Impact of Cellular Pathways of Fatty Acid Metabolism and Cholesterol Synthesis on Cardiomyocyte Cell Death

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Experimental Medicine Program

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

October 2001
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Date **Dec. 24, 2021**

DE-6 (2/88)
Fatty acids have long been implicated in increasing the severity and the extent of myocardial infarction. The common fatty acid palmitate, a 16C saturated fatty acid, was investigated for its role in myocardial cell death. Palmitate was observed to induce significant cardiomyocyte death, both apoptosis and necrosis/oncosis in a specific manner. Palmitate induced an increase in nuclear size, a decrease in intramitochondrial respiration, and a loss of mitochondrial potential. Palmitate was observed to induce an increase in mitochondrial permeability, evidenced by the release of cytochrome C from the mitochondria into the cytoplasm which was inhibitable by cyclosporin A, a drug that closes mitochondrial pores. Enhanced mitochondrial metabolism of palmitate with carnitine, a factor necessary for palmitate transportation into the mitochondria via the carnitine-palmitoyl transferase 1 (CPT-1) enzyme, also increased apoptosis. The CPT-1 inhibitor oxfenicine blocked this enhanced carnitine-palmitate death.

Shifting palmitate metabolism from mitochondria to peroxisomes with long term treatment with the peroxisome proliferators fenofibrate and WY 14643 resulted in a decrease in palmitate-induced apoptosis, suggesting that the location of palmitate oxidation is also responsible for apoptosis. Fenofibrate pretreatment also increased the expression of peroxisome proliferator activated receptors (PPARs): nuclear receptors known to be involved in the regulation of transcription of β-oxidation enzymes.

As palmitate can be incorporated into the apoptotic inducer ceramide, the effects of ceramide were investigated. The ceramide synthase inhibitor fumonisin B1 was ineffective at inhibiting palmitate-induced apoptosis by 24 h suggesting that de novo ceramide synthesis is not
involved in palmitate's action. Like palmitate, exogenous addition of ceramide induced apoptosis, a decrease in mitochondrial potential, and an increase in mitochondrial permeability. However, unlike palmitate, ceramide-induced an increase in intramitochondrial enzyme activity that was unaffected by cyclosporin A. Ceramide also induced a parallel decrease in ERK activation and an increase of SAPK activation. As well, phosphorylated p38 MAPK was observed to locate in the mitochondrial fraction and to be partially responsible for ceramide-induced apoptosis and loss of mitochondrial potential.

Another mechanism hypothesized to explain palmitate-induced death involves acetyl CoA, a byproduct of mitochondrial fatty acid oxidation, which eventually forms the starting substrate for cholesterol biosynthesis. Lovastatin, a HMG-CoA reductase inhibitor, was used in combination with palmitate to inhibit cholesterol biosynthesis during palmitate metabolism. The combination of palmitate and lovastatin resulted in apoptotic death greater than either palmitate or lovastatin alone. This observation suggests that palmitate's death action is not through increased cholesterol biosynthesis as lovastatin did not reduce palmitate-induced death. Further investigation showed that lovastatin co-treated with mevalonic acid, a product downstream of HMG-CoA reductase, blocked lovastatin-induced apoptosis suggesting that if allowed to continue, cholesterol biosynthesis is beneficial to the cardiomyocyte. Lovastatin activated caspase-2 and caspase-3 to a lesser extent and also induced dramatic changes in morphology. The morphological changes were distinct from those produced by palmitate. A physical association was observed to link changes in morphology and the apoptotic process, specifically the association of the small G-protein RhoB and caspase-2. This association appears constitutive and was unaffected by inhibition of caspase-2 activity.
In summary, this thesis unraveled the mechanism of action of palmitate-induced cell death in cardiomyocytes and indicated that palmitate acts via a mitochondrial mechanism involving palmitate metabolism and mitochondrial permeability. Palmitate does not induce apoptosis via ceramide as ceramide induces a separate distinct mechanism of cell death.
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<th>Description</th>
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<tr>
<td>ΔΨₐₘ</td>
<td>Mitochondrial potential</td>
</tr>
<tr>
<td>AcDEVD</td>
<td>Ac-DEVD-CHO (Ac-Asp-Glu-Val-Asp-aldehyde)</td>
</tr>
<tr>
<td>ACS</td>
<td>acyl-CoA synthase</td>
</tr>
<tr>
<td>ANT</td>
<td>adenine nucleotide translocator</td>
</tr>
<tr>
<td>CAD</td>
<td>caspase-activated-DNAse</td>
</tr>
<tr>
<td>CMX-Ros</td>
<td>Chloromethyl-X-Rosamine</td>
</tr>
<tr>
<td>cyt C</td>
<td>cytochrome C</td>
</tr>
<tr>
<td>DePsipher™</td>
<td>5'6,6’-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyaninine iodide</td>
</tr>
<tr>
<td>FATP</td>
<td>fatty acid transport proteins</td>
</tr>
<tr>
<td>FDA</td>
<td>fluorescein diacetate</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>JNK</td>
<td>cJun-activated protein kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinases</td>
</tr>
<tr>
<td>zVDVAD</td>
<td>z-VDVAD-fmk (Z-Val-Asp-Val-Ala-Asp-fluoromethylketone)</td>
</tr>
<tr>
<td>MTP</td>
<td>Mitochondrial transition pore</td>
</tr>
<tr>
<td>PARP</td>
<td>polyadenosine ribosylated protein</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress-activated protein kinases</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal dUTP nick-end labeling</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage dependent activated channel</td>
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<td>hours</td>
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SUMMARY OF HYPOTHESIS AND SPECIFIC AIMS

HYPOTHESIS

In this thesis, I will determine how palmitate induces apoptosis in cardiomyocytes by considering three mechanisms: 1) a direct effect of palmitate, 2) palmitate incorporation into de novo ceramide, and 3) palmitate linkage to cholesterol biosynthesis via the mevalonate pathway.

I will examine the following hypotheses:

I. The fatty acid palmitate induces apoptosis in cardiomyocytes through its metabolism in mitochondria. Increased production of palmitate metabolites damage mitochondria producing cell death.

II. Palmitate induced apoptosis does not occur through its incorporation into ceramide, a known apoptotic inducer. Rather, ceramide induces apoptosis utilizing a different mechanism than palmitate, transmitting its death signal in part by the MAPK pathways.

III. Palmitate-induced apoptosis does not occur through increased cholesterol biosynthesis. Rather, inhibition of cholesterol biosynthesis will induce death itself.

