Regulation of Cardiolipin Remodeling in Hypoglycemia-Induced Apoptosis

By

Maria Danos

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Pharmacology and Therapeutics

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Cardiolipin is a major mitochondrial membrane phospholipid in the mammalian heart and contains a primarily tetra-linoleoyl molecular species composition in this organ. The molecular remodeling of cardiolipin in the heart is essential to maintain this unique fatty acyl composition. The enzyme monolysocardiolipin acyltransferase catalyzes the acylation of monolysocardiolipin to cardiolipin in the molecular remodeling of cardiolipin in mammalian tissues. Monolysocardiolipin has been found to play a crucial role in apoptosis by binding to truncated Bid and causing the mitochondrial release of cytochrome *c*. Entry of a cell into hypoglycemia-induced apoptosis may be prevented when the cardiolipin molecular composition is maintained, and this maintenance may be mediated by monolysocardiolipin acyltransferase activity. The purpose of this project was to determine whether monolysocardiolipin acyltransferase activity plays a role in preventing hypoglycemia-induced damage to the cardiolipin molecule. Cardiolipin *de novo* biosynthesis and remodeling were examined in the surviving population of H9c2 cardiac myoblast cells exposed to metabolic hypoxia. H9c2 cells were induced to undergo apoptosis by addition of 2-deoxyglucose to induce hypoglycemia. H9c2 cells were incubated in the absence or presence of 2-deoxyglucose for 16 hours. Dead cells were removed and the surviving population of cells were incubated with either [1,3-^{3}H]-glycerol or [1-^{14}C]-linoleic acid (bound to albumin 1:1 molar ratio) and the radioactivity incorporated into cardiolipin was determined. The cardiolipin pool

size in H9c2 cells treated with 2-deoxyglucose was also determined. In addition, the activities of the cardiolipin biosynthesis and remodeling enzymes, monolysocardiolipin acyltransferase protein and mRNA levels were determined. The [1,3-^{3}H]-glycerol or [1-^{14}C]-linoleic acid incorporated into cardiolipin was unaltered in 2-deoxyglucose-treated cells compared to controls. Then too, the cardiolipin pool size, determined by phosphorous mass, was unaltered in these cells. In addition, the activities of the cardiolipin *de novo* biosynthetic enzymes were unaltered. Surprisingly, mitochondrial phospholipase A_2 activity was increased 33% ($p<0.05$) and monolysocardiolipin acyltransferase activity 1.7-fold ($p<0.05$) in 2-deoxyglucose-treated cells compared to controls. The increase in monolysocardiolipin acyltransferase activity was due to an elevated expression in monolysocardiolipin acyltransferase protein and this was due to increased mRNA expression. This shows that remodeling/reacylation of cardiolipin was increased in 2-deoxyglucose treated cells. Thus, a compensatory increase in monolysocardiolipin acyltransferase activity and expression occurs in order to maintain the cardiolipin pool size in H9c2 cells exposed to chemically induced hypoxia, in which phospholipase A_2 activity is elevated. Thus, the increase monolysocardiolipin acyltransferase is protective against accumulation of monolysocardiolipin and apoptosis.

ACKNOWLEGMENTS

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2-DG	2-deoxyglucose
AIF	Apoptosis-inducing factor
ATP	Adenosine-5'- triphosphate
CDP-DG	Cytidine-5'-diphosphate-1,2-diacylglycerol
CDS	CDP-DG synthetase
CL	Cardiolipin
CLS	Cardiolipin synthase
CPT-cAMP	8-(4-chlorophenylthio)-cAMP
CTP	Cytidine-5'-triphosphate
cyt *c*	Cytochrome *c*
DMEM	Dulbecco's modified Eagle's medium
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
G3P	Glycerol-3-phosphate
lyso-PG	Lysophophatydylglycerol
MLCL	Monolysocardiolipin
MLCL AT	Monolysocardiolipin acyltransferase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide
PA	Phosphatidic acid
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine

PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGP	Phosphatidylglycerolphosphate
PGPP	PGP phosphatase
PGPS	PGP synthase
PI	Phosphatidylinositol
PLA_2	Phospholipase A_2
PP_i	Pyrophosphate
PS	Phosphatidylserine
P/S	Penicillin/streptomycin
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
t-Bid	Truncated Bid
TNF-α	Tumor necrosis factor α

INTRODUCTION

Biological Membranes

Biological membranes of various kinds are fundamental to the organization of cells. For instance, the plasma membrane (cell membrane) encloses the cell, defines its boundaries, and maintains the essential differences between the cytosol and the extracellular environment (Campbell, 1987). In eukaryotic cells, biological membranes compartmentalize organelles within the cell, such as the nucleus, mitochondria, and endoplasmic reticulum (ER) maintaining their integrity. However, despite their differing functions, all biological membranes have a common general structure with the two main constituents being lipid and protein molecules, which together make up a thin film. The lipid molecules of biological membranes are arranged as a continuous double layer, known as the lipid bilayer. The lipid bilayer provides the basic membrane structure and serves as a relatively impermeable barrier to most water-soluble molecules (Alberts et al., 1983). The protein molecules are embedded in this lipid bilayer or are attached to its surfaces and mediate most of the other functions of the membrane. For example, proteins are responsible for the transport of specific molecules across the membrane and catalyzing membrane-associated reactions, such as adenosine-5'- triphosphate (ATP) synthesis. Each membrane has a unique composition of lipids and proteins appropriate to that membrane's specific functions (Alberts et al., 1983).

Phospholipid Function

The most abundant lipids in biological membranes are the phospholipids. Phospholipids are important structural and functional components of all biological membranes that define the compartmentalization of organelles within cells and the protective barrier, the cell membrane that surrounds cells (White, 1973). Phospholipids are small amphipathic molecules with hydrophilic heads and hydrophobic or lipophilic fatty acid tails (Campbell, 1987). The fatty acid tails can differ in length and normally contain between 14 and 24 carbon atoms. One of the fatty acid tails usually contains one or more double bonds (it is unsaturated), while the other tail does not (it is saturated). Differences in the length and saturation in the fatty acid tails of phospholipids are important and the degree of unsaturation influences the fluidity of the membrane (Alberts et al., 1983).

A typical phospholipid is composed of a glycerol bonded to two fatty acids and a phosphate group (Figure 1) (Alberts et al., 1983). Most phospholipids also have an additional chemical group bound to the phosphate group that differentiates the various phospholipids. For example, it may be connected with choline resulting in the phospholipid phosphatidylcholine (PC). Other phospholipids include phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylethanolamine (PE). The bipolar character of phospholipids is essential to their biological function in cell membranes and causes phospholipids to form bilayers spontaneously in

aqueous solution. Cell membranes contain a variety of different phospholipids that are distributed asymmetrically across the bilayer.

Cardiolipin

An important class of phospholipids are the polyglycerophospholipids (Hostetler, 1982). Polyglycerophospholipids are a class of phospholipids that contain 2 or 3 glycerol molecules joined together via a phosphodiester linkage. This class of phospholipids also contains 2-4 fatty acid chains of varying composition, joined via ester linkages to the glycerol moieties.

The major polyglycerophospholipid found in most mammalian tissues is bis-(1,2-diacyl-*sn*-glycero-3-phospho)-1',3'-*sn*-glycerol or cardiolipin (CL) (Hostetler, 1982). CL is a unique phospholipid dimer in which two phosphatidyl moieties are linked by a central glycerol group (Figure 2). Hence, unlike other phospholipids, CL consists of four fatty acid chains, which are occupied by monounsaturated and diunsaturated chains of 16-18 carbons in length (Schlame et al., 1993). However, mitochondrial CL is distinguished from all other phospholipids by the great abundance of linoleoyl molecular species (Schlame et al., 1993). In rat heart, cardiolipin contains 81%-88% linoleate and 8%-12% oleate as the major fatty acids (Ma et al., 1999; Mutter et al., 2000). This unique fatty acyl composition of the CL molecule is believed to be important for its proper biological functions. For instance, the hydrophobic double unsaturated linoleic diacylglycerol species appears to be the important structural requirement for the

Figure 1. Typical structure of a phospholipids; containing two fatty acid chains, a glycerol and a phosphate group. X represents various head groups differentiating numerous phospholipids.

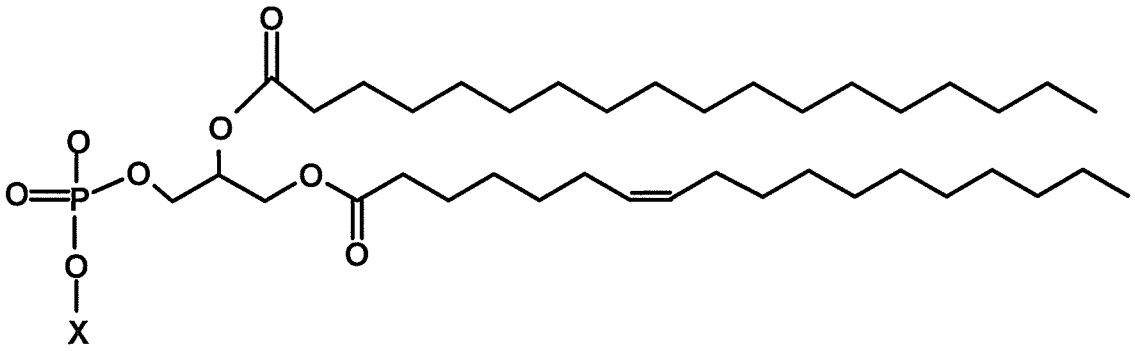
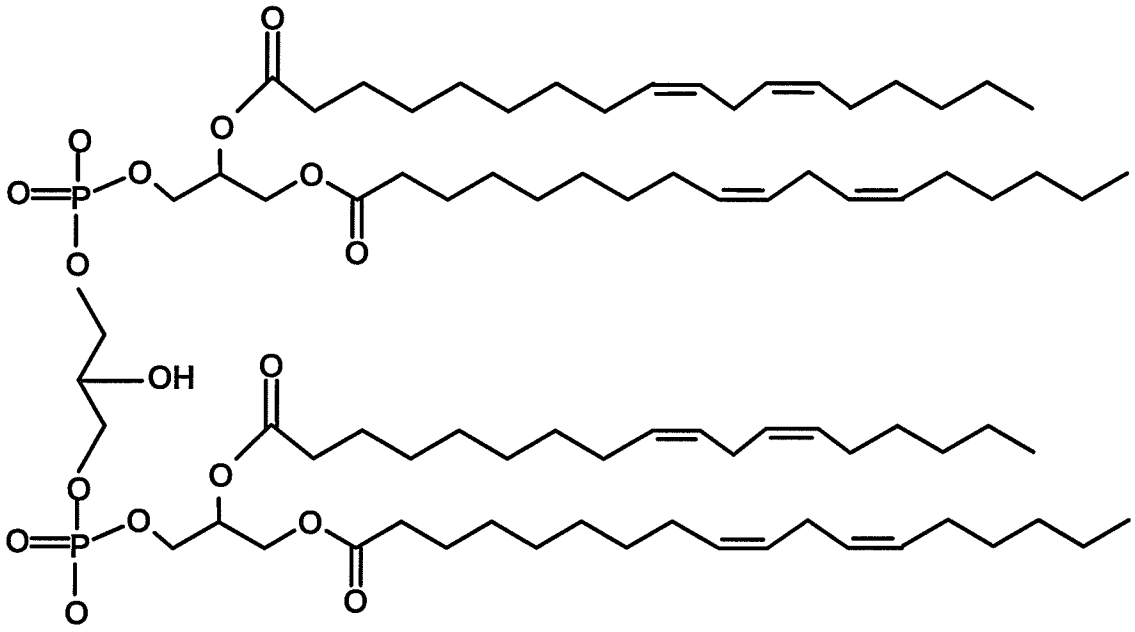


Figure 2. The structure of cardiolipin; containing 4 fatty acid chains, 3 glycerol molecules, and 2 phosphate groups.



high protein binding affinity of CL (Schlame and Rustow, 1990).

