON). All other chemicals were certified ACS grade or better obtained from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific Company (Fair Lawn, NJ). Scintillation fluid was EcoLite™ from ICN (Irvine, CA). Methyl ester standards for GC were obtained from Sigma Chemical Company (St. Louis, MO).

Preparation of TLC plates

Before use, TLC plates were scored into 2 cm wide lanes with 1 cm border lanes. The plates were activated by heating at 40°C for at least 30 min. Plates used for two-dimensional TLC were first treated with 0.4 M Borate, dried overnight, then activated at 40°C as before.

Treatment of animals

Treatment of rats conformed to the Guidelines of the Canadian Council on Animal Care. Male Sprague-Dawley rats (200-250 g) were made diabetic by injection of streptozotocin (65 mg/kg by tail vein). They were then treated daily with saline (100 µl ip) for five days. A separate group of rats was injected with insulin (12 units/rat ip) for five days. Control rats were treated with saline (100 µl ip). Another group of rats was treated with insulin (12 units/rat ip) for twenty-six days.

Isolation of mitochondria

Each rat was sacrificed by decapitation and the heart was immediately removed, rinsed, and placed in a 50 ml centrifuge tubes containing 8 ml of ice-cold homogenisation buffer (5 mM Tris, pH 7.4, 0.25 M sucrose, 2 mM EDTA). All subsequent steps were performed on ice as much as possible. A portion of the heart apex was removed and placed in 3 ml of 1:1 chloroform:methanol for CL acyl chain analysis. The remaining portion was homogenised using a Polytron® homogeniser (four 5-second full-speed bursts with 1 minute of cooling on ice) and the mitochondrial fraction was obtained by differential centrifugation. The homogenate was first centrifuged at 1000 × *g* (2000 rpm, JA-20 rotor, 4°C) for 5 minutes. The supernatant was placed into a fresh tube. The pellet was washed twice with 6 ml of homogenising buffer and the supernatants were pooled. The combined supernatants were centrifuged at 10 000 × *g* (12 000 rpm, JA-20 rotor, 4°C) for 15 minutes. The supernatant was discarded. The pellet (the mitochondrial fraction) was resupended in 1.5 ml of

homogenising buffer and subjected to 15 strokes in a tight-fitting Dounce homogeniser to aid in solubilisation. The protein concentration was determined using Bradford assays.

Protein determination

Bradford assays (Bradford, 1976) were performed according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

Monolysocardiolipin acyltransferase assay

Monolyso-CL acyltranserase activity was determined using [1-^{14}C] oleoyl-coenzyme A or [1-^{14}C] linoleoyl-coenzyme A as substrate as previously described (Ma *et al.*, 1999). To a 13 x 100 mm culture tubes, 35 µl of 1 mM MLCL, 11.7 µl of 0.5 M Tris, pH 8, 6.3 µl of 611.3 µM [1-^{14}C] linoleoyl-CoA (diluted with unlabelled linoleoyl-CoA to a specific activity of about 50 000 dpm/nmol), 50 µg of protein sample, and ddH_2O to a total volume of 116.7 µl. For assays using oleoyl-CoA, 6.3 µl of 611.3 µM [1-^{14}C] oleoyl-coenzyme A (diluted with unlabelled oleoyl-CoA to a specific activity of about 25 000 dpm/nmol) and 11.7 µl of 0.5 M Tris, pH 9.0 were used instead. Assays containing no monolyso-CL were also performed to assess the amount of incorporation of radiolabel into endogenous CL. The protein sample was added last and the mixture was incubated in a shaking water bath (25°C) for 10 minutes. Assays were terminated by addition of 3 ml of Solvent A (chloroform:methanol 2:1 v/v). Samples were washed with 0.8 ml 0.9% KCl, vortexed, and centrifuged for 5 min in a benchtop centrifuge (IEC) at full speed. The aqueous phase was then discarded and 2 ml TUP (theoretical upper phase; $CHCl_3$:CH_3OH:0.9% NaCl; 2:48:47

v/v/v) was added. The tube was vortexed and again centrifuged for 5 minutes. The aqueous layer was discarded and the samples were dried down under a stream of N_2 gas in a warm water bath.

Dried samples were dissolved in 25 µl of Solvent A and 20 µl was spotted along a single lane on a TLC plate. Ten µl of cardiolipin (4 mg/ml in Solvent A) and 10 µl of phosphatidyl glycerol (4 mg/ml in Solvent A) were spotted on each lane as markers. The plates were developed for 2 hours and 10 minutes using a solvent system of 50 ml $CHCl_3$, 30 ml C_6H_{14}, 10 ml CH_3OH, 5 ml CH_3COOH. Plates were allowed to dry and then visualised using iodine vapour. The band containing cardiolipin was scraped off the plate into scintillation tubes. Scintillation fluid (5 ml) was added and the amount of radiolabel incorporation was quantified using a Beckman LC 5801 scintillation counter (2 minute readings, quench corrected)

Analysis of CL fatty acyl groups

The heart apexes previously removed were homogenised in 3 ml chloroform:methanol (1:1 v/v) using a Polytron® homogeniser. The homogenate was then pelleted at full speed in a bench top centrifuge and the supernatant was recovered. Chloroform (1.5 ml) and 3 ml of 0.73% NaCl were added and the sample was vortexed and then pelleted. The aqueous phase was then discarded and 2 ml TUP was added. The mixture was then vortexed and centrifuged again. The aqueous phase was removed and the organic phase was dried down under N_2 gas.

The samples were dissolved in 50 µl Solvent A and spotted onto TLC plates (silica gel) previously treated with 0.4 M borate. The plates were developed in two-dimensions using

the following solvent system: 70 ml $CHCl_3$, 30 ml CH_3OH, 2 ml NH_4OH, and 3 ml ddH_2O for the first dimension; 65 ml $CHCl_3$, 35 ml CH_3OH, 5 ml ddH_2O for the second dimension. The developed plates were visualised using iodine vapour. The spots corresponding to CL were scraped into screw-cap test tube. CL was extracted from the silica according to the method of Arvidson (Arvidson, 1968). The extracted CL was dried down under N_2 gas and cleaved into methylesters (Tardi *et al.*, 1992). The methylesters were extracted and analysed by gas chromatography (Shimadzu GC-14A with Alltech 6' x 1/8" (O.D.) x 0.085" (I.D.)10% Silar 5CP steel column) and compared against standards.

Statistical analysis

The results of the experiments were averaged and compared with its respective control sample using a Student's *t* test. The results were considered significant if $P<0.05$.

Results

Effect of insulin on monolyso-CL acyltransferase activity

The effect of insulin on the monolyso-CL acyltransferase activity was examined. Rats were made diabetic or hyperinsulinemic. The monolyso-CL acyltransferase activities in the heart mitochondria were measured and are shown in Figure 2.1. From the results, both diabetes and hyperinsulinemia showed no effect on monolyso-CL acyltransferase activities. To ensure the negative results seen were not due to too short of a time-course, the duration of the hyperinsulinemic study was extended to 26 days (Figure 2.2). Again a slight decline was seen in monolyso-CL acyltransferase activity in the treated group, but these changes were insignificant.