SPECIFIC AIMS:

1. To determine that the fatty acid palmitate induces apoptotic cell death in cardiomyocytes.

2. To determine that palmitate-induced cell death involves a mitochondrial pathway because palmitate oxidation occurs within the mitochondria.
3. To determine whether inhibition of palmitate transport into the mitochondria can affect apoptosis by investigating the rate-limiting enzyme carnitine-palmitoyl transferase 1.

4. To determine whether fenofibrate, a peroxisome proliferator, will alter palmitate-induced apoptosis.

5. To determine whether palmitate induces apoptosis through its incorporation into the de novo synthesis of the apoptotic inducer ceramide.

6. To establish whether palmitate’s mechanism of action is via ceramide action on mitochondria by comparing palmitate and exogenous C₂-ceramide.

7. To determine whether MAPK pathways, particularly p38 MAPK, SAPK/JNK, and MEK/ERK, are involved in ceramide-induced apoptosis.

8. To determine whether palmitate-induced apoptosis involves increased downstream cholesterol biosynthesis. The inhibition of cholesterol synthesis with the HMG-CoA reductase inhibitor lovastatin should prevent palmitate-induced apoptosis by inhibiting increased cholesterol biosynthesis, if palmitate’s effect is due to increased cholesterol synthesis.

9. To determine whether apoptosis induced by HMG-CoA reductase inhibition by lovastatin operates via the cysteine proteases caspase-2 and caspase-3 using the specific inhibitors zVDVAD and AcDEVD, respectively.

10. To determine whether reactivation of cholesterol biosynthesis during HMG-CoA reductase inhibition with the addition of mevalonic acid will prevent lovastatin-induced cell death.

11. To determine whether lovastatin-induced changes to cellular morphology involve changes to cellular F-actin.
12. To determine whether there is a signaling molecule which is responsible for changes to cellular morphology and apoptosis during inhibition of cholesterol synthesis. Rho, a signaling molecule involved in the maintenance of cellular morphology will be selected to determine whether it interacts with caspase-2 to execute changes in morphology and play a role in apoptotic signaling.
Acknowledgements

This work would not have been possible without Dr. Simon Rabkin, whose kind support and thoughtful advice throughout my time in his laboratory is greatly appreciated. It is my hope that some of the work presented in this thesis will be useful as the basis for further experimental inquiries in his lab.

I am thankful to Carol Smythe who provided technical help and Lynann Burton who provided secretarial help. Others in Dr. Rabkin’s lab who participated in these experiments, to whom I am grateful, are Dino Vilimek, Dr. Scott Cowie, Michael Tsang, Shaun Klassen, and Tammy Huang.

I am thankful to Dr. Anthony Chow and his laboratory for their advice, shared laboratory equipment, and especially their computer equipment. In particular, I thank Ryan Hung, Scott Cameron, and Ernest Leung for their computer assistance. I would also like to thank the lab of Drs. Yossi Av-Gay, Neil Reiner, and Devki Nandan for their assistance.

This study was supported in part by a grant from the Heart and Stroke Foundation of British Columbia and the Yukon. I would also like to thank C.I.H.R. (formerly the Medical Research Council of Canada) and the Canadian Hypertension Society for granting me graduate studentships.

Lastly, I wish to thank my husband, Jonathan, and my family for their enduring support, patience, and understanding.
CHAPTER I

INTRODUCTION

I. CARDIomyocyte CELL DEATH IN CARDIAC DISEASE

Cell growth and death in many cell types are in equilibrium during the normal cell state, but under diseased conditions, this equilibrium is shifted (Bright and Khar 1994). In the heart where cardiomyocytes are terminally differentiated, this equilibrium is especially important as no new cell growth will occur to replace cardiomyocytes that die. Investigations into cell death in the myocyte element of the heart (cardiomyocytes) and ways to prevent it should prove helpful in the treatment of heart disease. By understanding how the cardiomyocyte dies, ways to circumvent the death process, or inhibit it, may reduce the morbidity and mortality of cardiac disease.

A. APOPTOSIS IN CARDIAC DISEASE

One form of cell death, apoptosis, has recently attracted considerable research interest. Various stimuli have been identified that trigger cardiomyocyte apoptosis. Stress conditions such as ischemia, especially when followed by reperfusion, and oxygen radicals can elicit apoptosis (Yue et al. 1998, Elasser et al. 2000, Harsdorf et al. 1999). Cytokines such as tumor necrosis factor α (TNFα) have been shown to induce apoptosis in cardiomyocyte culture which is clinically relevant as there are very high circulating levels of TNFα in advanced heart failure (for review, see Fiers et al. 1999; van Riemsdijk et al. 1999). Mechanical stress has been shown to elicit apoptosis in cardiac muscle in vitro that mimic the cardiac remodeling leading to dilated myopathy and heart failure (Cheng et al. 1995).
Apoptosis has been implicated in cardiac diseases such as cardiac hypertrophy (Bing et al. 1995), remodeling (Colucci et al. 1997), cardiomyopathy (Narula et al. 1996; Olivetti et al. 1997), arrhythmogenic right ventricular dysplasia (Mallat et al. 1996), myocardial infarction (MI) (Saraste et al. 1997), and arrhythmogenic right ventricular dysplasia (Mallat et al. 1996). Cardiac apoptosis was observed pathologically in heart failure after cardiac hypertrophy (Bing et al. 1995) and pathologic remodeling of the heart (for review, see Colucci et al. 1996; Colucci et al. 1997). Apoptosis has also been observed in the myocardium of patients with end-stage dilated cardiomyopathy (Narula et al. 1996; Olivetti et al. 1997) and in the infarct and peri-infarct regions of myocardium obtained from patients dying from a recent myocardial infarction (Saraste et al. 1997). Research into apoptosis in animal heart models also observed apoptosis in the myocardium such as ischemia-reperfusion (Gottlieb et al. 1994), MI (Kajstura et al. 1996), rapid ventricular pacing (Liu et al. 1995), mechanical stretch (Cheng et al. 1995), and aortic constriction-induced pressure overload (Teiger et al. 1996).

Regardless of the kind of cell death, there are serious consequences to the heart if the muscle element of the heart (cardiomyocytes) is lost. Loss of cardiomyocytes leads to a loss of cardiac mass and hence diminished pump power. Hence, loss of cells compromises cardiac contractility. Also, a significant loss of cells would influence cardiac remodeling due to disruption of the geometrical alignment of cardiomyocytes with its attendant effect on cardiac contractility. Another consideration is the site of apoptosis within the heart. If apoptosis occurs within the SA or AV nodes, there would be serious conduction disturbances leading to arrhythmias.