CL was first discovered and isolated from beef heart by Pangborn in 1942 (Pangborn, 1942). This phospholipid is present in all mammalian cells containing mitochondria, but it is most abundant in cells of tissues rich in mitochondria such as cardiac and skeletal myocytes (Hatch, 2004). For instance, in the heart, CL represents approximately 12–16% of the entire cardiac phospholipid mass (Hatch, 1994), and comprises 21% of the phospholipid mass of the inner mitochondrial membrane (Krebs et al., 1979). CL is synthesized and found exclusively in the mitochondria (Hostetler, 1982; Hatch, 1994). CL provides for diverse roles in the regulation of various mitochondrial processes including electron transport, mitochondrial lipid and protein import, and apoptosis (Hatch, 2004).

CL is associated with the inner mitochondrial membrane, where it has been shown to be required for the activation of a number of key mitochondrial membrane enzymes involved in the generation of ATP (Hoch, 1992; Hatch, 1998; Schlame et al., 2000). It is required for the reconstituted activity of key mitochondrial enzymes involved in cellular energy metabolism including cytochrome *c* oxidase (Vik et al., 1981), carnitine palmitoyltransferase (Fiol and Bieber, 1984), creatine phosphokinase (Muller et al., 1985), pyruvate translocator (Hutson et al., 1990), tricarboxylate carrier (Kaplan et al., 1990), glycerol-3-phosphate dehydrogenase (Belezani and Janesik, 1989), phosphate transporter

(Kadenbach et al., 1982), ADP/ATP translocase (Hoffman et al., 1994), and ATP synthase (Eble et al., 1990; Hoch, 1992). Under experimental conditions in which CL was removed or digested away from these proteins with phospholipases, denaturation and complete loss in activity of many of these proteins was observed (Sedlak and Robinson, 1999). The CL interaction with these mitochondrial proteins was specific since substitution with other phospholipids did not fully reconstitute activity. For example, the activity of delipidated rat liver cytochrome c oxidase was reconstituted by the addition of CL (Yamaoka et al., 1991). Depletion of CL from these enzymes resulted in a 60-70% decrease in activity, but enzyme function is restored after exogenous CL specifically bound to two high-affinity CL sites (Robinson et al., 1990). The ADP/ATP translocator has been shown to be tightly associated with six molecules of CL and this is an absolute requirement for translocator activity (Beyer and Klingenberg, 1995; Beyer and Nuscher, 1996).

An alteration in content and molecular species composition of CL has been shown to affect the activities of electron transport chain enzymes and will alter oxygen consumption in mitochondria (Yamaoka et al., 1990, 1991; Ohtsuka el al., 1993; Nomura et al., 2000). For example, in rat heart subjected to ischemia and reperfusion, the reduction in complex III activity is coupled with a reduction in cardiolipin (Petrosillo el al., 2003). Thus, it is suggested that CL is the 'glue' that holds the mitochondrial respiratory chain together (Zhang et al., 2002; Pfeiffer et al., 2003). Hence, maintenance of both the appropriate content

and fatty acid (molecular) composition of CL in mitochondria is critical for proper cellular function (Hatch, 2004).

Cardiolipin de novo Biosynthesis

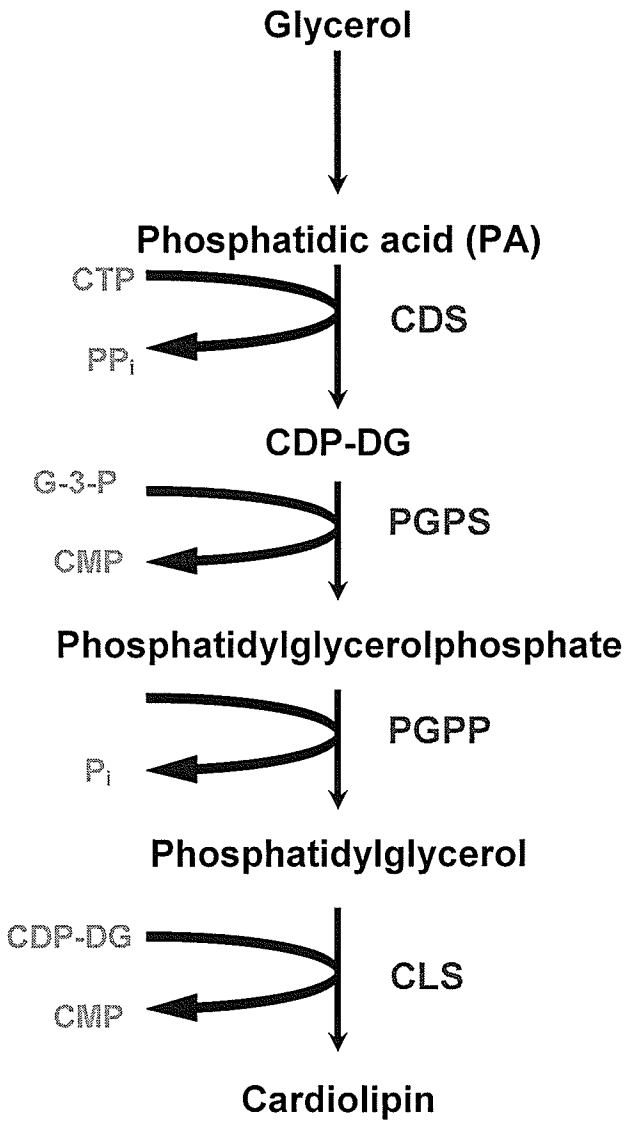
The *de novo* biosynthesis of CL proceeds on the inner leaflet of the inner mitochondrial membrane via the cytidine-5'-diphosphate-1,2-diacylglycerol (CDP-DG) pathway (Figure 3) (Schlame and Haldar, 1993; Hatch, 1994). In the first step of this pathway, phosphatidic acid (PA) is condensed with cytidine-5'-triphosphate (CTP) to form cytidine-diphosphate-1,2-diacyl-*sn*-glycerol (CDP-DG) with the release of pyrophosphate (PP_i) catalyzed by CDP-DG synthetase (CDS), also known as PA:CTP cytidylyltransferase (Kiyasu et al., 1963). Our laboratory demonstrated that this is a major rate limiting step of CL biosynthesis in myoblastic heart cells (Hatch, 1996). In the second step of the pathway, CDP-DG condenses with *sn*-glycerol-3-phosphate (G3P) to form phosphatidylglycerolphosphate (PGP) catalyzed by PGP synthase (PGPS). In several model systems, the level of PGPS activity may regulate the production of CL (Xu et al., 1999). The PGP formed from PGPS does not accumulate in cells and is rapidly converted into PG by PGP phosphatase (PGPP) (Kiyasu et al., 1963). In the final step of the CL biosynthetic pathway, PG is condensed with another molecule of CDP-DG to form a molecule of CL catalyzed by CL synthase (CLS) (Hostetler and van den Bosch, 1972). Our laboratory has recently cloned and characterized human CLS (CLS-1) (Lu et al., 2006). Biosynthesis of CL results in the unique di-phosphatidylglycerol structure.

CL and PG synthesis occur in the mitochondria (Hatch, 1994; Hostetler and van den Bosch, 1972; Schlame and Haldar, 1993), although PG may also be synthesized in microsomes (Hostetler and van den Bosch, 1972). PA is formed in the outer mitochondrial membrane and the ER, in addition to the inner mitochondrial membrane (Hostetler et al, 1982). PGPS and PGPP are found to be essential components of the inner mitochondrial membrane, which regulate levels of PGP and ultimately CL for growth and survival (Hatch, 2004). CDS and CLS are also associated with the inner mitochondrial membrane where the hydrophilic domains are exposed to the matrix side. Therefore, newly synthesized CL, PG and CDP-DG in the inner membrane are accessible to newly imported protein in the matrix (Schlame and Haldar, 1993).

Regulation of Cardiolipin Biosynthesis

H9c2 cardiac myoblast cells, derived from embryonic rat heart, appear to be a useful model to study mitochondrial polyglycerolphospholipid metabolism, since these cells actively take up and incorporate fatty acid and glycerol precursors into PG and CL (Hatch and McClarty, 1996). Incubation of H9c2 cardiac myoblast cells with 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) stimulates [1,3-^{3}H]-glycerol incorporation into PG and CL via activation of mitochondrial PGPS, indicating that the enzyme may be modulated by a protein kinase A mediated phosphorylation mechanism (Xu et al., 1999). Levels of ATP and CTP also regulate CL synthesis, given that production of CDP-DG requires CTP, and ATP is needed to produce CTP (Hatch and McClarty, 1996). In H9c2 cardiac

Figure 3. The *de novo* biosynthesis of mammalian cardiolipin. Condensation of PA with CTP to form CDP-DG with release of inorganic phosphate is catalyzed by CDP-DG synthetase (CDS). The committed step adds glycerol-3-phosphate (G3P) to CDP-DG yielding PGP catalyzed by PGP synthase (PGPS). The phosphate is removed by PGP phosphatase (PGPP). The final step is the condensation of PG and CDP-DG to form CL with release of CMP catalyzed by CL synthase (CLS).



myoblast cells a reduction of cellular CTP levels reduces *de novo* cardiolipin biosynthesis from glycerol, but not its resynthesis from linoleic acid (Hatch and McClarty, 1996). In addition, tumor necrosis factor α (TNFα) and short-chain ceramides (N-acetylsphingosine, N-hexanoylsphingosine) activate mitochondrial PGPS and promote [1,3- ^{3}H]-glycerol incorporation into PG and CL in H9c2 cells (Xu et al., 1999). The TNFα-mediated activation of mitochondrial phospholipase A_2 (PLA_2) activity in H9c2 cells occurs rapidly and could be an early event associated with the initiation of mitochondrial-mediated apoptosis (Xu et al., 1999). The above data in H9c2 cells clearly indicate that PG and CL metabolism may be regulated either directly or indirectly by a mechanism involving phosphorylation and dephosphorylation (Xu et al., 1999).