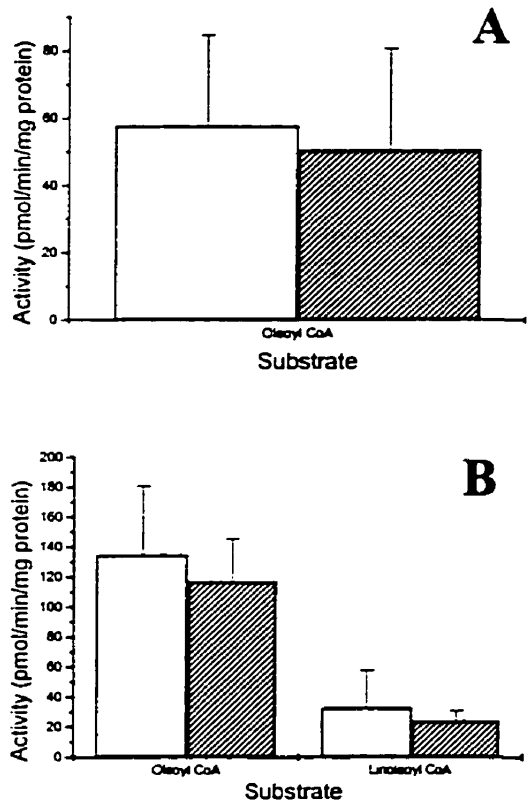

Figure 2.1: Effect of insulin on monolyso-CL acyltransferase activity. **A:** The heart mitochondria of streptozotocin-induced diabetic rats (hatched) were analysed for monolyso-CL:oleoy CoA acyltransferase activity and compared with control sample (white). **B:** The heart mitochondria of hyperinsulinemic rats (hatched) were analysed for monolyso-CL:oleoy CoA acyltransferase and monolyso-CL:linoleoyl CoA acyltransferase activities and compared with control (white). Mean ± SEM are shown. N=5.

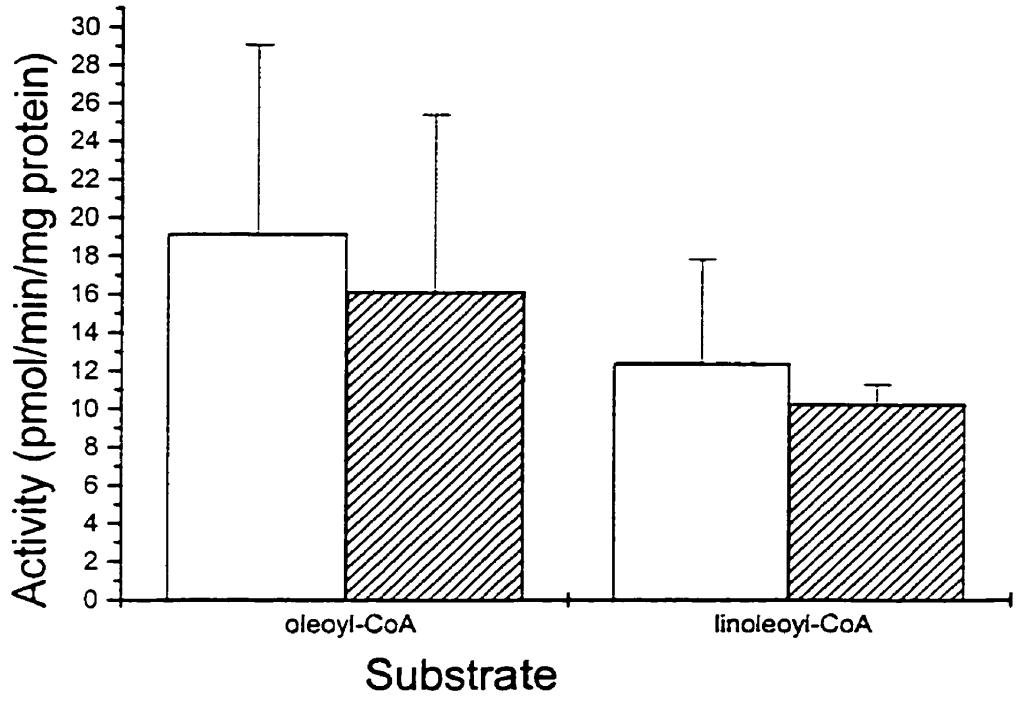

Figure 2.2: Effect of chronic hyperinsulinemia on monolyso-CL acyltransferase activity. Rats were treated daily with 12 U of insulin for 26 days (hatched) and the monolyso-CL acyltransferase activity was assayed and compared with saline treated rats (white). Mean ± SEM are shown. N=5.

Effect of insulin on CL fatty acyl groups

CL from the heart apexes of the above rats was isolated and the acyl groups were cleaved into methylesters. The acyl composition of CL from these rat hearts is shown in Figure 2.3. The results showed that the proportions of acyl groups in CL were unaltered in both diabetic and hyperinsulinemic rats compared to controls. The hyperinsulinemic studies were extended for 26 days (Figure 2.4). The results again showed that there was no significant alteration in CL acyl composition.

In sum, these experiments showed that insulin did not significantly alter the monolyso-CL acyltransferase activity. This was indicated by a lack of change in acyltransferase activity in both diabetic as well as hyperinsulinemic rats. In addition, studies into the acyl groups of CL showed no alteration in proportion of molecular species. This confirmed that insulin did not induce any changes in CL molecular remodelling.

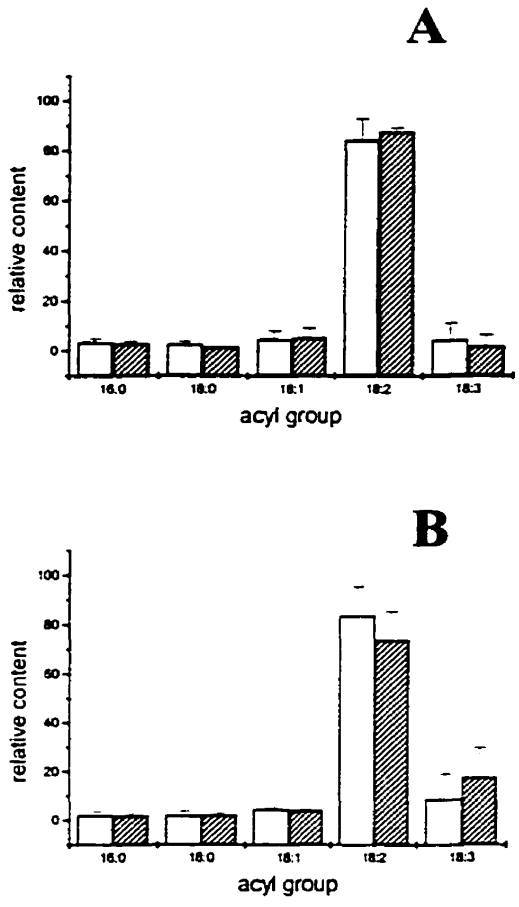

Figure 2.3: Effect of insulin on CL acyl composition. **A:** The CL of the hearts of streptozotocin-induced diabetic rats (hatched) was isolated and the acyl groups were cleaved and analysed by gas chromatography. This was compared with a control sample (white). **B:** The acyl groups of CL from the hearts of hyperinsulinemic rats (hatched) were compared with control (white). Mean ± SEM are shown. N=5.