II. DEFINITION OF CELL DEATH - APOPTOSIS vs. NECROSIS/ONCOSIS
A. NECROSIS/ONCOSIS (Figure 1)

Cell death can occur through apoptosis or necrosis/oncrosis. Necrosis was the term used to describe the form of death associated with structural and biochemical degradation of cell integrity due to accidental cellular damage whereas apoptosis is a functional type of cell death which is programmed genetically into every cell (Majno et al. 1995). However necrosis is an imprecise name as it not only refers to changes occurring during cell death but also to degradation after cells have died. Even apoptotic cells undergo secondary necrosis after the distinct apoptotic morphology has been accomplished. Hence, the term oncosis is recommended to distinguish this form of cell death from apoptosis (Levin et al. 1999). Oncosis is initiated by cellular damage that disrupts osmotic balance (Farber et al. 1990). Ions passively enter the cell resulting in swelling as water enters in response to the ion flux. Oncosis is characterized by dilation of the endoplasmic reticulum, increase in mitochondrial volume (swelling), destruction of nuclear chromatin, early membrane breakdown, and cell disintegration resulting in the release of lysosomal enzymes (Searle et al. 1982, Kerr et al. 1972). Oncosis occurs quickly, within a matter of minutes. Also, oncosis is a passive process that does not require active participation of the cell in its own death.

B. APOPTOSIS

Apoptosis is an active process in which the cell participates in its own destruction utilizing a set of genetic factors and a complex network of enzymes (for review, see Saraste and Pulkki 2000, Feuerstein et al. 2000, Green and Reed 1998, Fiers et al. 1999). Apoptosis occurs in stages over a period of hours or even days. Initially, an individual cell embedded in normal tissue loses contact with its neighbours. Chromatin within the nucleus condenses resulting in nuclear
Figure 1 - The diagram shows the relationship of oncosis and apoptosis and that both lead to secondary necrosis. The exact point at which death of the cell occurs and the postmortem phenomena recognized as necrosis ensue is somewhat arbitrary. From Levin et al. 1999
disintegration and DNA fragmentation into 180bp fragments. The cell shrinks due to loss of cytoplasmic volume and condensation of cytoplasmic proteins. Most of the intracellular organelles remain intact, although there is cytoplasmic condensation. The second stage is characterized by membrane ruffling and blebbing leading to cellular fragmentation and formation of apoptotic bodies (blebs). The formation of blebs appears to involve disruption of cytoskeletal-membrane interactions. Apoptotic bodies frequently contain whole organelles and nuclear remnants. In the final stage, macrophages phagocytose the cellular fragments for complete degradation of the cell. Apoptosis occurs without leakage of intracellular macromolecules therefore, it does not elicit any inflammatory response.

C. THE NEED FOR MORE THAN ONE FORM OF CELL DEATH

It is speculated that there is a need for two forms (different modes or mechanisms) of cell death to eliminate the cell. For example, oncotic cellular breakdown encourages phagocytosis and reutilization of the cell components by other cells. Unfortunately, this cellular recycling might aid the spread of potentially infective virus if the disassembled cell has been infected (Clemm and Miller 1993). In contrast, in apoptosis the apoptotic DNA fragmentation may deactivate the machinery needed for viral replication. However, this fragmentation may lead to the accumulation of these potentially harmful viral oligonucleotides (Clemm & Miller 1993). Also, apoptotic DNA fragmentation is important as it can inactivate already assembled viral DNA (Martz and Howell 1989). Apoptosis may be considered to be a functional form of cell death and is important to the cell as an essential regulator in ontogenesis and tissue homeostasis, embryogenesis, etc.. Thus, different types of death may function to deal with the containment and proper disposal of the injured or infected cell.
Necrapoptosis is a term currently in use to describe how one death insult can induce both apoptosis and necrosis. An example is acute myocardial ischemia-reperfusion injury (Kajstura et al. 1996). Myocardial necrosis is found in the central ischemic area during the first hours to days after the onset of ischemia. However apoptotic DNA fragmentation occurs mainly in the very early phases of the process in the central ischemic areas although it is found at later times in the border zones (Kajstura et al. 1996). Thus cell death may first be apoptosis and later recognized as necrosis. A possible mechanism for such a deciding factor for proceeding to apoptosis or oncosis may be the availability of ATP. As apoptosis is an energy-requiring process, sufficient levels of ATP are necessary for this path. In Jurkat and HeLa cells, when intracellular ATP levels drop below a critical value, CD95 triggering no longer elicits apoptotic cell death, but a pathway leading to oncosis (Eguchi et al. 1997; Leist et al. 1997). Presumably, if ATP depletion develops during the progression of apoptosis, necrotic cell death will intervene to produce the secondary necrosis that is so often associated with apoptosis. Along the same hypothesis, if an insult given to a cell results in a sudden opening of mitochondrial transition pores, there would be a rapid drop in cellular ATP producing oncosis. However if formation of the pores is slower, then profound ATP depletion is avoided allowing apoptotic signaling to proceed.

D. IMPORTANCE OF EXAMINING APOPTOSIS IN HEART DISEASE

The predominant form of cell death during cardiac disease is not always clear. Apoptosis was found to occur in less than 0.2% of cells in myocardial tissue obtained from patients with chronic end-stage heart failure (Olivetti et al. 1997). However, because apoptosis is an event lasting hours or days, it can occur several times after the initial injury. The death of even 0.2% of cells per day could, over a period of months or years, lead to the loss of a large fraction of the
cardiomyocyte pool. Hence, the current theory is that progressive myocardial failure reflects the continuing loss of viable myocytes due to the programmed cell death apoptosis.

III. APOPTOTIC EFFECTS ON THE CELL

A. STRUCTURAL CHANGES

1. The nucleus
   a) Apoptotic nuclear effects

Chromatin within the nucleus condenses resulting in nuclear disintegration. Nuclear condensation and disintegration are associated with the activation of an endogenous Ca$^{2+}$- and Mg$^{2+}$-dependent endonuclease which cleaves double stranded DNA at the most accessible internucleosomal inner region (Burgoyne et al. 1974). The only intact DNA remaining is that wrapped around the nucleosomes. This intact DNA is 180 bp in length, the size of the nucleosome. Because DNA degradation precedes plasma membrane breakdown, there is an accumulation of nuclei with low DNA content enriched with 180 bp DNA fragments (Duke and Cohen 1986).