In addition to phosphorylation regulation, cardiac mitochondrial PGPS activity appears to be under hormonal regulation. An increase in CL and PG synthesis and activity of PGPS and CLS are observed with elevated thyroid states (Cao et al., 1995). PGPS activity was increased in thyroxine-treated animals compared with controls and this may account for the elevated levels of PG observed in these organs (Cao et al., 1995). CLS activity also appears to be hormonally regulated. Thyroxine treatment of rats stimulates the activity of both liver and cardiac mitochondrial CLS approximately 2.5-fold and this may account for the elevated levels of CL observed in these organs (Hostetler, 1991; Cao et al., 1995; Mutter et al., 2000). In addition, CLS was shown to be elevated in heart mitochondria prepared from hyperthyroid rats and this was correlated with

an increase in cardiac CL content (Mutter et al., 2000). CLS requires Co^{2+} as a cofactor, presence of phosphatidylethanolamine and alkaline pH (Schlame and Haldar, 1993) for activation, and is inhibited by cations such as Cd^{2+}, Zn^{2+} Ca^{2+} and Ba^{2+} (McMurray and Jarvis, 1980). CLS activity is also dependent on the assembly of the mitochondrial membrane respiratory complex IV (Jiang et al., 1999), where studies in yeast demonstrated that mutants of cytochrome c oxidase showed significantly decreased CLS activity (Zhao et al., 1998). CLS activity is also dependent on the pH of the respiratory electron transport chain (Gohil et al., 2004).

Cardiolipin Remodeling

Remodeling of a phospholipid is defined as the change of one molecular species into another (Lands, 2000). Phospholipases and acyltransferases are generally regarded as the principal enzymes involved in phospholipid remodeling in mammalian tissues (MacDonald and Sprecher, 1991). Phospholipids can be catabolized through the action of various phospholipases. The different types of phospholipases are categorized according to the hydrolysis of specific bonds in a phospholipid. Phospholipase A_1 specifically cleaves the acyl group at the sn-1 position, while cleavage of the acyl group at the sn-2 position is catalyzed by phospholipase A_2 (PLA_2) (van den Bosch et al., 1965). Phospholipases C and D are responsible for cleavage at the phosphate group (Figure 4). William Lands (1960) first introduced a scheme for the deacylation/reacylation of phospholipids

based on the presence of both PLA_2 and acyltransferase activities in mammalian tissues.

The formation of the unique fatty acyl content of CL does not occur in the *de novo* biosynthesis, since the enzymes of this pathway lack appropriate substrate selectivity (Hostetler et al.,1975; Rustow et al., 1989; Schlame et al., 1986). The appropriate fatty acyl composition is particularly important for CL since an abnormal composition would alter the ability of CL to bind to proteins and stimulate the activity of enzymes in the mitochondrial membrane (Schlame et al., 1990; Vik et al., 1981). In rat heart, cardiolipin contains 81%-88% linoleate and 8%-12% oleate as the major fatty acids (Ma et al. 1999; Mutter et al. 2000). Hence, newly synthesized CL must be rapidly remodeled to obtain the observed enrichment of CL with linoleate (Hostetler et al. 1975).

Although, the deacylation/reacylation cycle was first described by William Lands several decades ago (Lands, 1960), it was not until 1990 that a deacylation-reacylation cycle for the molecular remodeling of endogenous CL in rat liver mitochondria was proposed (Schlame and Rustow, 1990). In the remodeling pathway, CL is deacylated by PLA_2 to from monolysocardiolipin (MLCL) (Figure 5). The resulting MLCL molecule is then reacylated by monolysocardiolipin acyltransferase (MLCL AT), utilizing linoleoyl-Coenzyme A to produce remodeled CL (Schlame and Rustow, 1990; Ma et al., 1999).

Figure 4. Types of phospholipases. Phospholipase A_1 specifically cleaves the acyl group at the sn-1 position, while phospholipase A_2 cleaves the acyl group at the sn-2 position. Phospholipases C and D are responsible for cleavage at the phosphate group.

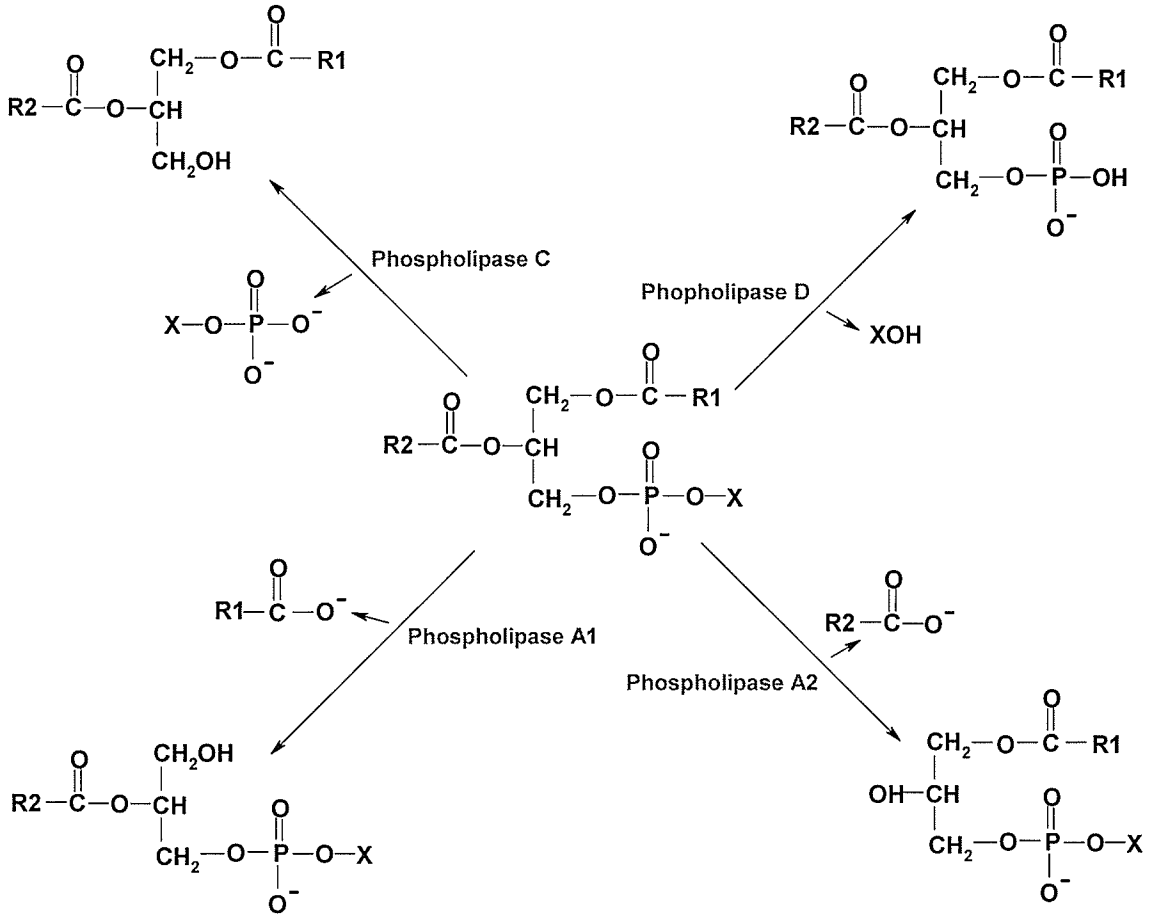
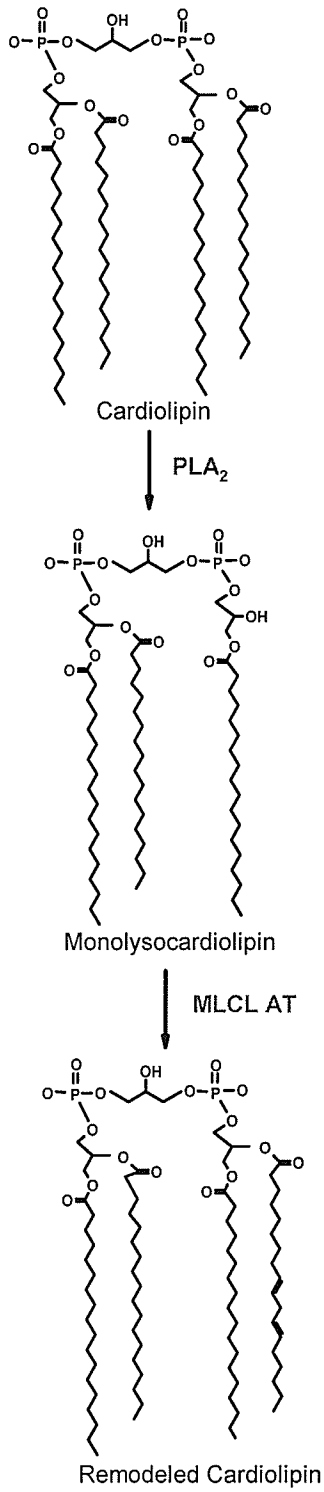


Figure 5. Cardiolipin remodeling pathway. Newly synthesized CL is deacylated by PLA_2 to produce the metabolite MLCL. MLCL is then reacylated with linoleoyl-CoA by MLCL AT.



Mitochondrial MLCL AT displays a molecular mass of 74 kDa and a ping-pong reaction mechanism with substrate specificity to MLCL (Taylor and Hatch, 2003).

Our laboratory purified a MLCL AT from pig liver mitochondria to apparent homogeneity, characterized its activity, and raised a polyclonal antibody to the protein (Taylor and Hatch, 2003). MLCLAT activity is observed in all tissues that contain mitochondria and exhibits a preference for unsaturated acyl-Coenzyme A substrates (Ma et al., 1999).

Regulation of Cardiolipin Remodeling

In rat heart, streptozotocin-induced diabetes and hyperinsulinemia appear not to affect MLCL AT activity or CL remodeling (Taylor et al., 2002; Hatch et al., 1995). However, in previous studies our laboratory observed that cardiac MLCL AT activity was elevated in hyperthyroid rats when CL biosynthesis and CL levels are elevated and reduced in hypothyroid rats in which CL biosynthesis and CL levels are reduced (Mutter et al., 2000; Taylor et al., 2002; Taylor and Hatch, 2003). In hyperthyroid rats, the elevation in cardiac MLCL AT activity and protein expression is likely required to maintain the appropriate fatty acid composition of CL, as the CL levels increased within the heart. Studies in rat heart and liver mitochondria indicate that MLCL AT may be a rate-limiting enzyme for the molecular remodeling of CL (Taylor and Hatch, 2003; Mutter et al., 2000). It is clear that the expression of MLCL AT is under thyroid hormone control in thyroid responsive tissues. Studies have demonstrated that MLCL AT activity may be

regulated by CLS activity and CL content in the membrane, where an increase or decrease in CL and CLS activity were accompanied by an increase or decrease in MLCL AT activity (Taylor et al., 2002). Maintenance of appropriate content and fatty acid composition of CL in mitochondria is essential for proper cellular function, and the CL *de novo* synthesis and remodeling are required for this maintenance (Hatch, 2004).