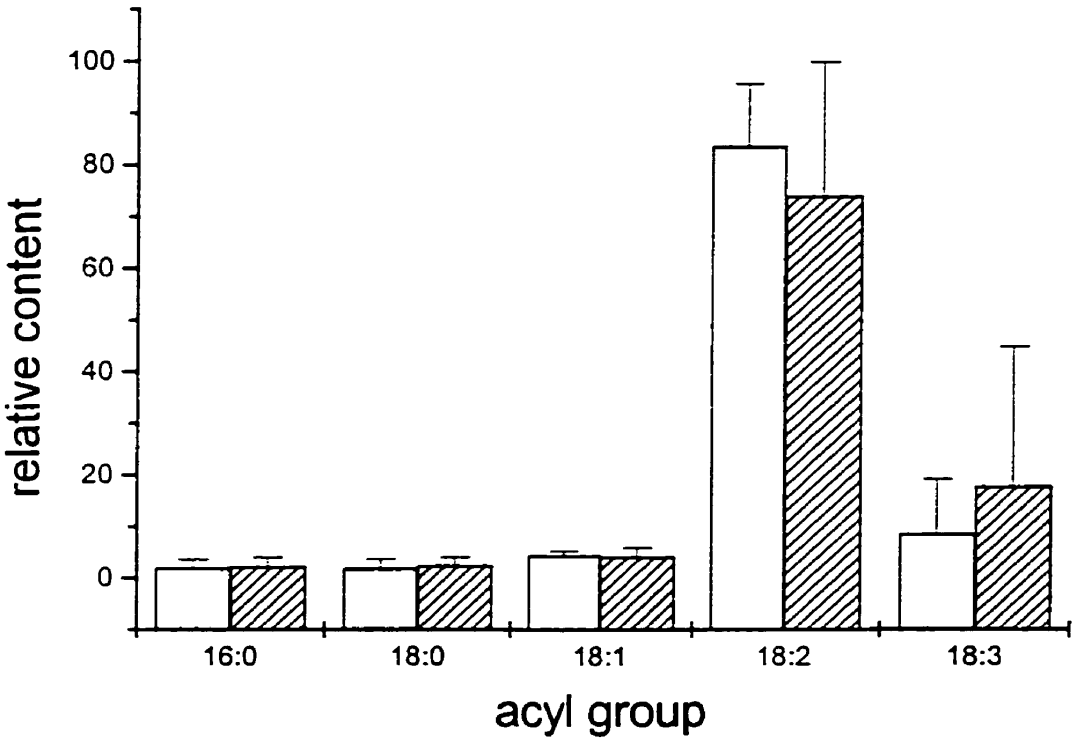

Figure 2.4: Effect of chronic hyperinsulinemia on CL acyl composition. Rats were treated daily with 12 U of insulin for 26 day (hatched). The CL of the hearts of was isolated and the acyl groups were cleaved and analysed by gas chromatography. This was compared with a control sample (white). Mean ± SEM are shown. N=5.

Discussion

Diabetes has complex effects on myocardial metabolism. These are related to systemic changes such as hyperglycemia and elevated levels of free fatty acids and ketone bodies. There are also changes in the cardiomyocyte such as down-regulation of glucose transporters and pyuvate dehydrogenase activity (Stanley *et al.*, 1997). The shift in fuel from glucose to fatty acids for energy production may have implication on CL remodelling activity. CL is synthesised exclusively in the mitochondria where β-oxidation of fatty acids also occurs. The reliance on fatty acid oxidation for energy may affect the availability of specific fatty acids for CL remodelling.

Import of long-chain fatty acids into the mitochondria requires three carnitine-dependent enzymes. The first is carnitine palmitoyl transferase I (CPT-I). This converts long-chain acyl-CoA to acylcarnitine on the cytosolic side of the inner mitochondrial membrane. The second enzyme is carnitine:acylcarnitine translocase. This transports acylcarnitine across the inner mitochondrial membrane in exchange for a carnitine from the matrix. The final enzyme is carnitine palmitoyl transferase II (CPT-II) which is in the matrix and regenerates the long chain acyl-CoA from acylcarnitine. Malonyl-CoA is a potent inhibitor of CPT-I (Paulson *et al.*, 1984). Streptozotocin-induced diabetic swine hearts have been demonstrated to have less malonyl-CoA suggesting that import of fatty acids into the mitochondria can occur at a greater rate (Hall *et al.*, 1996). Malonyl-CoA is synthesised by carboxylation of acetyl-CoA catalysed by acetyl-CoA carboxylase. In strepozotocin-induced diabetic rats, it was found that acetyl-CoA carboxylase activity was inhibited (Stanley *et al.*, 1997). Further,

it was found that diabetes leads to the phosphorylation of the enzyme leading to inhibition (Gamble and Lopaschuk, 1997). Insulin leads to a dephosphorylation of the enzyme and inhibition of fatty acid import into the mitochondria. These effects of insulin on the carnitine-dependent translocase system may affect CL.

There has been a previous association between the carnitine-dependent pathway and CL. In aged rats, there is a loss of CL content as well as mitochondrial function. The administration of acetyl-L-carnitine restores CL and mitochondrial function back to levels of young rats (Paradies *et al.*, 1995; Paradies *et al.*, 1994). Presumably, acetyl-L-carnitine acts by increasing the rate of fatty acid import into the mitochondria. Thus, the import of fatty acids into the mitochondria may be important for CL synthesis and perhaps remodelling.

The effect of diabetes on fatty acid oxidation could inhibit the linoleoyl-CoA available for remodelling. Thus, we hypothesised that excess or lack of insulin would alter the acyl composition of CL. However, the results show that the molecular species of CL is unaltered.

Insulin is important in cellular metabolism. Certain specific molecular species of CL were previously shown to activate various mitochondrial enzymes responsible for bioenergetics (Yamaoka-Koseki *et al.*, 1991; Rusnak *et al.*, 1997). It was thus hypothesised that since insulin plays a role in energy production, the increased presence or lack of insulin should alter the activity of enzymes that remodel CL. It was previously shown that increased thyroxin, another hormone involved in bioenergetics, was able to increase monolyso-CL acyltransferase activity (Mutter *et al.*, 2000). However, the results shown here indicate that this is not true for insulin. Thus the changes in metabolism caused by insulin are not mediated by an alteration in CL molecular species composition.

Previous studies with hyperthyroid rats show that both CL sythase (Hostetler, 1991; Cao *et al.*, 1995) and monolyso-CL acyltransferase activity (Mutter *et al.*, 2000) are elevated. Studies with streptozotocin-induced diabetic rats show that CL synthase activity is unaltered (Hatch *et al.*, 1995). Coupled with the finding in this study that monolyso-CL acyltransferase activity is unaltered suggests that perhaps increased synthesis of CL must precede increased remodelling activity. It has been proposed that only newly synthesised CL is remodelled (Schlame and Rüstow, 1990). Thus, the upregulation of CL synthase activity may be accompanied by an elevation in monolyso-CL acyltransferase activity in order to maintain the appropriate molecular species composition of CL. The molecular nature of this proposed tight balance in both enzymatic activities is currently unknown and warrants further study.

CHAPTER 3: CHARACTERISTICS OF MONOLYSOCARDIOLIPIN ACYTRANSFERASE ACTIVITY

Introduction

Monlyso-CL acyltransferase activity, which is responsible for reacylating monolyso-CL to CL in remodelling, has not been fully characterised. The studies presented in this chapter demonstrated some of the characteristics of monolyso-CL acyltransferase activity. Monolyso-CL acyltransferase has not been completely purified yet. Therefore, the following discussion of the characteristics of this enzyme mainly involved the activity measured in the mitochondrial fraction. Linoleic acid and oleic acid comprise the two most abundant tails found on CL in the heart at 88% and 8% of all CL acyl tails, respectively (Ma *et al.*, 1999). Thus, the reacylation of monolyso-CL with either linoleoyl and oleoyl acyl tails was studied.