These endonucleases include DNA fragmentation factor (DFF 40) (Liu et al. 1998) and caspase activated DNase (CAD) (Enari et al. 1998; Sakahira et al. 1998), and NUC70 (Urbano et al. 1998). DFF40 and CAD are present in normal cells as inactive heterodimers with the inhibitor proteins DFF45 (Liu et al. 1997) and ICAD (inhibitor of CAD) (Sakahira et al. 1998). These enzymes are selectively activated upon cleavage by caspase 3 (Sakahira et al. 1998, Liu et al. 1997) or by other members of the caspase family (Tang et al. 1998). Exposure of nuclei to activated CAD or DFF40 is sufficient to induce the nuclear fragmentation typical of apoptosis (Liu et al. 1998; Enari et al. 1998)
Apoptotic DNA fragmentation was also observed in heart failure. Assessed by TUNEL and electrophoretic separation of fragmented DNA, DNA fragmentation was observed in ischemic and idiopathic dilated cardiomyopathy associated with clinical heart failure, acute myocardial infarction, congenital arrhythmogenic dysplasias, myocarditis, and arrhythmias (for review, see Anversa et al. 1998; Narula et al. 1996).

b) Nuclear enlargement

In heart tissue, the cellular signaling pathways activated during excess cardiac stretch as the heart dilates to adjust to the loss of cardiomyocytes (Yamazaki et al. 1995), lead to nuclear enlargement (Cluzeaud et al. 1984). Increased nuclear size was observed in patients with hypertrophic obstructive cardiomyopathy (Maron et al. 1975; Noda et al. 1980) and in biopsies from patients with idiopathic hypertrophic or dilated cardiomyopathies (Baandrup et al. 1981; Popovich et al. 1995). Increased nuclear size was also observed in hearts explanted for end stage heart failure (Scholz et al. 1994). Similarly, there was an increase in nucleolar size in myocardial biopsies from patients with adriamycin-induced cardiomyopathy (Unverferth et al. 1983). Increased nuclear size may be an adaptation to the loss of viable myocytes due to cardiomyopathy or may be an integral part of its pathogenesis.

2. The mitochondria

As mitochondrial function is affected by apoptosis, it is necessary to review the importance of the mitochondria to the cell, specifically with regards to energy production. The respiratory chain is the final step in fatty acid/carbohydrate metabolism in which the bulk of ATP is generated (for a review of the respiratory chain, see Nelson and Cox 2000). Reduced electron carriers such as NADH and FADH$_2$ are generated early in metabolic pathways such as glycolysis
(oxidation of glucose, lactate, or pyruvate) and fatty acid oxidation. NADH$_2$ and FADH$_2$ enter the respiratory chain and the electrons are transported to various protein electron carriers in the respiratory chain with production of ATP. This transfer of electrons in mitochondria from reduced carriers in a step-wise manner to several protein electron carriers eventually react with molecular oxygen to generate water. This step-wise reoxidation involves several protein electron carriers, firmly embedded in the inner membrane of mitochondria, in the sequence: NADH dehydrogenase (Complex I), Coenzyme Q10, succinate dehydrogenase (Complex II), cytochromes B and C1 (Complex III), cytochrome C, cytochrome C oxidase and cytochromes A and A3 (Complex IV), and ATP synthase (Complex V) to the final oxygen molecule. The overall chain sequence is quite exergonic: one pair of reducing equivalents generated from NADH$_2$ is sufficient to drive the coupled synthesis of 2.5 moles of ATP by oxidative phosphorylation. The transfer of electrons within the mitochondria, in conjunction with proton pumps, help create a negatively charged environment within the mitochondria, often termed a "negative sink". The disparity between inside the mitochondria and outside is often referred to as an "electrochemical gradient" or "mitochondrial potential" (ΔΨ$^\text{m}$). It is imperative that the mitochondria remain intact to generate this electrochemical gradient to aid electron transfer across the mitochondria. Any increase to mitochondrial permeability will have dramatic effects on mitochondrial respiration as the electrochemical gradient can not be maintained to ensure proper electron transport.

Although the proton carriers may be firmly embedded in the mitochondrial membrane, components to the electron transport chain can be mobile. Cytochrome C is an example of a mitochondrial marker which can be lost during an increase in mitochondrial permeability.
Mitochondria play a critical role in the production of apoptosis through several different mechanisms (for review, see Lemasters et al. 1998b; Kroemer et al. 1998). At least three general mechanisms are known: (i) alteration of mitochondrial potential ($\Delta \Psi_m$) via increased mitochondrial permeability (Tatton et al. 1999, Budd et al. 1998), (ii) disruption of mitochondrial respiration and the generation of ATP (Di Lisa et al. 1998), and (iii) release of proteins from mitochondria that trigger caspases. The proximity of mitochondria to various caspases further links mitochondria to apoptosis (Krajewski et al. 1999, Samali et al. 1998).

a) Loss of $\Delta \Psi_m$ through an increase in mitochondrial permeability caused by mitochondrial transition pores (MTP) (Figure 2)

Apoptotic stimuli lower mitochondrial membrane potential, the driving force of the cell's energy generation (for review, see Lemasters et al. 1998), that is attributed to an increase in mitochondrial permeability. This makes it difficult to maintain the electrochemical gradient needed for mitochondrial respiration (for review, see Lemasters et al. 1998).

Increased mitochondrial permeability can occur by nonspecific injury to the mitochondrial membrane lipids or by a specific mechanism causing the opening of the mitochondrial transition pores (MTPs). (for review, see Lemasters et al. 1998). MTP is a proteinaceous megapore located on the inner mitochondrial membrane that allows small solutes (<1.5 kDa) to move freely in and out of the mitochondria (for review, see Lemasters et al. 1998). Increased MTPs produce a loss of ions and metabolites from the mitochondrial matrix and induces extensive swelling as a result of the colloidal osmotic pressure exerted by the matrix proteins (Halestrap et al. 1998). MTPs are located between the outer and inner mitochondrial
Figure 2 - Mitochondrial transition pore formation. ROS = Reactive Oxygen Species; Ca\(^{2+}\) = Calcium; VDAC = Voltage dependent activated channel From Green and Reed 1998.
membranes and the association between its components dictate whether it remains open or closed (for review, see Lemasters et al. 1998a&b; Halestrap et al. 1998). Major components of MTP include cyclophilin-D, adenine nucleotide translocase (ANT) (inner), voltage-dependent channel (outer), and a member of the Bcl-2 family (Halestrap et al. 1990; Zoratti and Szabo. 1995; Beutner et al. 1996). Minor components such as creatine kinase, Bax and hexokinase have also been identified (Halestrap et al. 1990; Zoratti and Szabo 1995; Beutner et al. 1996).