Cardiolipin Function

Role of Cardiolipin in Aging

CL composition is altered during the aging process. Reduced CL levels and altered molecular composition are associated with mitochondrial decay in aging (Shigenaga et al., 1994). For example, rat heart CL decreased with age and cardiac cytochrome c oxidase and adenine nucleotide translocase activities were correspondingly reduced (Paradies et al., 1992, 1994). The hearts from aged rats exhibit decreased carnitine palmitoyltransferase I activity correlating with an apparent decrease in CL content compared with young animals (McMillin et al., 1993). Also, with aging CL becomes more susceptible to oxidation (peroxidation). In rodents, the sensitivity of CL to peroxidation increases with age and this appears to be in part attributable to the replacement of 18:2 with the more readily peroxidizable 22:4 and 22:5 (Langaniere and Yu, 1993). Also, in patients with complex I deficiency, muscle cytochrome c oxidase activity decreases with age, possibly because of peroxidation of unsaturated fatty acids in CL (Scholte et al., 1995).

Cardiolipin and Apoptosis

In addition to its key role in support of mitochondrial oxidative phosphorylation, CL metabolism has been implicated in mitochondrial-mediated apoptosis (McMillin and Dowhan, 2002; Wright et al., 2004; Esposti, 2002, 2004; Fariss et al., 2005). The role that cardiolipin plays in the apoptotic process has been extensively reviewed (McMillin and Dowhan, 2002; Esposti, 2002). Previous evidence has shown that mitochondria have a crucial role in apoptosis by releasing apoptotic factors such as cytochrome *c* (cyt *c*) and apoptosis-inducing factor (AIF) from the intermembrane space into the cytoplasm (McMillin and Dowhan, 2002). Cyt *c* released from the mitochondria is an important pro-apoptotic signal in the mitochondrial death pathway (Liu et al., 2004). The liberation of cyt *c* and that of AIF from mitochondria are irreversible in the process that leads to apoptosis (Liu et al., 2004).

CL shows a strong affinity for cyt *c* and the binding of cyt *c* to the inner mitochondrial membrane is known to involve CL (Brown, 1977). It has been shown that incubation of cardiomyocytes with palmitate reduces CL levels and this is associated with an increase in cyt *c* release from the inner mitochondrial membrane (Ostrander et al., 2001). In addition, oxidative damage to the CL molecule is associated with cyt *c* release and apoptosis (Nomura et al., 2000). Reactive oxygen species (ROS), which are free radicals produced by the mitochondrial electron transport chain, have been demonstrated to affect complex I activity by causing oxidative damage to CL (Paradies et al., 2002). A

strong positive correlation is established between fatty acid unsaturation of CL and antioxidant production in cells (Watkins et al., 1998). Peroxidation of CL induces release of cyt *c* from mitochondria into the cytosol and this is associated with the induction of apoptosis (Nomura et al., 1999). It has been well documented that a decreased level of CL in the inner mitochondrial membrane due to accelerated break-down or oxidative stress, leads to impaired cyt *c* binding, and increases the susceptibility of cells to undergo mitochondrial-mediated apoptosis (Shidoji et al., 1999; Ushmorov et al., 1999). In addition, the generation of CL hydroperoxides in mitochondria was found in apoptosis induced with 2-deoxyglucose (2-DG) (Nomura et al. 2000). Thus, the association of cyt *c* with CL is lost upon CL peroxidation.

Based upon these and several other studies, it appears that CL metabolism may play a central role in the pathway leading to cellular apoptosis. It was reported that CL was required for the specific targeting of truncated Bid (t-Bid) to mitochondria (Lutter et al., 2000). However, it has been shown that it is actually the metabolite MLCL, the key intermediate in CL remodeling that is crucial in apoptosis (Esposti et al., 2003). The molecule MLCL is generated by PLA_2 hydrolysis of CL during induction of apoptosis (Sorice et al., 2004). It is located on the outer membrane of the mitochondria where the remodeling of CL occurs (Eichberg, 1974; Hambrey and Mellors, 1975) and displays a high affinity for t-Bid (Esposti et al., 2003; Liu et al., 2005). Bid is a pro-apoptotic protein of the Bcl-2 family that is cleaved by caspase 8 to allow the active C-terminus end

of it (t-Bid) to move to the mitochondria and promote the release of cyt *c* triggering a cascade of caspases (Esposti et al., 2001; Liu et al., 2005).

It was previously demonstrated that addition of the proapoptotic factor tumor necrosis factor α (TNF- α) to H9c2 cells stimulated mitochondrial PLA_2 activity, which cleaves CL, disrupting energy metabolism, leading to apoptosis (Xu et al., 1999). Also, MLCL accumulates during Fas-mediated apoptosis as a byproduct of CL degradation by PLA_2 and enhances t-Bid binding to membranes (Esposti et al., 2003). When t-Bid binds to MLCL, it forms a complex (t-Bid-MLCL complex) that acts as a ligand for other Bcl-2 proteins Bax/Bak, causing mitochondrial membrane permeabilization (Zamzami and Kroemer, 2003; Crompton, 2000). Bax/Bak insert into the mitochondrial membrane, forming pores (Zamzami and Kroemer, 2003; Liu et al., 2004), allowing cyt *c* to be released from the intermembrane space to the cytoplasm (Epand et al., 2002; Scorrano et al., 2002). The released cyt *c* activates caspase 9 in concert with the cytosolic factors ATP and Apaf-1, and, as a result, caspase 3 is activated leading to apoptosis (Liu et al., 2004; Hardwick and Polster, 2002). Thus, apoptosis is thought to progress via binding of t-Bid to MLCL, followed by CL oxidation, which results in cyt *c* release. The released cyt *c* from mitochondria activates caspase 9 in concert with cytosolic factors ATP and Apaf-1, and then caspase 3 is activated (Jemmerson et al., 1999).

Objective and Hypothesis

Given the importance of loss of CL, and accumulation of its immediate metabolic product MLCL, in mitochondria-mediated apoptosis, it is possible that rapid CL synthesis and/or its resynthesis from MLCL is required in response to proapoptotic stimuli-mediated CL degradation to restore cellular homoeostasis and prevent the entry of cells into apoptosis. Hence, increased CL biosynthesis and resynthesis may be a protective mechanism against apoptosis.

We hypothesize that a cell's entry into hypoglycemia-induced apoptosis may be prevented or attenuated when the CL molecular composition is maintained, and this maintenance may be mediated by MLCL AT activity, the enzyme that catalyzes the acylation of MLCL to CL in the molecular remodeling of CL in mammalian tissues. In the present study we used the hypoglycemia model to determine whether MLCL AT activity plays a role in preventing hypoglycemia-induced damage to the cardiolipin molecule in H9c2 cardiac myoblast cells.

MATERIALS AND METHODS

Materials

Rat heart H9c2 cardiac myoblast cells derived from embryonic rat heart were obtained from American Type Culture Collection. 2-deoxy-D-glucose (2-DG) and Thiazolyl Blue Tetrazolium Bromide (MTT) were purchased from Sigma Chemical Company, St. Louis, USA. [U-^{14}C]glycerol-3-phosphate, [5-^{3}H]cytidine 5'-triphosphate, [1,3-^{3}H]glycerol, and [1-^{14}C]linoleic acid were obtained from either Dupont, Mississauga, Ontario, or Amersham, Oakville, Ontario, Canada. [1-^{14}C]linoleoyl-CoA was obtained from American Radiolabeled Chemicals Inc., St. Louis, USA. Phosphatidyl[^{14}C]glycerol ([^{14}C]PG) was synthesized from [U-^{14}C]glycerol-3-phosphate as described previously (Hatch, 1994). MLCL (a mixture of 1'(1-acyl-*sn*-glycerol-3-phosphoryl)-3'-(1",2"-diacyl-*sn*-glycerol-3-phosphoryl)glycerol and 1'-(1,2-diacyl-*sn*-glycerol-3-phosphoryl)-3'-(1"-acyl-*sn*-glycerol-3-phosphoryl) glycerol), produced by PLA_2 hydrolysis of bovine heart CL, was obtained from Avanti Polar Lipids, Alabaster, USA. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Canadian Life Technologies (GIBCO), Burlington, Ontario, Canada. Lipid standards were obtained from Serdary Research Laboratories, Englewood Cliffs, New Jersey, USA. Thin layer chromatographic plates (silica gel G, 0.25 mm thickness) were obtained from Fisher Scientific, Winnipeg, Canada. Ecolite scintillant was obtained from ICN Biochemicals, Montreal, Quebec, Canada. Western blotting analysis system was used for protein expression studies and

was obtained from Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England. Kodak X-OMAT film was obtained from Eastman Kodak Co., Rochester, NY., USA. QIAGEN OneStep RT-PCR kit was used for PCR studies, which included enzyme mix (containing Omniscript™ Reverse Transcriptase, Sensiscript™ Reverse Transcriptase, and HotStarTaq® DNA Polymerase), RT-PCR buffer, Q-Solution, dNTP Mix and RNase-free water. Mitochondrial MLCL AT and actin primers were obtained from Invitrogen, Burlington, Ontario, Canada, and were used in PCR studies. All other chemicals were certified ACS grade and obtained from either Fisher Scientific, Winnipeg, Manitoba, Canada or Sigma Chemical Company, St. Louis, USA.

Hypoglycemia-Induced apoptosis

H9c2 cells were induced to undergo apoptosis by exposure to hypoglycemia with 2-DG (Nomura et al., 2000). Glucose depletion was achieved by incubating cells in the presence of 100 mM 2-DG in low glucose DMEM, supplemented with 10% fetal bovine serum, for up to 16 h (Nomura et. al., 1999).

Culture, Radiolabeling and Harvesting of H9c2 Cells

H9c2 cells were cultured in DMEM supplemented with 10% (v/v) FBS, and 1% (v/v) penicillin/streptomycin (P/S). Cell cultures were maintained at 37°C in humidified air, containing 5% CO_2 until 80% confluence. Each dish of cells was subcultured at a 1:4 ratio and confluence was usually obtained after 4 days of incubation. Cells were maintained at a logarithmic growth phase for all

experiments. Cells were then incubated for up to 16 h with DMEM in the absence or presence of 100 mM 2-DG in the absence or presence of 0.1 mM [1,3-^{3}H]glycerol (10 µCi/dish) or 0.1 mM [1-^{14}C]linoleic acid (bound to albumin 1:1 molar ratio) (1 µCi/dish). Subsequent to incubation the medium was removed and the cells washed twice with 2 ml of ice-cold phosphate-buffered saline (PBS). The PBS was removed and 2 ml of methanol:water (1:1 v/v) was added. The cells were harvested using a rubber policeman into test tubes. The mixture was vortexed, and a 50 µl aliquot was taken for protein determination. 2 ml of chloroform was added to the tubes followed by 0.5 ml 0.9% NaCl to initiate phase separation. The tubes were vortexed and then centrifuged at 500 x g for 10 minutes (bench top centrifuge), the aqueous phase was removed, and 2 ml theoretical upper phase (48 ml methanol, 47 ml 0.9% NaCl, 3 ml chloroform) was added to wash the organic phase. The tubes were vortexed and centrifuged as described above and the aqueous phase removed. The organic phase was dried under a stream of N_2 gas and resuspended in 50 µl of chloroform:methanol (2:1 v/v). A 40 µl aliquot of organic phase was placed onto a thin-layer plate and phospholipids were separated by two-dimensional thin-layer chromatography on silica gel 60 plates developed with chloroform-methanol-water-ammonium hydroxide (70/30/3/2) in the first dimension and chloroform-methanol-water (65/35/5) in the second dimension. In some experiments, phospholipid standards were placed onto plates prior to chromatography. The phospholipids were detected with iodine vapour, scraped off into scintillation vials, and 5 ml Ecolite scintillant was added. Radioactivity incorporated into CL and other

phospholipids were determined by liquid scintillation using a Beckman scintillation counter.