Kinetic studies

Previous studies conducted in our lab have characterised the apparent kinetic values of monolyso-CL acyltransferase in the crude rat heart mitochondrial preparation (Ma *et al.*, 1999). The optimal pH for the conversion of monolyso-CL and oleoyl-CoA to CL was around 8.0-9.0. The optimal pH for the conversion of monolyso-CL and linoleoyl-CoA to CL was around 7.5-8.5. With oleoyl-CoA as substrate, the apparent K_m for the catalysis of the reacylation reaction for oleoyl-CoA was 12.5 μM and for monolyso-CL was 128.9 μM. With linoleoyl-CoA as substrate, the apparent K_m for linoleoyl-CoA was 6.7 μM and for

monolyso-CL was 59.9 μM. These studies showed that there are different affinities for each of two substrates. Not surprisingly, since it is the most abundant acyl group found on CL, the enzyme for the reacylation reaction showed higher affinity for linoleoyl-CoA than for oleoyl-CoA. The affinity for monolyso-CL also appeared higher with linoleoyl-CoA indicating that oleoyl-CoA may have some inhibitory effects on acyltransferase activity.

Substrate specificity

The differences in affinity for monolyso-CL above may also indicate that there are separate enzymes, one more specific for linoleoyl-CoA and another more specific for oleoyl-CoA. If this were the case, it would be expected that the monolyso-CL acyltransferase that is more specific for linoleoyl-CoA would be more active or be present in larger amounts. However, this cannot be proven unless the activities can be separated by a purification process.

In this study, monolyso-CL acyltransferase was characterised. The specificity of the enzyme for acyl-CoA species was examined. The effect of fatty acid binding proteins, lipid binding proteins, and acyl-CoA binding proteins on activity was studied. Finally, the ability of other acyl-CoA to compete with either linoleoyl-CoA or oleoyl-CoA for reacylation of monolyso-CL was determined. These studies are important in gaining insights into the process of CL remodelling. They may also reveal properties that may be exploited for purification of the enzyme.

Materials and Methods

Materials

Rat heart fatty acid binding protein (hFABP) and mouse adipocyte FABP (aFABP) were obtained from Dr. Judith Storch (Rutgers University, NJ). Adipocyte lipid binding protein (aLBP) and keratinocyte lipid binding protein (kLBP) were obtained from Dr. David Bernlohr (University of Minnesota, MN). Rat liver acyl-CoA binding protein (ACBP) was obtained from Dr. Jens Knudsen (Odense University, Denmark). Myristoyl-CoA, palmitoyl-CoA, stearyl-CoA, and linoleoyl-CoA were obtained from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ). Palmitoleoyl-CoA, BSA (Fraction V), ATP, and coenzyme A was obtained from Sigma (St. Louis, MO). [1-^{14}C] Oleic acid, [1-^{14}C] oleoyl-coenzyme A and [1-^{14}C] linoleic acid were obtained from Dupont NEN (Boston, MA). [1-^{14}C] linoleoyl-coenzyme A was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO).

Mitochondria sample

Twenty male Sprague-Dawley rats weighing 150-175 g were housed in cages of two or three and fed rat chow and water *ad libitum*. Prior to harvesting of hearts, the rats were fasted overnight. Rats were sacrificed by decapitation and a total of 15.42 g of hearts was collected. The mitochondria fraction was isolated as described in Chapter 2.

Competition assays

Monolyso-CL acyltransferase assays were performed similar to that described in Chapter 2, except for the addition of 6.3 µl of a 611.3 µM unlabelled fatty acyl CoA to a final concentration of 33 µM. The fatty acyl CoA's used with [1-^{14}C] oleoyl-CoA were myristoyl-CoA, palmitoyl-CoA, palmitoleoyl-CoA, stearyl-CoA, and linoleoyl-CoA. With [1-^{14}C] linoleoyl-CoA, the fatty acyl CoA's used were myristoyl-CoA, palmitoyl-CoA, palmitoleoyl-CoA, stearyl-CoA, and oleoyl-CoA. Control assays were performed containing either [1-^{14}C] oleoyl-CoA or [1-^{14}C] linoleoyl-CoA alone in the absence of an unlabelled fatty acyl CoA.

Monolyso-CL acyltransferase assays with FABP, ACBP, and LBP

Monolyso-CL acyltransferase assays were performed similar to that described in Chapter 2 with the addition of either 16.5 µM or 33 µM of hFABP, aFABP, ACBP, aLBP, or kLBP to the assay mixture. Control assays were performed using BSA.

Specificity for acyl-coenzyme A assays

To a 13 x 100 mm culture tubes, 35 µl of 1 mM MLCL, 11.7 µl of 0.5 M Tris, pH 8, 6.3 µl of 611.3 µM [1-^{14}C] linoleic acid (diluted with unlabelled linoleic acid to a specific activity of about 25 000 dpm/nmol), 11.7 µl of 100 mM ATP, 11.7 µl of 10 mM coenzyme A, 50 µg of protein sample, and ddH_2O to a total volume of 116.7 µl were added. For assays

using oleic acid, 6.3 µl of 611.3 µM [1-^{14}C] oleic acid (diluted with unlabelled oleic acid to a specific activity of about 12 000 dpm/nmol) and 11.7 µl of 0.5 M Tris, pH 9.0 were used instead. In other experiments using either linoleic acid or oleic acid, both ATP and coenzyme A were omitted. Two positive control experiments were performed: One using the monolyso-CL acyltransferase assay described in Chapter 1; the other as previously with the addition of ATP and coenzyme A.

Results

Effect of competing acyl-CoA on monolyso-CL acyltransferase activity

Monolyso-CL acyltransferase assays were performed on rat heart mitochondria using either radiolabelled linoleoyl-CoA or oleoyl-CoA with an unlabelled acyl-CoA as listed in Table 3.1. The amount of radiolabel incorporated into CL was measured. From the results, we see that all fatty-acyl CoA species tested were able to decrease the amount of radiolabel incorporation into CL. The only exception was myristoyl-CoA. This substrate had no effect on the amount of linoleic acid incorporation into CL. The fact that all competitors except one were able to decrease the incorporation of radiolabel suggested general non-selectivity for substrate. However, the observation that myristoyl-CoA did not interfere with incorporation of linoleic acid suggested that there might be some subtle differences in selectivity. Certainly the difference in radiolabel incorporation profile for [1-^{14}C] oleoyl-CoA and [1-^{14}C] linoleoyl-CoA did suggest some selectivity. Perhaps there are isoforms of monolyso-CL acyltransferase that demonstrate different preferences for acyl-CoA species.

Table 3.1: Effect of other acyl-CoA species on monolyso-CL oleoyltransferase and monolyso-CL linoleoyltransferase activity.