i) Opening of MTP

MTPs open during oxidative stress, high calcium, inorganic phosphate, adenine nucleotide depletion, and ischemia/reperfusion where there is a cellular acidic environment due to lactic acid accumulation (Halestrap et al. 1998; Lemasters et al. 1998b). Conductance of this pore is so great that the opening of only a few pores is sufficient to cause mitochondrial depolarization, uncoupling of oxidative phosphorylation, and large-amplitude mitochondrial swelling (Zoratti M and Szabo 1995). A Ca^{2+}-triggered conformational change of ANT bound to cyclophilin-D was responsible for the formation of MTP (Halestrap et al. 1990). Cyclophilin D binds strongly and specifically to the ANT (Woodfield et al. 1998). Calcium sensitivity affects MTP by two mechanisms: 1) by increasing cyclophilin D binding to the ANT and 2) by greatly reducing the affinity of the ANT. Binding of adenine nucleotides to this site was shown to inhibit MTP competitively with respect to Ca^{2+} (Halestrap et al. 1998).

ii) Closing of MTP

The opening of MTP involves various components that have so far been observed to be partially inhibited by the immunosuppressive cyclic oligopeptide cyclosporin A, the phospholipase inhibitor trifluoperazine and Bcl-2 (Petronilli et al. 1994; Bernardi et al. 1996).
1994; Zamzani and Szabo 1996; Ghafourifar et al. 1999). Cyclosporin A is effective as it inhibits cyclophilin D (Halestrap et al. 1990). Cyclosporin A also affects MTP by its action on the Bcl-2 component (Halestrap et al. 1990). Cyclosporin A is also a potent inhibitor of the calcium-sensitive phosphatase calcineurin which is involved in the dephosphorylation, hence activation, of BAD, a pro-apoptotic member of the Bcl-2 family (Green and Reed 1998). Mg\(^{2+}\), ADP, acidic matrix pH, and a high membrane potential favor the closed state of MTP (for review, see Lemasters et al. 1998b).

b) Loss of mitochondrial respiration and generation of ATP

Oxidative phosphorylation requires that the mitochondrial inner membrane be impermeable to all but a few selected metabolites and ions (for review, see Wojtczak and Wieckowski 1999). If this permeability barrier is lost, mitochondria become uncoupled and hydrolyze ATP rather than synthesize it (for review, see Wojtczak and Wieckowski 1999). Eventually, if this persists, cell death will be a result.

c) Release of activators of caspases (Figure 3)

MTPs allow the release of mitochondrial elements, such as cyt C (cyt C), into the cytosol (Narula et al. 1999; Cai et al. 1998; Jiang L et al. 1999; Yang and Cortopassi 1998). The released cytosolic cyt c, together with dATP, binds to apoptosis-activating factor-1 (Apaf-1), which in turn activate an initiator caspase, caspase-9 (Cai et al. 1998; Li P et al. 1997; Jiang L et al. 1999). Caspase-9 then activates one of the apoptotic effector caspase, caspase-3, hence stimulating the effector pathway of apoptosis leading to DNA degradation, cell shrinkage, and cleavage of a variety of apoptotic substrates such as polyadenosine ribosylated protein (PARP) (for review, see Lemasters 1998a&b). In some cells, procaspase-3 is located within the mitochondria and liberated into the cytosol during apoptosis, ready for activation (Mancini et al.
Figure 3 - The involvement of mitochondria in ceramide-induced apoptosis and necrosis. Cyto C = cytochrome C; $\Delta \Psi$ = mitochondrial potential; ROS = Reactive oxygen species; APAF = apoptotic protease activating factor. From Green and Reed 1998.
1998). This process has been observed clinically in the heart. Translocation of cyt C from mitochondria into cytoplasm has been observed in explanted failing hearts (Narula et al. 1999) and in response to oxygen radical treatment (Harsdorf et al. 1999)

3. The Cytoskeleton

Apoptosis induces a change in morphology to produce cell rounding and membrane blebbing (Wyllie et al. 1980; Orrenius et al. 1992). Any changes to cell morphology must involve a change to the cytoskeleton as actin in its filamentous, polymerized form (F-actin) is the backbone of microfilaments which partly comprise the cytoskeleton (Hinshaw et al. 1993). Hence, alteration of cellular morphology will result from changes in actin polymerization and depolymerization. Broad spectrum stresses, such as oxidant stress and ATP depletion, cause depolymerization of F-actin that is associated with changes in cellular morphology (Hinshaw et al. 1991; Hinshaw et al. 1993). Actin polymerization has been observed to be disrupted during apoptosis in HL-60 cells (Levee et al. 1996) and rat renal proximal tubular cells (van de Water et al. 1996). This process may involve active caspases as caspase 3 rapidly cleaves gelsolin, a recognized actin-modulating protein (Kothkota et al. 1997).

In order for the nucleus to break down during apoptosis, the nuclear lamina, the supporting structure of the nuclear envelope, undergoes disassembly (Lazebnik et al. 1993). This process depends on caspase-mediated degradation of nuclear lamins A and B (Orth et al. 1996; Takahashi et al. 1996). As well, proteins involved in the regulation of chromatin structure (Nicholson et al. 1997) or interactions between chromatin and nuclear matrix proteins are also cleaved (Casiano et al. 1996).
B. ENZYMATIC CHANGES (CELLULAR SIGNALLING) IN APOPTOSIS

There is a need for signal transmission from the extracellular sensor to the intracellular apoptotic mechanisms to produce nuclear disintegration, alteration of cellular morphology, and other apoptotic effects within the cell. There are five major signaling pathways that have been suggested to convey apoptotic stimuli in cardiac myocytes (Feuerstein and Young 2000). However, I re-classify these pathways into 3 major pathways which may interact with each other:

(i) Receptor-mediated pathway.

Apoptosis is sometimes initiated by “death receptors”. The Fas/TNFα family of cytokine receptors operate via unique ‘death domains’ that are linked to several intracellular signaling pathways resulting in apoptosis (Pulkki 1997). Apoptosis is also sometimes initiated by G-protein-coupled receptors (eg. angiotensin II) (Adams 1998). Also, stimulation of an apoptotic receptor can induce the generation of an apoptotic molecule such as ceramide which propagates the apoptotic signal (Hofmann and Dixit 1998);

(ii) Effects on mitochondria

Changes to mitochondrial permeability or direct damage to mitochondria can induce apoptosis by the release of cytochrome C (cyt C) which activates caspases (see below).