Assessment of Cell Viability

Cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay (Scudiero et al., 1988). In this assay, a mitochondrial dehydrogenase enzyme from viable cells cleaves the tetrazolium rings of MTT and forms purple formazan crystals, which are largely impermeable to cell membranes. Thus, the number of surviving cells is directly proportional to the amount of formazan product created by the surviving cells. MTT was prepared at a concentration of 5 mg/ml in PBS and stored at 4°C. H9c2 cells were cultured as described above in the absence or presence of 100 mM 2-DG for 16 h. Subsequent to incubation 200 µl of MTT was added to the control and 2-DG treated dishes. After 1 h incubation with MTT at 37 °C, all medium was removed and 500 µl isopropanol/HCl solution (13.2 mls 2-propanol and 1.8 mls 0.2 HCl) was added to solubize the MTT-formazan product. These dissolved solutions were then pipetted into 96 well plates and read with an absorbance multiwell scanning spectrophotometer reader at 570 nm.

Lowry's Protein Assay

Protein was determined by Lowry protein assay (Lowry et al., 1951). Standard curve was prepared using bovine serum albumin (1mg/ml) in volumes of 0, 10, 20, 40 and 80 µl. In each sample, 100 µl 1% deoxycholic acid was

added and the tube vortexed. A 50:1 ratio mixture of 2% Na_2CO_3 in 0.1M NaOH and 0.5% $CuSO_4$ in 1% sodium citrate was prepared, and 2ml of this mixture was added to the samples, vortexed and allowed to sit for 10 minutes. A 1:1 H_2O: Folin reagent mixture was prepared and 200 µl was added to each sample, vortexed and allowed to sit for 30 minutes. The absorbance of each sample was measured at 660nm (A_{660}).

Determination of Phospholipid Pool Sizes

In order to determine the pool size of CL in H9c2 cells treated with 2-DG, the phosphorous mass of CL and other phospholipids were measured (Rouser et al., 1970). H9c2 cells were incubated in 100 mM 2-DG for 16 h and cells harvested as described above. A 50 µl aliquot of organic fraction was spotted on a borate-treated thin-layer plate. The plate was subjected to two-dimensional chromatography and placed in an iodine tank in order to stain and identify the location of the phospholipids on the silica gel. Phospholipids of interest on the silica gel were removed and placed in 13 x 100 mm test tubes. Standard curve was prepared using potassium phosphate (KH_2PO_4) (1 mM) in volumes of 0, 25, 50, 100, and 200 µl. Perchloric acid (70%) was added to each tube containing organic sample (500 µl) and the tubes were covered and heated at 180 °C for 90 min in a heater block. After allowing the tubes to cool, 2.5 ml of water and 0.5 ml 2.5 % ammonium molybydate were added and the tubes were vortexted. Next, 0.5 ml of 10% ascorbic acid was added, the tubes vortexed and then incubated in a hot water bath (95 °C) for 15 min. The tubes were allowed to cool to room

temperature before being centrifuged at 500 x g in order to sediment the silica gel. The absorbance of each sample was then measured on a spectrophotometer at 820nm (A_{820}). Absorbance values were converted to phosphorous mass by comparing readings to those obtained from the standard curve.

Preparation of Subcellular Fractions and Assay of Enzyme Activities

All isolation procedures were preformed on ice at 4°C. Subcellular fractions were prepared from H9c2 cells incubated plus or minus 100 mM 2-DG for 16 h as described above. Cells were harvested using PBS and pipetted into test tubes. The tubes were centrifuged at 500 x g for 10 min and the PBS removed. Addition of 1 ml homogenizing buffer (0.25 M sucrose, 10 mM Tris-HCl, 0.145 M NaCl, pH 7.4) was followed by homogenization by 50 strokes of a tight fitting Dounce A homogenizer. The homogenate was centrifuged at 1,000 x g for 10 min and the resulting supernatant centrifuged at 12,000 x g for 15 min. The resulting pellet was resuspended in 0.5 ml of homogenizing buffer by a tight fitting Dounce A homogenizer and used as the source of mitochondrial fraction for assay of mitochondrial enzyme activities.

CDS was assayed by a modification of the method of Carman and Kelley (1992). 0.02 mg of mitochondrial protein was added to a 16 mm x 100 mm glass test tube (all test tubes were treated with dimethyldichlorosilane, 20% in 1,1,1,-trichloroethane). Then, the following chemicals were added in order, 0.145 M

NaCl to a volume of 60 µl, 10 µl of 0.5 M Tris/maleate (pH 6.5; 0.5M Tris buffered with 1.0 M maleic acid solution), 10 µl of 10 mM [5-^{3}H]CTP (specific radioactivity 12000 DPM/nmol), 10 µl of 0.15 M Triton X-100, 5 mM PA (prepared by sonicating for 20 min a weighed sample of PA in the Triton solution). The reaction was started by addition of 10 µl of 0.2 M $MgCl_2$,$6H_20$. The mixture was incubated at 30 °C for 2 h. The reaction was then terminated by addition of 0.5 ml of 0.1 M HCl in methanol. For lipid extraction, 2 ml of chloroform and 0.7 ml of 0.9 % NaCl were added to cause phase separation. The mixture was centrifuged and the aqueous phase removed by suction. The lower phase was washed with 3 x 5 ml of the theoretical upper phase (chloroform/methanol/0.9 % NaCl, 1:45:47, by vol.), recentrifuged and the aqueous phase removed by suction. The lower phase was dried under a stream of N_2 gas and resuspended in 0.25 ml chloroform:methanol (2:1). Lipids were applied to silica gel plates and separated by two-dimensional thin layer chromatography with chloroform/methanol/water (65:25:4) in the first dimension and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5) in the second dimension. Lipids were stained with iodine and after evaporation of iodine, spots of silica gel were scraped and transferred into scintillation vials. Radioactivity was measured by liquid scintillation counting.

PGPS was assayed by a modification of the method of Carman and Belunis (1983). The incubation mixture contained 0.1 mg mitochondrial enzyme and 0.145 M NaCl added to a volume of 50µl, 10 µl of 0.5 M Tris/HCl (pH 7.0), 10

µl of β-mercaptoethanol and 10 µl of 5.0 mM [U-^{14}C]glycerol-3-phosphate (specific radioactivity 12500 DPM/nmol). The reaction was initiated by addition of 10 µl of 100 mM $MgCl_2$,$6H_20$. The mixture was incubated at 30 °C for 1 h. The reaction was then terminated by addition of 0.5 ml of 0.1 M HCl in methanol. For lipid extraction, 2 ml of chloroform and 0.7 ml of 0.9 % NaCl were added to cause phase separation. The mixture was centrifuged and the aqueous phase removed by suction. The lower phase was washed with 3 x 5 ml of the theoretical upper phase (chloroform/methanol/0.9 % NaCl, 1:45:47, by vol.), recentrifuged and the aqueous phase removed by suction. The lower phase was dried under a stream of N_2 gas and resuspended in 0.25 ml chloroform:methanol (2:1). Lipids were applied to silica gel plates and separated by two-dimensional thin layer chromatography with chloroform/methanol/water (65:25:4) in the first dimension and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5) in the second dimension. Lipids were stained with iodine and after evaporation of iodine, spots of silica gel were scraped and transferred into scintillation vials. Radioactivity was measured by liquid scintillation counting.

CLS was assayed exactly as described by Schlame and Hostetler (1992), except that the assay contained 0.1 mg of mitochondrial protein, the pH of the assay was 8.5 and the samples were sonicated for 10 seconds in a Branson model 1200 sonicator before incubation. Incubation was at 37 °C for 1 h with [^{14}C]PG (specific radioactivity 45000 DPM/nmol). The reaction was terminated by addition of 0.5 ml of 0.1 M HCl in methanol. For lipid extraction, 2 ml of

chloroform and 0.7 ml of 0.9 % NaCl were added to cause phase separation. The mixture was centrifuged and the aqueous phase removed by suction. The lower phase was washed with 3 x 5 ml of the theoretical upper phase (chloroform/methanol/0.9 % NaCl, 1:45:47, by vol.), recentrifuged and the aqueous phase removed by suction. The lower phase was dried under a stream of N_2 gas and resuspended in 0.25 ml chloroform:methanol (2:1). Lipids were applied to silica gel plates and separated by two-dimensional thin layer chromatography with chloroform/methanol/water (65:25:4) in the first dimension and chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5) in the second dimension. Lipids were stained with iodine and after evaporation of iodine, spots of silica gel were scraped and transferred into scintillation vials. Radioactivity was measured by liquid scintillation counting.

PLA_2 activity was determined as described using 50 µg of mitochondrial protein (Cao et al., 1995). The reaction mixture contained 100 µl of 0.25 M Tris/HCl, pH 8.5, 100 µl of 25 mM $CaCl_2$, 100 µl of 0.2 mM [U-^{14}C]PG (specific radioactivity 0.3 µCi/pmol), water and 50 µg of protein. The radioactive substrate PG was synthesized as described above, mixed with stock PG and dried under a stream of N_2. Water was added to this mixture and it was then vortexed and then sonicated for 10 min in a bath sonicator before use. The reaction was initiated by addition of [^{14}C]PG. The mixture was incubated at 37 °C for 30 min and terminated by the addition of 2 ml of chloroform, followed by the addition of 1 ml of 0.1 M HCl in methanol and 1 ml of 0.73% NaCl. The mixture was centrifuged

at full speed in a bench-top centrifuge, and the aqueous phase was removed by suction. The organic phase was dried under a stream of N_2 gas and resuspended in 25 µl chloroform:methanol (2:1, v/v). A 20 µl sample was separated on thin layer plates in the solvent system chloroform/methanol/7M NH_4OH (30:15:2, by vol.). A 5 µl sample of lysophophatIdylglycerol (lyso-PG) was placed on each plate as standard, and, after development of the plates, the lyso-PG spot detected with iodine was removed and the radioactivity in lyso-PG was determined.

For assay of MLCL AT activities, 25 µg fractions of protein were incubated for 1h at 37 °C in 50 mM Tris-HCl, pH 8.0, 33 µM [1-^{14}C]linoleoyl-CoA (specific radioactivity 14200 DPM/nmol) at pH 8.0, and 35 µL MLCL substrate in a final volume of 0.35 ml. The MLCL substrate in chloroform was dried under a stream of N_2 gas and resuspended in double-distilled water via sonication in a bath sonicator for 45 min prior to addition to the assay mixture. The reaction was initiated by the addition of the radioactive acyl-CoA substrate and was terminated by the addition of 3 ml of chloroform:methanol (2:1, v/v). To facilitate phase separation, 0.8 ml of 0.9% KCl was added. The aqueous phase was then removed and the organic phase was washed with 2 ml of theoretical upper phase (chloroform/methanol/0.9% NaCl, 3:48:47, by vol.). The resulting organic fraction was dried under nitrogen and resuspended in 50 µl of chloroform:methanol (2:1, v/v) and 40-µl was placed on a thin-layer plate. CL was separated from other phospholipids by two-dimensional chromatography with chloroform/

methanol/ammonium hydroxide/water (70/30/2/3) in the first dimension and chloroform/methanol/water (65/35/5) in the second dimension. Plates were stained with iodine and after evaporation of iodine, the silica gel spot corresponding to CL was removed and placed in a plastic scintillation vial, and 5 ml of scintillant was added. Radioactivity incorporated into CL was examined approximately 24 h later using a liquid scintillation counter.