Competitor	Oleoyltransferase (pmol/min/mg protien)	% control	Linoleoyltransferase (pmol/min/mg protien)	% control
control	129.8	-	280.4	-
myristoyl-CoA	69.2	53.3	297.9	106.3
palmitoyl-CoA	48.0	37.0	142.8	50.9
palmitoleoyl-CoA	63.3	48.9	193.5	69.0
stearyl-CoA	62.1	47.9	146.0	52.1
linoleoyl-CoA	64.4	49.6	ND	-
oleoyl-CoA	39.9	30.7	186.2	66.4

ND – not determined

Effect of FABP, ACBP, and LBP on monolyso-CL acyltransferase activity

It was previously observed that long-chain fatty acyl-CoA binding proteins such as FABP could increase the incorporation of [1-^{14}C] oleoyl-CoA into phosphatidic acid up to 18-fold (Jolly *et al.*, 1997). Monolyso-CL acyltransferase assays were performed with the addition of FABP, ACBP, or LBP isolated from various sources. FABP, ACBP, and LBP were added at a 1:1 and 1:2 molar ratio to the amount of acyl-CoA. The results are shown in Table 3.2 and expressed as a percentage of the control value. The results with [1-^{14}C] linoleoyl-CoA showed a decrease in incorporation, but this decrease was similar to what was seen with BSA. Thus, the effects seen were attributed to non-specific events. The results with [1-^{14}C] oleoyl-CoA again showed a similar amount of radiolabel incorporation as with BSA, suggesting a non-specific interaction. The results with aLBP and kLBP were quite elevated, suggesting a possible increase in incorporation. Unfortunately, this phenomenon could not be further investigated due to the limited amount of aLBP and kLBP available.

Table 3.2: Effect of BSA, FABP, ACBP, and LBP on monolyso-CL oleoyltransferase and monolyso-CL linoleoyltransferase activity.

Competitor	Oleoyltransferase (% control)	Linoleoyltransferase (% control)
16.5 μM hFABP	91.1	68.3
33.0 μM hFABP	117.4	75.0
16.5 μM aFABP	97.0	67.4
33.0 μM aFABP	96.8	68.0
16.5 μM ACBP	120.3	66.1
33.0 μM ACBP	76.6	49.2
33.0 μM aLBP	145.3	ND
33.0 μM kLBP	143.1	ND
16.5 μM BSA	87.6	74.1
33.0 μM BSA	86.5	67.6

ND – not determined

Monolyso-CL acyltransferase activity requires acyl-CoA species

Many reacylation reactions of lipids require a fatty acid activated by CoA. These include acyl-CoA:glycerol-3-phosphate acyltransferase (Haq *et al.*, 1987), diacylglycerol acyltransferase (O'Doherty and Kukis, 1975), and lysophosphatidic acid acyltransferase (Bordewick *et al.*, 1985). In this experiment, we demonstrated that monolyso-CL acyltransferase activity is CoA-dependent. Monolyso-CL and mitochonrial sample were incubated with the substrates listed in Table 3.3. From the results, we saw that maximal incorporation of radiolabel into CL only occurred when acyl-CoA is in the substrate mixture. When linoleic acid was used alone, no radiolabel incorporation was detected. Likewise, with oleic acid alone, only a very minute amount of radioactivity was detected in CL. With the addition of ATP and CoA to the assay mixture, this allowed the conversion of linoleic acid and oleic acid to its corresponding acyl-CoA via acyl-CoA synthetase (also located in the mitochondria). As expected, we saw an increase in radiolabel incorporation into CL as compared to using the fatty acid alone. The addition of ATP and CoA to acyl-CoA showed a decrease in activity from control values. This may be attributed to the activation of other free fatty acids in the crude mitochondrial sample which could then compete with the radiolabelled substrate for incorporation.

Table 3.3: Requirement of a CoA-bound species.

Substrate(s)	Radiolabel incorporation into CL (pmol/min/mg protein)	% control
[1-^{14}C] linoleoyl-CoA	280.4	
[1-^{14}C] linoleic acid, ATP, CoA	14.6	5.2
[1-^{14}C] linoleic acid only	ND	-
[1-^{14}C] linoleoyl-CoA, ATP, CoA	81.5	29.1
[1-^{14}C] oleoyl-CoA	41.3	
[1-^{14}C] oleic acid, ATP, CoA	6.8	16.5
[1-^{14}C] oleic acid only	3.5	8.5
[1-^{14}C] oleoyl-CoA, ATP, CoA	31.5	76.3

ND – not detected

Discussion

Heart, skeletal muscle, and liver are tissues that are known to contain CL with a high composition of linoleic acid. This almost homogeneous molecular species of CL indicated that remodelling of CL likely occurs in these tissues. We characterised the monolyso-CL acyltransferase activity in the rat heart mitochondria.

The results showed that there was no doubt that an acyl-CoA species is required for the reacylation of monolyso-CL to CL. Previous studies had shown that monolyso-CL could be reacylated using PC (Schlame and Rüstow, 1990). This suggested the possibility that perhaps reacylation could occur by direct transacylation from PC. However, studies in our laboratory have shown that transacylation does not occur (Ma *et al.*, 1999). More likely, an acyl group was cleaved off the PC molecule and was esterified to an acyl-CoA by acyl-CoA sythetase. This then became the substrate for monolyso-CL acyltransferase. This would be consistent with what was found in our study. The reaction is shown in our study to be acyl-CoA dependent.

The effects of various binding proteins on monolyso-CL were characterised. Fatty acid binding proteins (FABPs) are a family of small molecular weight proteins that are abundantly found in the cytoplasm. FABPs are believed to have a wide range of cellular functions some of which are only recently being elucidated (Kaikaus *et al.*, 1990). Some of the functions of FABP include the uptake of fatty acids from the plasma (McCormack and Brecher, 1987; Renaud *et al.*, 1978), translocation of fatty acids to between membranes (McCormack and Brecher, 1987), and stimulation of a large number of enzymes involved in fatty acid metabolism (Kaikaus *et al.*, 1990). FABPs are also thought to modulate cell

growth and differentiation by various mechanisms such as modulating the availability of arachidonic acid and oleic acid to activate protein kinase C (Kaikaus *et al.*, 1990).

FABPs are known to bind acyl-CoA (Hubbell *et al.*, 1994) and have been proposed to play an important role in both the metabolism and transport of long-chain acyl-CoA (Færgeman and Knudsen, 1997). Acyl-CoA binding proteins (ACBPs) have a 3-4 fold higher affinity for acyl-CoA than FABP (Færgeman and Knudsen, 1997). This class of binding proteins are also thought to be important for the metabolism and transport of long-chain acyl-CoA. As ACBP was initially found as an impurity of a FABP preparation (Mogensen *et al.*, 1987), many of the functions attributed to FABP binding of long-chain acyl-CoA may actually be due to ACBP binding.

Lipid binding proteins (LBPs) are another large multigene family of low-molecular weight intracellular proteins. The primary function of LBP is thought to be the solubilisation and trafficking of hydrophobic compounds in the cell (Kane and Bernlohr, 1996). LBPs bind lipophilic molecules such as long chain fatty acids and retinoids. Some members of the family can bind prostaglandins and lysophosphlipids (Matarese *et al.*, 1989). Little is known about whether or not LBPs have a metabolic function.

FABPs have been shown to increase the activity of many acyl-CoA-utilising enzymes (Færgeman and Knudsen, 1997). These include acyl-CoA:glycerol 3-phosphate acyltransferase (Haq *et al.*, 1987), diacylglycerol acyltransferase (O'Doherty and Kuksis, 1975) and lysophospholipid acid acyltransferase (Bordewick *et al.*, 1985). In the current study, it did not appear that FABP, ACBP, nor LBP had any appreciable effect on monolyso-CL acyltransferase activity. Thus the reacylation of monolyso-CL was speculated to involve free floating acyl-CoA substrates. Monolyso-CL acyltransferase activity occurs at the

mitochondria where fatty acids are normally directed. Perhaps the supply of acyl-CoA at the mitochondria may normally be abundant enough that the monolyso-CL acyltransferase does not have to depend on FABP, ACBP, or LBP for its activity or activation.