(iii) Caspases, a family of cysteine proteases operating in a cascade that is activated either by receptor originating signals or mitochondrial-associated cyt c (for review, see Thornberry and Lazebnik 1998; van Riemsdijk 1999).

Some of these pathways were examined in this thesis and will be discussed in greater detail.
1. Caspases (Figure 4)

Caspases play a central role in apoptosis as they comprise a group of highly regulated molecules that mediate cell death (for reviews see Thornberry and Lazebnick 1998; Wolf and Green 1999; Budihardjo et al. 1999). To date, there are 13 caspases identified and it is still unknown as to whether all caspases are present in the heart.

a) Biology of caspases

Caspases are activated during apoptosis in a self amplifying cascade. Caspases, or cysteine proteases, cleave their substrate proteins specifically after an aspartate residue (for review, see Thornberry and Lazebnik 1998). They are formed continuously and are normally present as inactive proenzymes. The proenzyme has three domains: an amino terminal domain, a large subunit (~20kDa), and a small subunit (~10kDa). Activation involves proteolytic processing between domains followed by association of the large and small subunits to form a heterodimer. Upon activation, the large and small subunits become intimately associated and both contribute residues necessary for substrate binding and catalysis. The pro-caspases, when converted to their mature, active enzyme form, can then activate further these caspases directly induce cell death (Enari et al. 1998). A proapoptotic signal culminates in activation of the upstream initiator caspases (eg. caspase-2,-8,-9,-10) leading to proteolytic activation of the downstream or effector caspases-3,-6, or -7. The effector caspases actually cleave a set of vital proteins and thus, initiate and execute the apoptotic degradation phase including DNA degradation and the typical morphologic features.

Caspases induce death upon activation by several different mechanisms: either by death receptor activation or by the mitochondrial release of an apoptosis inducing factor such as cyt c (Narula et al. 1999; Cai et al. 1998; Jiang S et al. 1999; Yang and Cortopassi 1998). In the case
Figure 4 - Summary of caspase nomenclature and function. From Green and Reed 1998
of death receptors, ligand stimulation causes an activation of an initiator caspase (caspase 8) which directly activates the effector caspases (eg. caspase-3 or -7). As well, the initiator caspase (eg. caspase-8) interacts with cytosolic Bid, a member of the Bcl-2 family, which acts on the mitochondria resulting in the release of cyt c. Upon release, cyt c leads to the activation of caspase-9 and the eventual activation of caspase-3 (Narula et al. 1999; Cai et al. 1998; Jiang L et al. 1999; Yang and Cortopassi 1998)

b) Caspases and the heart

Active forms of caspases-2, -3, and -7 are generated in the ischemic-reperfused heart (Holly et al. 1999). Caspase-3 substrate cleavage has been observed under oxidative stress, in ischemic-reperfused myocardium, during acute cardiac allograft rejection and in explanted failing human hearts (Holly et al. 1999; Harsdorf et al. 1999; Koglin et al. 1999; Narula et al. 1999). Moreover, activated caspase 3 has been shown to co-localize with the TUNEL positive cardiomyocytes in ischemic/reperfused heart (Black et al. 1998). Caspase inhibitors have been shown to prevent cardiomyocyte death in response to simulated ischemia in vitro (Gottlieb et al. 1996) and in ischemic-reperfused heart, together with reduction of myocardial infarct size (Holly et al. 1999; Yaoita et al. 1998). As the initiator, caspase-2, and the effector, caspase-3, figure prominently in heart disease, the thesis will investigate these two caspases. Caspase-2 is also of interest because it has two subtypes: Ich-1_L which induces apoptosis and Ich-1_s which suppresses apoptosis (Duan & Dixit 1997).

c) Caspase-2 and Caspase-3

Procaspase-2 uses its long N-terminal prodomain to anchor itself within the cell membrane (Yang et al 1998). Activation of procaspase-2 occurs upon oligomerization of its long N-terminal prodomain via autoproteolysis (Yang X et al 1998; Martin et al 1998; Butt et al
1998; Muzio et al 1998; Thornberry et al. 1997). In contrast, caspase 3 has a short N-terminus allowing it to remain in the cytosol. As mentioned previously, caspase-3 can be activated by the initiator caspase-9.

The cellular localization of these caspases also must be considered. Caspase-2 has a nuclear localization, suggesting it acts directly or in close proximity to effectors of the nuclear fragmentation of apoptosis (Zhivotovsky et al. 1999). In some cells, procaspase-3 is located within the mitochondria and is liberated into the cytosol during apoptosis, ready for activation (Mancini et al. 1998).

2. MAPKs - SAPK/JNK, p 38, and ERK (Figure 5)

The name MAPK, or mitogen-activated protein kinases or microtubule-associated-protein kinase, comes from the observation that the first discovered member responded to mitogens (for review, see Feuerstein & Young 2000; Paul et al. 1997). MAPKs are activated in response to the appropriate stimulus by phosphorylation on both a threonine and tyrosine that appear in a threonine-X-tyrosine motif close to the active site. Activated MAPKs phosphorylate target proteins on serine or threonine residues adjacent to a proline. MAPKs fall into three main families: the ERKs (extracellular regulated kinases), the SAPK/JNK (stress-activated protein kinases/c-jun amino-terminal kinase), and the p38 MAPKs (Feuerstein & Young 2000; Paul et al. 1997). Each family contains multiple isoforms encoded by different genes and splice variants, and differs from other family members in the amino acid X in the threonine-X-tyrosine activation motif (Feuerstein & Young 2000; Paul et al. 1997).
Figure 5 - Overview of MAPK signaling cascades. MEK = MAPK kinase; MKK = MAPK kinase kinase; ERK = extracellular response kinase; SAPK = stress-activated protein kinase; JNK = cJun activated kinase. From Cell Signaling Technology catalogue 2000-2001
a) ERK

ERKs respond primarily to mitogenic stimuli such as growth factors and PMA (for review, see Paul et al. 1997; Feurstein & Young 2000). ERKs and its upstream activating kinase mitogen activated protein kinase kinase (MEK), in general, seem to be anti-apoptotic (Xia et al. 1995). Xia et al (1995) reported that for apoptosis to occur, there must be concurrent activation of SAPK and p38 with inactivation of ERK. A greater amount of ERK activity relative to that of JNK or p38 may promote neuronal cell survival whereas more JNK/p38 activity relative to that of ERK may trigger apoptosis (Xia et al. 1995). An active MEK/ERK pathway was observed conferring protection against apoptosis (Erhardt et al 1999). These data demonstrate that direct and selective activation of ERK prevents apoptosis and promotes survival.

b) SAPK/JNK

SAPK/JNK activation occurs in response to physiological stresses such as heat, chemical, oxidative, osmotic, pH, hypoxia, growth factor withdrawal and UV light (Cohen et al. 1997). Activation of SAPK/JNKs lead to the activation of nuclear transcriptional mechanisms, especially c-jun. Once activated, c-jun stimulates its own expression by interacting with two AP-1 sequence elements within its promoter (for review, see Paul et al. 1997).