Western Blot Analysis

H9c2 cells at 80% confluence were incubated in the absence or presence of 2-DG for 16 h. The cells were harvested, homogenized and mitochondrial fractions of H9c2 cells were obtained as above. Aliquots containing 25 µg of mitochondrial fraction protein were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% acrylamide gel with molecular weight standards, using a BioRad Mini-Protean® II Dual Slab Cell electrophoresis unit. Protein from the separating gel was transferred onto polyvinylidene fluoride (PVDF) membranes for 90 min at 15 volts using a BioRad Trans-Blot SD Semi-Dry Transfer Cell. The expression of MLCL AT was examined by immunoblotting in 10 ml Tris-buffered saline containing 0.1% Tween-20 with anti-MLCL AT polyclonal antibody (1:250 dilution) or (40 µl antibody dissolved in 10 ml Tris-buffered saline containing 0.1% Tween-20) at 4°C overnight. Subsequently, the membrane was washed and incubated with peroxidase labeled anti-rabbit IgG secondary antibody (1:1000 dilution) for 1 h at room temperature. Expression of β-actin was examined by incubating the PVDF membrane with anti-rat β-actin

antibody as described previously (Webster et al., 2005). Identification was according to ECL Western blotting analysis system. Protein bands in the membrane were visualized by enhanced chemiluminescence.

RT-PCR Analysis

The cDNA for MLCL AT was amplified with specific primers synthesized by Invitrogen™ Life Technologies. Total RNA was isolated from H9c2 cells using the TRIZOL Reagent according to the manufacturer's instructions. The treated cells were then pipetted into a small capped tube, 0.2ml of chloroform was added, and each sample was shaken vigorously and centrifuged at 14000 x g for 15min. The supernatant was removed to another tube and 0.5ml of isopropyl alcohol was added, causing the RNA to precipitate. This was centrifuged again at 14000 x g for 10min to obtain the RNA pellet, which was then washed with 75% ethanol and centrifuged for 5min. The ethanol was then removed and the RNA pellet was resuspended in autoclaved, double-distilled water and quantitated by absorbance at 260 nm using the 260:280 nm ratio as an index of purity. The integrity of the RNA was confirmed by denaturing agarose gel electrophoresis of the isolated RNA sample. The first strand cDNA from 1 μg total RNA was synthesized by employing 150 units of Moloney-murine leukemia virus reverse transcriptase (RT), 25 pmol of random hexamer primer, 20 units of ribonuclease inhibitor, 1 mM dithiothreitol, and 10 pmol each of the four deoxynucleotides, in a total volume of 15 μl. The reaction mixture was incubated at 37°C for 1 h and terminated by heating the sample to 95°C for 5 min. An

aliquot of the resultant cDNA preparation was used directly for each amplification reaction. The primers for rat MLCL AT: Forward 5'-AAC ACG GTC TTC GAT GCC AAG CGC CTC-3'; Reverse 5'-GGT CAA ACA CAA GGA TGT TCT TCT CTC-3'. PCR product length is 400 bp. The primers for rat β-actin: Forward 5'-GTG GGG CGC CCC AGG CAC CA-3'; Reverse 5'-CTC CTT AAT GTC ACG CAC GAT TTG-3'. PCR product length is 540 bp. Polymerase chain reaction (PCR) was performed in 20 μl reaction mixtures containing 8 pmol of primer, 8 pmol of each dNTP, and 0.4 unit of *Taq* DNA polymerase. The mixture was overlaid with 30 μl of mineral oil to prevent evaporation and was incubated in a Perkin-Elmer DNA Thermal Cycler under the following conditions for MLCL AT: denaturation 94^0C, 1 min; annealing 55^0C, 1 min; extension 72^0C, 2 min; repeat for 25 cycles. The amplified RT-PCR product was analyzed by 1.5% agarose gel electrophoresis in 1 X TAE buffer (40 mM Tris acetate and 2 mM sodium EDTA) and visualized by staining with 0.5 μg/μl ethidium bromide. The relative intensities of the bands were analyzed by scanning the film, and subsequently determined by Scion Image software.

Statistical Analysis

Student's t-test was used for determination of statistical significance. The level of significance was defined as $p<0.05$.

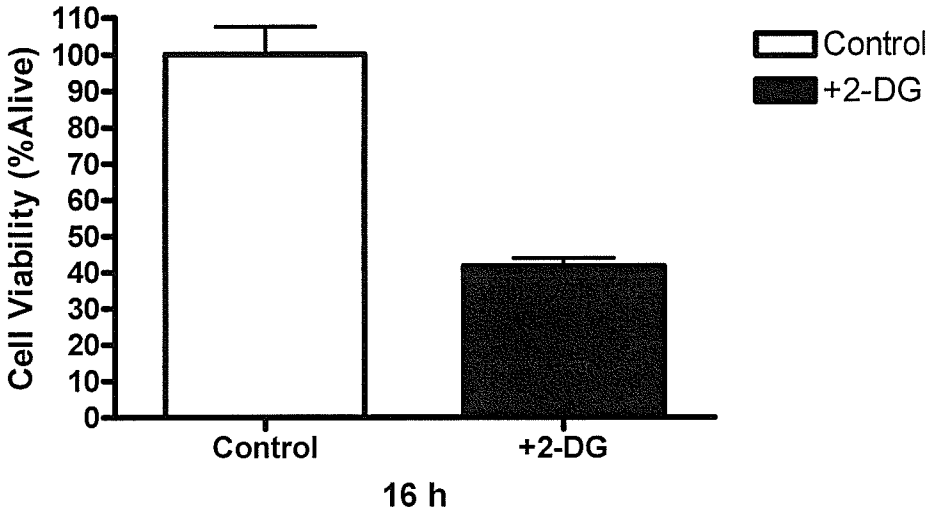
RESULTS

2-Deoxyglucose Induces Apoptosis in H9c2 Cells

To examine the effect of metabolic hypoxia on cardiolipin *de novo* biosynthesis and remodeling, H9c2 cells were exposed to 2-DG. 2-DG is a glucose analog which potently inhibits glycolysis leading to a metabolic hypoxia (Nomura, 1999). 2-DG has previously been shown to induce apoptosis by stimulating intracellular ROS production, cardiolipin oxidation, and the release of cyt *c*, a marker of apoptosis (Nomura, 2000; Imai et al., 2003). 2-DG incubation of H9c2 cells induces a rapid depletion of intracellular ATP and causes the release of cyt *c* from mitochondria and the induction of cell death after 16 h (Nomura, 1999). The cytotoxic effect of 2-DG has been previously demonstrated and the effect was both time- and dose-dependant with 16 h being optimal time, when the cells were exposed to 100 mM 2-DG (Nomura, 1999). Thus, in all experiments, the same dose and time period was used that has been previously observed to be optimal to induce cellular apoptosis.

Cell viability was examined by MTT assay to confirm apoptosis occurs after 16 h with 100 mM 2-DG. As seen in Figure 6, cell viability was decreased by 55% in the 2-DG treated cells as compared to the control cells. Thus, 16 h exposure to 100 mM 2-DG results in marked cell death in H9c2 cells.

Figure 6. MTT Assay for cell viability. H9c2 cells ±2-DG for 16 h were incubated with MTT for 1 h at 37 °C and cell viability was determined as described in Materials and Methods. Cell viability of control cells was set to 100% and cell viability in 2-DG treated cells is expressed as percentage of control cells.



Incorporation of Radioactivity into Cardiolipin

To determine if 2-DG treatment altered *de novo* biosynthesis of CL in the surviving population of H9c2 cells, cells were incubated for up to 16 h with 0.1 mM [1,3-^{3}H]glycerol in the absence or presence of 100 mM 2-DG and the radioactivity incorporated into CL in the surviving population of cells was determined as described in the Materials and Methods. As seen in Figure 7, there were no significant differences, at any time point, in the incorporation of [1,3-^{3}H]glycerol into CL between the control and the 2-DG treated cells. This data indicated that *de novo* CL biosynthesis from glycerol might not be affected in cells that survived 2-DG treatment.

To examine if CL remodeling was altered by 2-DG treatment in the surviving population of H9c2 cells, cells were incubated for up to 16 h with 0.1 mM [1-^{14}C]linoleic acid (bound to albumin 1:1 molar ratio) in the absence or presence of 100 mM 2-DG and the radioactivity incorporated into CL in the surviving population of cells was determined as described in the Materials and Methods. As seen in Figure 8, there were no significant differences, at any time point, in the incorporation of [1-^{14}C]linoleic acid into CL between the control and the 2-DG treated cells. This data initially indicated that remodeling of CL with [1-^{14}C]linoleic acid was not affected in cells that survived 2-DG treatment.

Figure 7. Incorporation of [1,3-^{3}H]glycerol into cardiolipin via the CDP-DG pathway. H9c2 cells ±2-DG were incubated with 0.1 mM [1,3-^{3}H]glycerol for up to 16 h and the radioactivity incorporated into CL determined as described in Materials and Methods. Values are expressed as DPM/mg of protein and represent the means of three separate experiments.