Finally, the effect of other acyl-CoA species on the incorporation of linoleoyl-CoA or oleoyl-CoA into CL was characterised. The results show that the other acyl-CoA species with the exception of myristoyl-CoA are able to interfere with both linoleoyl-CoA or oleoyl-CoA incorporation into CL. These results were rather unexpected. The acyl composition of CL in the heart is about 88% linoleic acid. The enzymes that synthesise CL have been shown to be non-selective for acyl groups as discussed in Chapter 1. Therefore, in order to maintain such a high proportion of linoleic acid, there must be specific remodelling. However, the results presented here indicate that perhaps monolyso-CL acyltransferase is not as specific for linoleoyl-CoA as one might expect. The implications of this are unknown at this time. However, since these competing acyl-CoA species were not labelled, it is uncertain at this time whether they were actually incorporated into CL or they simply acted as an inhibitor to monolyso-CL acyltransferase activity.

CHAPTER 4: SUBMITOCHONDRIAL LOCALISATION OF MONOLYSOCARDIOLIPIN ACYLTRANSFERASE ACTIVITY

Introduction

Localisation of cardiolipin metabolism

CL is synthesised exclusively in the mitochondria (Hostetler and van den Bosch, 1972) as one of the few phospholipids that the mitochondrion is capable of synthesising. The steps of CL synthesis as outlined in Figure 1 are localised in the following areas of the mitochondria: The first step, phosphatidic acid (PA) synthesis, was found to be localised on the outer mitochondrial membrane (Carroll *et al.*, 1982; Hesler *et al.*, 1985). The second step, activation of PA to CDP-DG, was found to occur both on the inner and outer mitochondrial membrane (Jelsema and Moore, 1978). The third and fourth steps of CL synthesis, leading to the formation of phosphatidyl glycerol (PG), occur on the inner membrane (Hostetler and Van den Bosch, 1972; Jelsema and Moore, 1978). The final step, condensation of CDP-DG and PG to form CL, is localised to the matrix side of the inner mitochondrial membrane (Schlame and Haldar, 1993).

Localisation of monolyso-CL acyltransferase activity

Prior to this study, the submitochondrial location of monolyso-CL acyltransferase was not known. Since the monolyso-CL acyltransferase activity appeared to be increased only in instances where cardiolipin synthase activity is elevated (Mutter *et al.*, 2000), it was

postulated that monolyso-CL acyltransferase activation may be tied to cardiolipin synthase activation. Cardiolipin synthase is known to be associated with a large complex in the inner mitochondrial membrane (Zhao *et al.*, 1998). Therefore suggesting the possibility that monolyso-CL acyltransferase could be part of this complex. Thus, it was hypothesised that monolyso-CL acyltransferase activity would be localised in the inner mitochondrial membrane. In this study, rat livers were used instead of hearts because a larger amount of mitochondria, which was necessary to perform these experiments, could be harvested per rat.

Materials and Methods

Materials

Digitonin, rotenone, cytochrome *c*, and trypsin (EC 3.4.21.4, type IX from porcine pancreas) were obtained from Sigma Chemical Company (St. Louis, MO). All other chemicals were certified ACS grade or better and purchased from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific Company (Fair Lawn, NJ).

Isolation of mitochondria

The livers of ten male Sprague-Dawley rats (175-250 g) were harvested immediately after decapitation. Ten rats yielded approximately 50 g of liver. The mitochondria were isolated from the mitochondria based on a published method (Greenawalt, 1974). The livers were placed in 2 ml of ice-cold isolation medium (0.22 M D-mannitol, 0.07 M sucrose, 0.5 mg/ml BSA, 0.02 M HEPES, pH 7.4) per 1 g of liver. All procedures were performed at 4°C or on ice. Using scissors, the livers were chopped into smaller pieces and rinsed twice with isolation medium. A suspension of approximately 1 volume liver mince and 2 volumes isolation medium was made. This was portioned out and homogenised using a tissue grinder with a radially serrated teflon pestle, a 30 ml tube, and 4 passes at 1500 rpm. The homogenate was diluted 1:3 with isolation medium. Subsequently, it was centrifuged at 850 $\times$ g_{max} (2200 rpm, Beckman JA-10 rotor, 4°C) for 10 minutes in a superspeed centrifuge (Beckman J2-HS). The supernatant was transferred to fresh tubes and centrifuged at 11 325

× g_{max} (8000 rpm, Beckman JA-10 rotor, 4°C) for 15 minutes. The supernatant was discarded and the pellet was gently muddled into a paste using a cooled homogeniser pestle. It was then resuspended in half the original volume prior to initial centrifugation. This was then centrifuged at 16 300 × g_{max} (9500 rpm, Beckman JA-10 rotor, 4°C) for 15 minutes. The supernatant was discarded and the pellet was resuspended in isolation medium with two strokes of the pestle in a 40 ml dounce homogeniser. The concentration was adjusted to give approximately 100 mg/ml mitochondrial protein.

Protein determination

The protein concentration was determined using biuret method as described previously (Layne, 1957). In a 1 ml solution containing 1 to 10 mg of protein, 4 ml of biuret reagent (6.0 mM $CuSO_4 \cdot 5H_2O$, 21.3 mM $NaKC_4H_4O_6 \cdot 4H_2O$, and 750 mM NaOH) was added, vortexed, and allowed to 30 minutes at room temperature (20-25°C) before the absorbance was read at 560 nm. Readings were compared with standards containing known concentrations of BSA. Miniature assays were also used which consisted of 200 µl of protein sample and 800 µl of biuret reagent.

Preparation of digitonin solution

Digitonin was recrystallised once from hot absolute ethanol and dried. Sixty mg of recrystalised digitonin were redissolved in 5 ml of almost boiling isolation medium without BSA. The solution was allowed to cool and 50 µl of 50 mg/ml BSA solution was added.

Separation of mitoplasts and outer mitochondrial membrane

Mitochondrial outer membrane and mitoplasts were isolated from mitochondria using methods previously described (Greenawalt, 1974). In a pre-cooled 20-ml beaker 5 ml of mitochondrial suspension was added and gently stirred using a miniature Teflon stir bar. Five ml of digitonin solution was added and the stirring was continued for 15 minutes. The suspension was diluted by adding 15 ml of isolation medium. The mixture was the homogenised using a dounce homogeniser and then centrifuged at 11 000 × g_{max} (9500 rpm, Beckman JA-20 rotor, 4°C) for 10 minutes. The supernatant, which contained the outer membrane fragments, was collected. The pellet was resuspended in half the original volume (i.e. 12.5 ml) and centrifuged again at 11 000 × g_{max}. The supernatant was removed and combined with the first collection. The pellet, designated as the mitoplast fraction, was resuspended in a minimal amount of isolation medium and the concentration was adjusted to about 80 mg/ml using biuret method of protein determination. The pooled supernatants were pelleted in an ultracentrifuge (Beckman L8-80M) at 145000 × g_{max} (38 000 rpm, Ti60 rotor, 4°C) for 1 hour. The supernatant was discarded and the pellet, designated the outer membrane fraction, was resuspended in isolation medium to yield a concentration of about 30 mg/ml.