SAPK/JNK has been determined to be pro-apoptotic as demonstrated in several studies (for review, see Basu and Kolesnick 1998). In cultured neuronal cells, SAPK is activated with a concurrent inhibition of ERK to induce apoptosis. (Xia et al. 1995). Tournier et al (2000) suggested that mitochondria are influenced by pro-apoptotic signal transduction through the SAPK/JNK pathway. As well, there have been reports of SAPK/JNK activation during loss of ΔΨm concomitant with apoptosis in B lymphoma cells and human conjunctiva epithelial Chang cells (Takada et al 2001, Jendrossek et al. 2001). Co-activation of both SAPK/JNK and p38...
MAPK induced apoptosis in the rat heart (Bogeyevitch et al. 1996). In addition, SAPK/JNK phosphorylation in response to ceramide is robust as ceramide’s lethality is associated with a strong stimulation of SAPK/JNK in some cell lines (Jarvis et al. 1997). Taken together, SAPK/JNK activation induces apoptosis.

c) p38 MAPK

p38 is activated by dual phosphorylation on a Thr-Gly-Tyr motif in response to endotoxin, cytokines, physical stress such as hyperosmolarity, and chemical stress such as hydrogen peroxide (Han et al. 1994; Raingeaud et al. 1995; Rouse et al. 1994; Freshney et al. 1994, Lee et al. 1994). The p38 family has at least 4 isoforms identified: α and β being the best characterized (Lee et al. 1994). p38 downstream targets include a number of transcription factors including ATF-2 which can also interact with the SAPK product c-Jun. As well, p38 has been implicated in F-actin reorganization and vinculin recruitment to the focal adhesion complex, implying a role for p38 regulation of the cytoskeleton (Hout 1997).

There are previous reports of a pro-apoptotic role for p38 MAPK. Zhuang et al (2000) reported that, in human leukemia cells, inhibition of p38 phosphorylation also inhibited DNA fragmentation, decrease in ΔΨm, and caspase-3 activation. In addition, Zhuang et al (2000) hypothesized that p38 signals to activate caspase-3 resulting in apoptosis. They also reported a role for p38 in cadmium-induced apoptosis in promonocytic U937 cells. Galan et al (2000) reported that there was a rapid phosphorylation of p38 concomitant with apoptotic DNA laddering and that p38 inhibition with SB 203580 decreased apoptotic DNA degradation, phosphatidyl serine exposure, and loss of ΔΨm. Apoptosis was attenuated by p38 inhibition with SB 203580 (Galan et al. 2000).
d) Interaction between MAPKs

MAPKs influence each other's activity. Most notably, co-activation of SAPK and p38, with a concurrent deactivation of ERK, suggests that there is a convergence of SAPK/p38 pathways that ultimately leads to apoptosis (Xia et al. 1995; Bogoyevitch et al. 1996; Yin et al. 1997; Mackay et al. 1999). Iwama et al. (2001) reported, in arsenic-treated U937 cells, a concomitant p38 activation and a deactivation in ERK during the loss of ΔΨ, cytochrome C release, and apoptosis. As well, there are common points which serve as a mechanisms for integration of the SAPK and p38 pathways. For example, the SAPK/JNK target, c-Jun, can function as a homodimer or interact with the partner protein ATF2, which is activated by p38 (Raingeaud et al. 1995). Hence, there are common points which serve as a mechanisms for integration of the SAPK and p38 pathways.

e) MAPKs and the heart

There have been several previous studies detailing the role of MAPKs in the heart, in health and disease. As mentioned, MAPKs are affected in the heart and the signaling response differs based on the trigger (for review, see Feuerstein & Young 2000). ERKs respond primarily to mitogenic stimuli as evident in hypertrophy (Robinson & Cobb 1997) whereas SAPK and p38 MAPKs respond to physiological stresses such as pH and hypoxia as evident in ischemia/reperfusion injury (Wang et al. 1998a).

SAPK/JNK has effects within the heart. SAPK/JNK activation induces characteristic features of cardiomyocyte hypertrophy, increased expression of atrial natriuretic factors, and changes in sarcomeric organization (Bogoyevitch et al. 1996). Ceramide has also been known to activate the stress-response kinase cascade resulting in the activation of SAPK (Verheij et al. 1996). In contrast, co-activation of SAPK/JNK and p38 induced phenotypic alterations
compatible with apoptosis without hypertrophy (Wang et al. 1998a; Bogeyevitch et al. 1996),
suggesting a convergence of the SAPK/MAPK pathways which ultimately lead to a crucial
decision between apoptosis or hypertrophy.

p38 has also been documented to have a role in heart disease. Myocardial ischemia and
reperfusion were shown to activate p38 in vivo which led to apoptosis (Wang et al. 1998b;
Bogoyevitch et al. 1996; Yin et al. 1997). Ischemia alone caused a moderate increase in p38
activity which was greatly accentuated upon reperfusion after ischemia (Ma et al. 1999). p38
inhibition accelerated the recovery of coronary flow, cardiac contractility, and left ventricular
pressure as well as attenuated apoptosis after ischemia (Ma et al. 1999). Wang et al. (1998b)
showed that the p38 MAPK pathway is activated in murine hearts subjected to chronic transverse
aortic constriction coincident with the onset of ventricular hypertrophy (Wang et al. 1998a&b).
Furthermore, the p38α isoform appears to be pro-apoptotic whereas the p38β isoform is
antiapoptotic as well as evidence of differential expression of these isoforms (Wang et al.
1998a&b). These data suggest a highly differentiated p38 pathway that includes divergent
functions for different molecules of the p38 MAPK family.