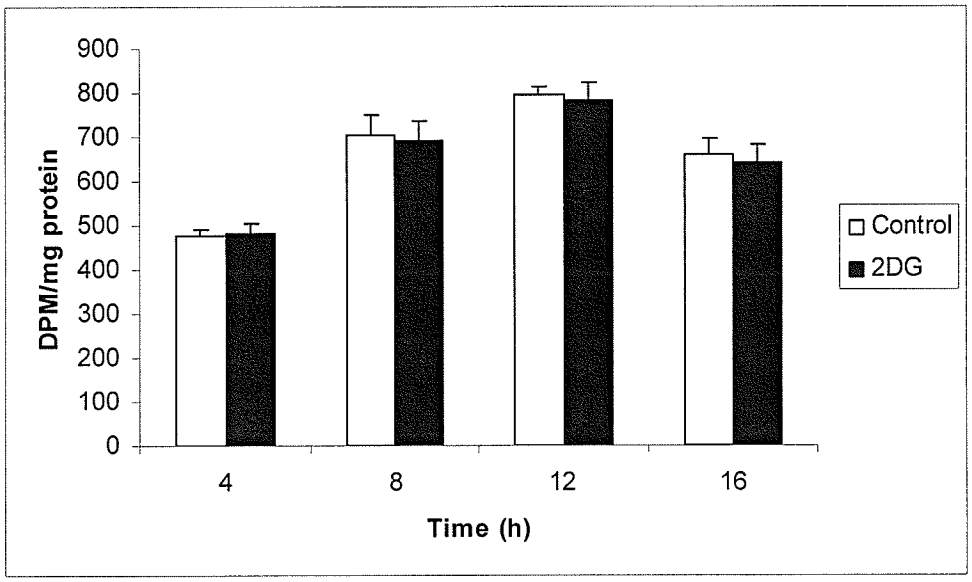
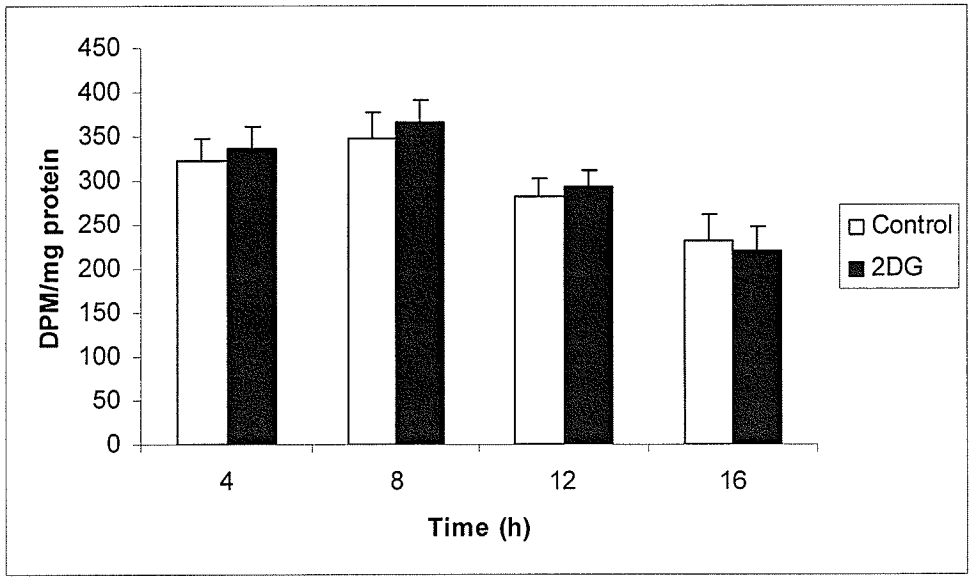


Figure 8. Incorporation of [1-^{14}C]linoleic acid into cardiolipin via the remodeling pathway. H9c2 cells ±2-DG were incubated with 0.1 mM [1-^{14}C]linoleic acid for up to 16 h and the radioactivity incorporated into CL determined as described in Materials and Methods. Values are expressed as DPM/mg of protein and represent the means of three separate experiments.



Cardiolipin Pool Size

To examine if the pool size of CL was altered by 2-DG treatment in the surviving population of H9c2 cells, phosphorous mass of CL in control and 2-DG treated cells was determined in the surviving population of cells as described in Materials and Methods. As seen in Table 1, there are no significant differences, between control and 2-DG treatment, in the pool sizes of any of the phospholipids. More specifically, the pool size of CL was 2.75 nmol/mg protein in control and 2.34 nmol/mg protein in the 2-DG treated cells. These data indicate that the CL pool size was unaltered in cells that survived 2-DG treatment.

Activities of Enzymes Involved CL Metabolism

To confirm that *de novo* CL biosynthesis was not affected by 2-DG treatment, the activities of the enzymes involved in the biosynthetic pathway of CL were determined in mitochondrial fractions prepared from the control cells and cells treated with 100 mM 2-DG for 16 h (as described in Materials and Methods). CDS, PGPS, and CLS activities were unaltered by 2-DG treatment compared to control (Table 2). These data confirmed that 2-DG treatment had no effect on the *de novo* biosynthesis of CL in the surviving population of H9c2 cells subjected to 2-DG mediated hypoxia.

To confirm that remodeling of CL with [1-^{14}C]linoleic acid was not affected by 2-DG treatment in surviving cells, the activities of the enzymes involved in CL remodeling were determined. Mitochondrial fractions were prepared from control

Table 1. Pool Size of phospholipids in H9c2 cells treated with 2-deoxyglucose. H9c2 cells were incubated ±2-DG for 16 h and the pool sizes for the phospholipids were determined as described in Materials and Methods. Values are expressed in nmol/mg of protein and represent the means and standard deviations of three dishes *p<0.05.

Phospholipid	**Control** nmol/mg protein	**2-DG**
CL	2.7 ± 0.4	2.3 ± 0.3
PE	39.3 ± 0.8	38.9 ± 0.9
PS	4.4 ± 0.3	3.9 ± 0.5
PC	79.9 ± 0.6	76.4 ± 0.6

and H9c2 cells treated with 100 mM 2-DG for 16 h and mitochondrial PLA_2 and MLCL AT activities determined as described in Materials and Methods. Remarkably, PLA_2 and MLCL AT activities were elevated 33% ($p<0.05$) and 1.6-fold ($p<0.05$), respectively, by 2-DG treatment compared to controls in the surviving population of H9c2 cells (Table 2). These results indicate that there was an elevation in the remodeling of CL in the surviving population of H9c2 cells treated with 2-DG and this was due to an increase in mitochondrial PLA_2 and MLCL AT activities.

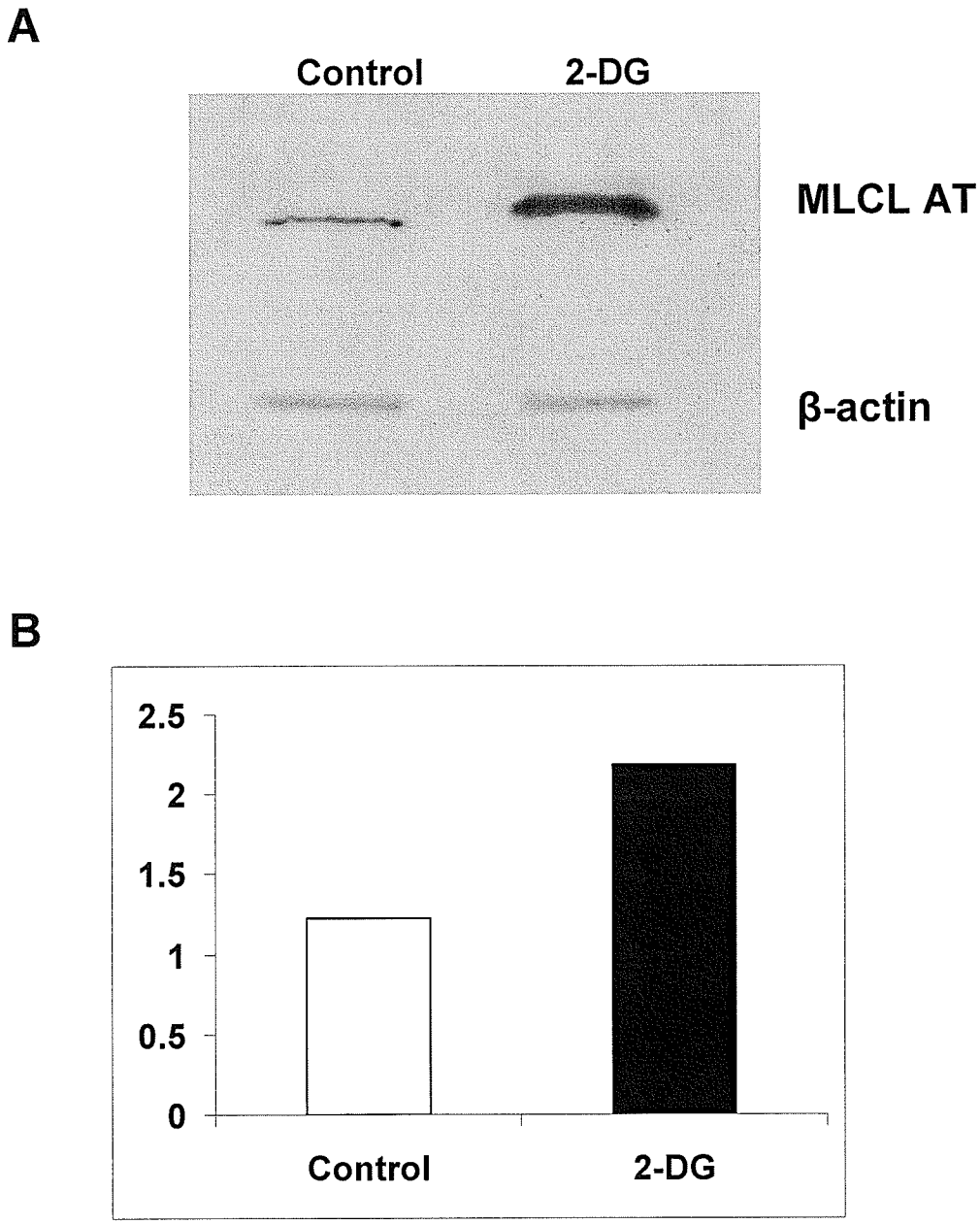
Western Blot Analysis

To determine the reason for the increase in mitochondrial MLCL AT activity Western blot analysis of mitochondrial MLCL AT protein expression was performed in mitochondrial fractions using anti-MLCL AT antibody. H9c2 cells were incubated for 16 h in the absence or presence of 100 mM 2-DG. Mitochondrial fractions were prepared from the surviving population of H9c2 cells and MLCL AT protein levels determined using western blot analysis as described in Materials and Methods. As seen in Figure 9, the protein level of mitochondrial MLCL AT was elevated in 2-DG treated cells compared to control. MLCL AT protein was increased 1.8-fold in 2-DG treated cells compared to controls relative to the expression of β-actin (Figure 9). Thus, the increase in MLCL AT activity in the surviving population of H9c2 cells treated with 2-DG was due to an increase in protein expression of MLCL AT.

Table 2. Activities of the CL *de novo* biosynthesis and remodeling enzymes. Enzyme activities in mitochondrial fractions prepared from H9c2 cells ±2-DG were determined as described in Materials and Methods. Values are expressed in pmol/min/mg of protein and represent the means and standard deviations of three dishes *p<0.05.