Enzyme assays

Samples of mitochondria, mitoplasts, and outer membrane fraction were diluted 1/15th, 1/10th, and 1/5th, respectively. Monolyso-CL acyltransferase activity was determined in each of these fractions as described in Chapter 1. Monoamine oxidase was assayed as a marker

for the outer mitochondrial membrane and succinate dehydrogenase was assayed as the marker for the inner mitochondrial membrane. Monoamine oxidase was assayed as previously described (Tabor *et al.*, 1955). The assay mixture contained 300 µl 0.2 M potassium phosphate buffer, pH 7.2, 30 µl 0.1 M benzylamine sulfate, 520 µl ddH_2O, and 50 µl enzyme sample. The mixture was vortexed and incubated in a 37°C water bath for 5 min. Change in absorbance at 250 nm was measured every second for 5 minutes using a spectrophotometer with a thermostated chamber at 37°C (Pharmacia Biotech Ultrospec 4000 with Swift II Reaction Kinetics v1.00 analytical software). Succinate dehydrogenase was assayed as previously described (Hovius *et al.*, 1990). The assay consisted of 200 µl of 4 x reaction mix (400 mM potassium phosphate buffer, pH 7.6, 20 mM EDTA, 4 mM KCN, 2 µM rotenone, 25 mM succinate), 480 µl ddH_2O, and 20 µl sample. This was incubated for 5 minutes in a 37°C water bath. One hundred µl of 0.4 mM cytochrome *c* (in 10 mM potassium phosphate buffer, pH 7.6) was added. The change in absorbance at 550 nm was measured every second for 2 minutes using a spectrophotometer with a thermostated chamber at 37°C.

Protease treatment

Mitochondria and mitoplasts were also treated with 0.1 mg/ml trypsin for 30 min at 32°C. The reaction was stopped with 6 mM PMSF (phenylmethylsulphonyl fluoride). Control samples were treated the same except trypsin was replaced with buffer.

Results

The enzymatic activity of monolyso-CL acyltransferase was localised. Rat liver mitochondria were used for these studies due to the larger amount of mitochondria available per rat as compared to the heart. Mitochondria were isolated and then treated with digitonin to release the outer membrane. The outer membrane and the resulting mitoplasts were separated and assayed for monolyso-CL acyltransferase activity as well as membrane marker activity (Table 4.1). The results show that there is an increase in the specific activity of monolyso-CL:oleoyl CoA acyltransferase in the outer membrane fraction. This corresponds with an increase in monoamine oxidase activity and a decrease in succinate dehydrogenase activity. This indicates that this fraction is indeed enriched with outer membrane and that monolyso-CL:oleoyl CoA acyltransferase is associated with this fraction.

In a separate study, mitoplasts and mitochondria were treated with trypsin to assess the susceptibility of monolyso-CL acyltransferase activity to digestion. The results are shown in Table 4.2. Monolyso-CL acyltransferase activity was susceptible to protease digestion in both samples.

The above two studies indicate that monolyso-CL acyltransferase activity may be localised on the outer membrane.

Table 4.1: Marker enzyme activity and monolyso-CL:oleoyl CoA acyltransferase activity in rat liver mitochondria and subfractionates.

Fraction	Oleoyltransferase (pmol/min/mg protien)	MAO (units*/mg protein)	SDH (nmol cyt c oxidised/min/mg protein)
Mitochondria	318.5	120.4	112.1
Mitoplasts	297.0	125.3	131.7
Outer Membrane	393.1	201.1	13.1

*one unit of activity represents a 0.001 change in absorbance at 250 nm in one minute

MAO = monoamine oxidase (outer membrane marker); SDH = Succinate dehydrogenase (inner membrane marker)

Table 4.2: The effect of protease treatment on marker enzyme activity and monolyso-CL acyltransferase activity in rat liver mitochondria and subfractionates.

Sample (treatment)	Oleoyltransferase (pmol/min/mg protein)	MAO (units*/mg protein)	SDH (nmol cyt c oxidised/min/mg protein)
Mitochondria (control)	597.6	123.3	82.0
Mitochondria (protease)	19.1	88.8	82.6
Mitoplast (control)	117.0	122.4	95.7
Mitoplast (protease)	151.5	101.1	7.2

*one unit of activity represents a 0.001 change in absorbance at 250 nm in one minute

MAO = monoamine oxidase (outer membrane marker); SDH = Succinate dehydrogenase (inner membrane marker)

Discussion

It was previously demonstrated that the synthetic machinery for CL synthesis is on the matrix side of the inner mitochondrial membrane (Schlame and Haldar, 1993). The results shown indicate that monolyso-CL acyltransferase activity is definitely present on the outer membrane. This is interesting considering that CL is synthesised on the inner membrane. The exact implication for this arrangement is unknown at this time.

Speculations as to why monolyso-CL acyltransferase would be located on the outer membrane may be related to the localisation of phospholipase A_2. Phospholipase A_2 is the enzyme that first deacylates CL in the remodelling pathway. Studies have indicated that phospholipase A_2 is localised in the inner membrane and on the outer membrane contact sites (Levrat and Louisot, 1992). Thus it is possible that CL could be remodelled on the outer membrane at the contact sites. In fact, CL is known to be highly concentrated at the mitochondrial contact sites (Ardail *et al.*, 1990).

Another reason why monolyso-CL would be located on the outer membrane may stem from the source of acyl-CoA for remodelling. Linoleic acid is an essential fatty acid, which means the mitochondria cannot synthesise it. This means that this fatty acid must be imported. Figure 4.1 shows the most recent proposed model of long-chain fatty acid import into the mitochondria. (Kerner and Hoppel, 2000). Fatty acid is converted to acyl-CoA on the cytosolic side of the outer membrane. It is then brought into the intermembrane space through a porin channel. Once in the intermembrane space, it is then converted into acylcarnitine by CPT-I. Acylcarnitine is then transported across the mitochondrial inner

membrane by carnitine:acylcarnitine translocase where acyl-CoA is regenerated by CPT-II. If localised on the outer membrane, monolyso-CL acyltransferase may have an advantage in obtaining substrate. The enzyme might possibly draw on a pool of acyl CoA in the intermembrane space of the mitochondria or from the cytosol, thus avoiding the competition for substrates with the β-oxidation enzymes in the mitochondrial matrix.

These studies in no way discount the fact that monolyso-CL acyltransferase activity may still have a localisation in the inner mitochondrial membrane. Therefore, the possibility of an association with the cardiolipin synthase complex is still possible. Future studies involving an isolation of the cardiolipin synthase complex (Zhao *et al.*, 1998) and assaying for monolyso-CL acyltransferase activity may answer this question.

Overall, the results show that remodelling of CL can occur on the mitochondrial outer membrane. Since it has been proposed that only newly synthesised CL is remodelled (Schlame and Rüstow, 1990), it brings up the question of how newly synthesised CL is transported from the inner membrane to the outer membrane. The mechanism of lipid export from the mitochondria is currently unknown. Nonetheless, the findings of this study present a property that can be exploited for future purification of this enzyme.