Enzyme	Control	+2-DG
	pmol/min/mg protein	
CDS	0.05 ± 0.01	0.05 ± 0.01
PGPS	415 ± 9	464 ± 4
CLS	2.9 ± 0.3	2.8 ± 0.3
PLA_2	82.3 ± 5.1	108.8 ± 5.5*
MLCL AT	13.7 ± 1.6	22.3 ± 1.8*

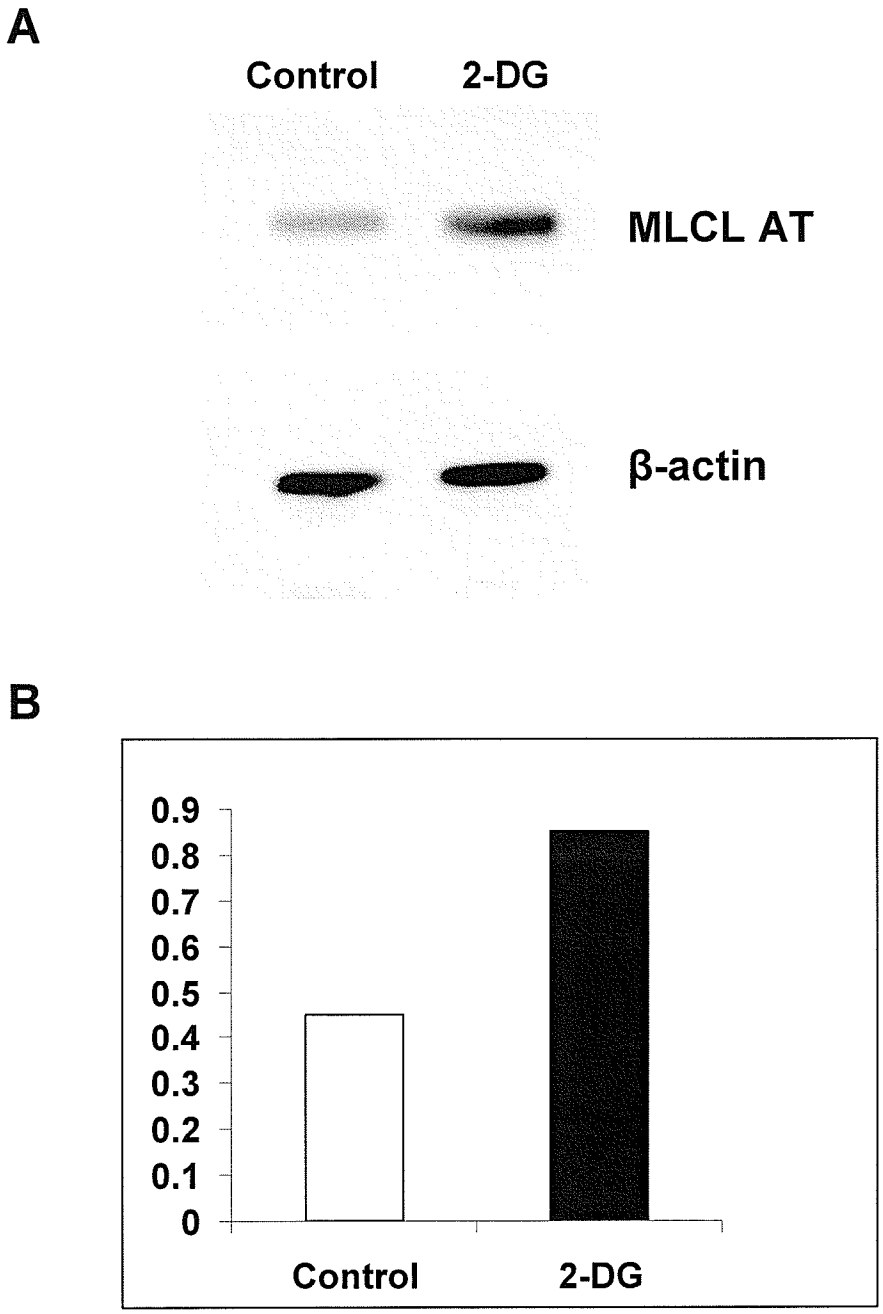
Figure 9. Western blot analysis of MLCL AT. **A:** Western Blot analysis was performed to determine MLCL AT protein expression in control H9c2 cells (Lane 1) and 2-DG treated H9c2 cells (Lane 2) as described in Materials and Methods. **B:** Ratio of expression of MLCL AT relative to β-actin expressed in arbitrary units.



RT-PCR Analysis

RT-PCR experiments were performed to determine if 2-DG treatment affected the relative mRNA levels of MLCL AT. Total RNA was isolated from H9c2 cells and the relative expression of MLCL AT mRNA was determined by RT-PCR as described in Methods and Materials. When compared to β-actin, there was a significant elevation in MLCL AT mRNA expression in 2-DG treated cells compared to control cells (Figure 10). A 53% increase in the expression of MLCL AT mRNA was observed in 2-DG treated cells compared to control. This corresponded to the observed increases in enzyme activity in these cells.

Figure 10. RT-PCR analysis of MLCL AT mRNA in H9c2 cells treated with 2-DG. **A:** Cellular fractions were prepared and RT-PCR analysis of MLCL AT in control H9c2 cells (Lane 1) or 2-DG treated H9c2 cells (Lane 2) performed as described in Materials and Methods. **B:** Ratio of expression of MLCL AT relative to β-actin expressed in arbitrary units.



DISCUSSION

The objective of this study was to investigate whether MLCL AT activity plays a role in preventing 2- DG mediated hypoglycemia-induced damage to the CL molecule. Studies using H9c2 cardiac myoblast cells, which are highly active in CL *de novo* biosynthesis and remodeling, allowed for examination of these pathways. Various radioactive lipid precursors can be incorporated into CL through the CDP-DG pathway (*de novo* biosynthesis pathway of CL) and the CL remodeling pathway in H9c2 cells (Hatch and McClarty, 1996).

Incorporation of [1,3-^{3}H]glycerol into CL was found to be unaltered in the surviving population of H9c2 cells treated with 2-DG, indicating that CL biosynthesis from the *de novo* biosynthetic precursor glycerol was unaltered by 2-DG mediated hypoglycemia. This observation coincided with the results of the *de novo* biosynthesis enzyme studies, where the enzyme activities of CDS, PGPS and CLS were unaltered by 2-DG treatment compared to controls. Thus, CL *de novo* biosynthesis was unaltered under our experimental conditions.

The incorporation of [^{14}C]linoleic acid into CL was also found to be unaltered in the surviving population of 2-DG treated cells compared to the controls, initially indicating that CL remodeling was not affected by 2-DG treatment. However, the enzyme activities for both remodeling enzymes, PLA_2 and MLCL AT, were increased in the 2-DG treated cells when compared to

control. This indicates that CL remodeling is elevated by 2-DG mediated hypoglycemia. Our laboratory has previously demonstrated that addition of the pro-apoptotic factor TNF-α to H9c2 cells stimulated mitochondrial PLA_2 activity towards mitochondrial phospholipids (Xu et al., 1999). Here we found that hypoglycemia-induced apoptosis by 2-DG treatment also resulted in an increase in PLA_2 activity. MLCL AT enzyme activity was also elevated in the surviving population of 2-DG treated cells compared to control, which would normally produce an elevation in [1-^{14}C]linoleate incorporation into CL in these cells. However, we did not observe an increase in the incorporation of [1-^{14}C]linoleic acid into CL in the 2-DG treated cells when compared to the control. In fact, there were no significant differences between the two groups, control versus 2-DG treated, in the incorporation of [1-^{14}C]linoleic acid initially indicating that CL remodeling was not affected by 2-DG treatment. However, the enzyme activities, for both remodeling enzymes, PLA_2 and MLCL AT, were increased in the 2-DG treated cells when compared to control. This indicates that CL remodeling is indeed elevated by 2-DG mediated hypoglycemia. The reason there is no observed increase in the incorporation of [1-^{14}C]linoleic acid into CL in the 2-DG treated cells when compared to the control is due to the simultaneous increase in both PLA_2 and MLCL AT activities. We found that when cells were induced to undergo apoptosis, PLA_2 activity increased. This would result in an increased deacylation of CL and thus an accumulation of MLCL on the outer membrane of mitochondria. This generation of MLCL may bind to t-Bid and begin the cascade of caspases that end in cellular apoptosis. It has previously been shown that

MLCL accumulates during Fas-mediated apoptosis as a byproduct of CL degradation by PLA_2 and enhances t-Bid binding to membranes (Esposti et al., 2003). Although, PLA_2 activity is increased, MLCL AT activity is also increased. The MLCL produced is likely quickly being reacylated back to CL in the 2-DG treated cells. This would explain why [1-^{14}C]linoleic acid label does not decrease or accumulate in CL. Thus, we do not see an altered incorporation of [1-^{14}C]linoleic acid into CL as might be expected to be observed when remodeling of CL is altered.

In support of the elevation in CL remodeling in the 2-DG treated cells was the observed increase in MLCL AT protein expression in 2-DG treated cells compared to controls. Protein expression of MLCL AT was increased in 2-DG treated cells compared to controls demonstrating that the observed elevation in resynthesis (MLCL AT activity) in these cells was due to an increase in the protein levels of this enzyme within the mitochondria. In addition, RT-PCR analysis indicated that there was an increase in mRNA expression of MLCL AT in H9c2 cells treated with 2-DG compared to control. Thus, the observed increase in mitochondrial MLCL AT activity in 2-DG treated cells was due to an increase in both its protein and mRNA expression.

The pool size of CL was found to be unaffected by 2-DG treatment. Since the activities of the CL biosynthetic enzymes were unaltered in the surviving population of H9c2 cells treated with 2-DG, it appears that MLCL AT activity may be up regulated in H9c2 cells treated with 2-DG as a compensatory mechanism

for the elevation in PLA_2 activity in order to maintain the CL pool. Previous studies in our laboratory showed that the CL pool size was elevated when PGPS and CLS activities were elevated in liver and heart cells (Mutter et. al., 2000; Cao et. al., 1995). This up regulation of MLCL AT activity and MLCL AT protein and mRNA expression could serve as a compensatory mechanism during hypoglycemia-induced apoptosis by 2-DG in an effort to protect cells against the accumulation of MLCL caused by the increase in PLA_2 activity. Thus, 2-DG mediated hypoglycemia causes an increase in MLCL AT activity in order to maintain the CL pool size and prevent or even attenuate hypoglycemia-induced apoptosis.

Given the importance of the accumulation of MLCL in mitochondria-mediated apoptosis, it is possible that rapid CL synthesis and/or its resynthesis from MLCL is required in response to proapoptotic stimuli-mediated CL degradation to restore cellular homeostasis and prevent the entry of cells into apoptosis. Hence, increased CL resynthesis may be a protective mechanism against apoptosis. However, the direct role of CL and its metabolite MLCL in apoptosis remains controversial. Several studies indicate that CL plays the main role in mitochondrial-mediated apoptosis and is required for the specific targeting of t-Bid to mitochondria, mediating the release of cyt *c* (Lutter et al., 2000). In contrast, evidence also supports a role for the CL metabolite, MLCL, in apoptosis (Esposti et al., 2003). The molecule MLCL is generated by PLA_2 hydrolysis of CL during induction of apoptosis (Sorice et al., 2004) and enhances t-Bid binding to membranes (Esposti et al., 2003; Liu et al., 2005). In fact, one study has shown

that CL itself is not even required for Bax-mediated cyt *c* release from mitochondria (Iverson et. al., 2004). These authors showed that in a CHO cells defective in PG and CL synthesis mitochondrial-mediated apoptosis proceeded normally. Thus, the role of CL in apoptosis still remains controversial.

In future studies our hypothesis that MLCL AT activity plays a role in preventing hypoglycemia-induced damage to the cardiolipin molecule, may be tested more directly by transfection of H9c2 cells with MLCL AT to up regulate MLCL AT protein followed by treatment with 2-DG. One would expect that up regulation of MLCL AT should delay or even prevent cell entry into apoptosis. In summary, 2-DG hypoglycemia-induced apoptosis results in an increase in CL remodeling and this is due to an increase in both MLCL AT and PLA_2 activity. In addition, MLCL AT expression may be up regulated as a compensating mechanism in order to maintain that CL pool and thus attenuate apoptosis during 2-DG mediated hypoglycemia.

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