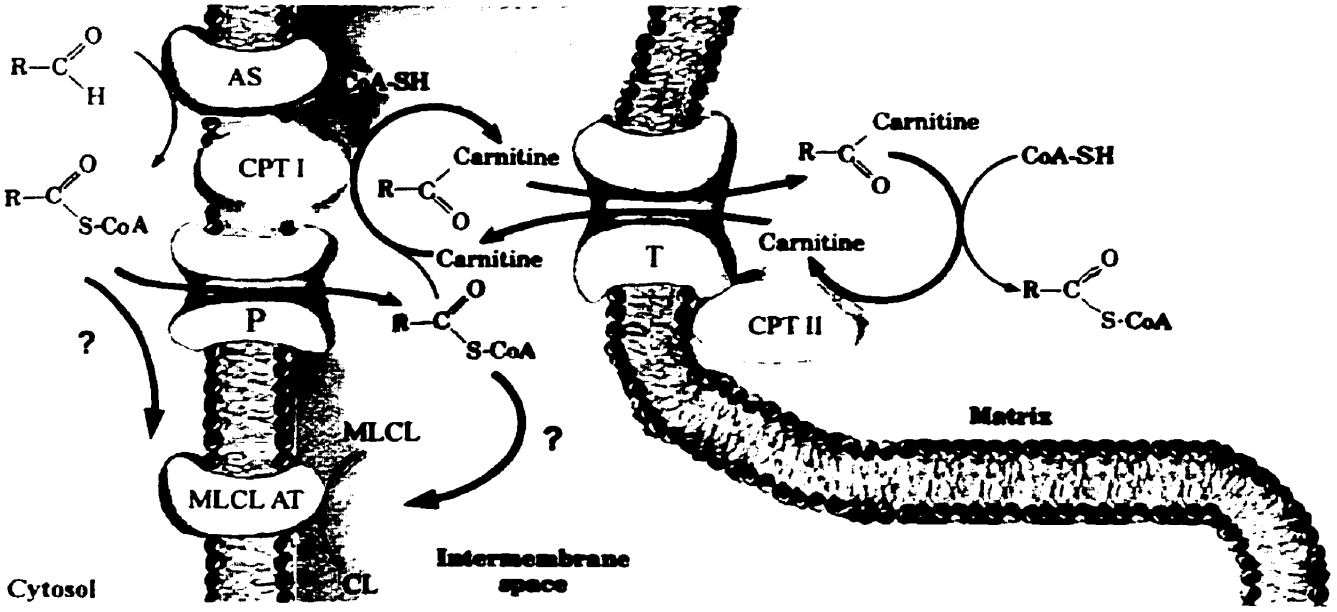

Figure 4.1: Proposed relationship between monolyso-CL acyltransferase activity and fatty acid import into mitochondria. MLCL AT – monolyso-CL acyltransferase; AS – acyl-CoA synthetase; CPT I – carnitine palmitoyltransferase I; T – carnitine:acylcarnitine translocase; CPT II – carnitine palmitoyltransferase II; P – porin. Free fatty acids are brought to the mitochondria with the help of FABP and converted by AS to acyl-CoA. Acyl-CoA then crosses the inner mitochondrial membrane into the intermembrane space via a P molecule. Once in the intermembrane space, CPT I converts acyl-CoA to acylcarnitine. Acylcarnitine is transported across the inner mitochondrial membrane via T. Once in the matrix, acyl-CoA is regenerated with the help of CPT II. Monolyso-CL acyltransferase on the outer mitochondrial membrane may be able to obtain acyl-CoA either from the cytosol or from the pool in the intermembrane space.

CHAPTER 5: SIGNIFICANCE OF RESULTS

The studies presented above showed that monolyso-CL acyltransferase activity in the rat heart was not affected by insulin. It was also demonstrated that the reacylation of monolyso-CL to CL was acyl-CoA-dependent. FABP, ACBP, and LBP had no effect on monolyso-CL acyltransferase activity and other acyl-CoA species were able to interfere with incorporation of both linoleoyl-CoA and oleoyl-CoA into monolyso-CL. The only exception was that myristoyl-CoA did not affect linoleoyl-CoA incorporation. In the liver, monolyso-CL acyltransferase activity was found on the outer membrane. However, this in no way discounted the possibility that it may be also located in the inner membrane.

These studies have given further insight into the dynamics of CL molecular remodelling. It was speculated that remodelling might be closely associated with CL synthase activity. We would expect monolyso-CL acyltransferase activity would be upregulated at the same time that CL synthase activity was increased. This would ensure that newly synthesised CL is remodelled to the appropriate molecular species. The fact that insulin did not increase CL synthase activity (Cao *et al.*, 1995) and that this was followed by no increase in monolyso-CL acyltransferase activity lend credence to this theory. It is known that the decline in CL in aged rats could be reversed upon addition of acetyl-L-carnitine (Paradies *et al.*, 1995; Paradies *et al.*, 1994). It would be interesting to study whether this is due to an increase in CL synthase activity. If it was indeed due to an increase in CL synthase activity, it would be of further interest to see if this is accompanied by an increase in monolyso-CL acyltransferase activity.

These studies have also provided insight into the importance of remodelling. As was discussed in Chapter 1, CL displays an almost homogeneous acyl composition in the liver, skeletal muscle, and heart. This specificity in species is thought to be important for optimal activity of mitochondrial enzymes. However, the study of interference with incorporation of linoleoyl-CoA and oleoyl-CoA into monolyso-CL by other acyl-CoA's show that the enzyme may not be particularly selective for either linoleoyl-CoA or oleoyl-CoA. The question of why this can be the case and yet CL still can maintain an extremely high amount of linoleic acyl tails is puzzling. As the majority of phospholipids already contain an unsaturated species at the *sn-2* position, perhaps it is not as necessary to remodel CL at this position and therefore selectivity is not necessary to maintain a particular molecular species. The *sn-1* positions of phospholipids generally contain a saturated acyl species. Thus, it may be of interest to study remodelling at the *sn-1* position as selectivity at this site may be more important.

CL is believed to be involved with the mitochondrial contact sites (Ardial *et al.*, 1990). The contact sites are important for the import of proteins and other molecules into the mitochondria. The finding that monolyso-CL acyltransferase activity is found on the outer membrane leads to a speculation that the specific molecular species of CL may be important either for import of molecules or for the formation of the contact sites.

Why is acyl composition of CL seemingly so homogeneous? Evidence has shown that molecular species is important for the optimal activity of oxidative phosphorylation (Yamaoka-Koseki *et al.*, 1991; Rusnak *et al.*, 1997). However, only heart, liver, and skeletal muscle are among the tissues that show this near homogeneity of linoleic acyl tails. Why do

other tissues not show such high levels of linoleoyl tails? The question of how this high level of linoleic acyl tails is maintained remains unanswered.

It possible that monolyso-CL acyltransferase on the outer membrane may play roles other than the specific remodelling of CL. For example, the recent finding that lysophosphatidic acid acyltransferase acylation of lysophosphatidic acid is important to induce fission of Golgi membranes (Weigert *et al.*, 1999). As CL is found in large amounts in the contact sites, perhaps reacylation may be important in the association and dissociation of the inner and outer membranes. Perhaps monolyso-CL acyltransferase may even play an important role in fission during mitochondrial replication.

Whether or not defects in this enzyme have any clinical significance have yet to be shown. One may anticipate that the mitochondrial enzymes may show lower activity with a defect in monolyso-CL acytransferase. Purification of the enzyme may be the next logical step in studying this enzyme. Purification may allow for more precise characterisation of the importance of the enzyme through immunoprecipitation and knockout experiments. It will also allow more sensitive assays to be developed to determine whether the increase in activity seen with thyroxin was due to an increase in enzyme synthesis or in enzyme activation.

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