3. G-proteins

The effects of many hormones, growth factors, and other ligands can only be produced
by transmission of information across the plasma membrane by receptors because the ligands
themselves are excluded by the hydrophobic nature of the membrane bilayer. G-protein coupled
receptor (GPCR) bind specific ligands and transmits signals. As a consequence of ligand
binding, the heterotrimeric guanine nucleotide-binding protein (G-protein) is activated and
transmits its message within the cell (for review, see Milligan and Grassie 1997).
a) Structure of GPCR

All GPCRs consist of single polypeptides in which seven largely hydrophobic regions form helices that traverse the plasma membrane (for review, see Milligan and Grassie 1997). The architecture of GPCR is such that the GPCR provides a binding site for the ligand. As the GPCR is a transmembrane polypeptide, the G-protein is closely associated to the GPCR and membrane to allow G-protein activation (for review, see Milligan and Grassie 1997).

b) Structure and activation of G-proteins

Heterotrimeric G-proteins consist of three non-identical polypeptide subunits named α, β, and γ (for review, see Milligan and Grassie 1997). The α-subunit is the largest of the complex, followed by β, and then γ. G-proteins are activated upon ligand binding to the GPCR. The inactive G-protein α-subunit which is GDP bound, binds GTP and dissociates from the βγ complex resulting in α being released from the GPCR/membrane to execute its effect (for review, see Milligan and Grassie 1997). It is suggested that the N-terminal region of the α-subunit is responsible for the connection to the membrane: the N-terminal is myristoylated (attachment of a 14C fatty acyl group) which allows the N-terminal to be incorporated into the membrane (for review, see Milligan and Grassie 1997). The βγ subunits have also been reported to have effector functions (for review, see Milligan and Grassie 1997).

c) G-proteins and apoptosis

G-protein mediated apoptosis involves signaling partners to execute the nuclear effects of apoptosis. In normal Gαq expressing cardiomyocytes, p38 MAPK activity was significantly increased in hypertrophy (Adams et al. 1998). However, expression of constitutive active Gαq in cardiomyocytes was reported to stimulate robust apoptosis by concomitant activation of SAPK/JNK and p38 MAPK (Adams et al. 1998). Similar results were seen in noncardiac cells.
transfected with constitutive active Goq (Althoefer et al. 1997): high levels of Goq signaling can stimulate apoptosis.

d) G-proteins and heart disease

A growing body of evidence supports an important role for Goq11/Gon11 pathways for cardiac hypertrophy (Akhter et al. 1998; for review, see Dorn & Brown 1999; Post et al. 1999), severe heart failure with marked dilated chambers and death (Adams et al. 1998) in humans and in the scar and peri-infarcted myocardium in rat hearts (Ju et al. 1998). Also, dilated congestive heart failure is associated with increased left ventricular Goq content (Bohm et al. 1994; Eschenhagen et al. 1992).

e) Small G-proteins and cell morphology

When cells become apoptotic, there is a dramatic change in cellular morphology, related to changes with the cytoskeleton. The small G-proteins Ras, Rho, and Rac have been implicated in regulating changes in cell structures during oncogenic cellular transformation (Qiu et al. 1995). In this situation, cellular transformation is mediated by small G-proteins resulting in a decrease in stress fiber formation and focal adhesions (Dartsch et al. 1994; Khosravi-Far et al. 1994). The Rho family, in particular, is remarkable in the ability of each family member to control a specific organizational state of the actin cytoskeleton in response to a common stimulus (Hall et al. 1998; Ridley and Hall 1992). For example, upon EGF or PDGF stimulation in fibroblasts, Rac1 is activated to promote ruffling of plasma membrane while activated RhoA and RhoB regulate the stress fibers and focal adhesion sites. (Ridley and Hall 1992).

f) The G-protein Rho

The Rho p21 family is part of the ras-related small G protein superfamily (for review, see Hall et al. 1990; Takai et al. 1992). The Rho family consists of A,B, and C, and more recently,
Rac1 and Cdc 42. Rho is regulated by both GEP (GDP/GTP exchange protein) and GAP (GTPase activating protein) (Hall et al. 1990; Takai et al. 1992). The conversion from the GDP-bound inactive form to the GTP-bound active form is regulated by GEP and the reverse is regulated by GAP. There are 2 types of GEP for Rho: one stimulatory (rhoGDS) and the other inhibitory (rhoGDI) (Ueda et al. 1990). These regulatory proteins are present in most cells and the intracellular amount and inhibitory action of rhoGDI is respectively larger and stronger than that of rhoGDS (Kikuchi et al. 1992).

Rho can undergo three kinds of post-translational modifications at the C-terminus: i) geranylgeranylation (see following section), ii) removal of the three C-terminal amino acids, and iii) carboxymethylation (Katayama et al. 1991). Only the post-translationally processed Rho is sensitive to GDI and GDS actions, although modified and unmodified forms of Rho are sensitive to GAP (Takai et al. 1992; Mizuno et al. 1991; Hori et al. 1991).

Rho is present in the GDP-bound inactive form complexed with rhoGDI in the cytosol at rest in such cells as insulinoma cells and smooth muscle cells (Regazzi et al. 1992). In addition to inhibition by GDI association, Rho can also be ADP-ribosylated by C3 exoenzyme of Clostridium botulinum (Sugai et al. 1992). Addition of C3 to NIH/3T3 cells makes the cells round and refractile (Rubin et al. 1988) whereas in PC-12, abnormally short neurites are generated (Rubin et al. 1988). Microinjection of rhoGDI was shown to induce morphological changes and the disappearance of stress fibres in Swiss 3T3 cells (Miura et al. 1993) that are similar to those changes induced by C3 exoeyme treatment which inhibits G-proteins (Chardin et al. 1989, Paterson et al. 1990). Also, HMGCoA reductase inhibitor (statins) makes NIH/3T3 cells round and refractile (Fenton et al. 1992). Rac1 induces the assembly of focal adhesions and stress fibres, which cause morphological change through RhoA (Ridley and Hall 1992). These
data suggests that active Rho is needed for normal cell morphology. These results also suggest that Rho regulates these cell functions through the actin-myosin system.

Post-translational modification is also important for Rho activity and cellular placement. Post-translational modification of Rho is essential for Rho action as only modified Rho reacts with GDI (Takai et al. 1992; Mizuno et al. 1991; Hori et al. 1991). Rho has been found in the cytosol, inactivated, but reversibly associated with GDI, which allows Rho to re-enter the membrane (Seabra et al. 1998).

Dissociation of GDI from Rho is a prerequisite for GDP/GTP exchange and this step correlates with the translocation of RhoA to the plasma membrane (Bokoch et al. 1994) because of Rho's lipid modifications (Casey et al. 1996). RhoA has been shown to concentrate in caveolae which are plasma membrane domains that are clustered in regions of the cell surface rich in actin filaments (Michaely et al. 1999). Here, Rho regulates stress fiber formation at focal adhesions, cell surface structures where stress fibers terminate and where many signal transduction molecules are localized (for review, Ingber et al. 1994; Qiu et al. 1995). Rho B has also been localized to late endosomes (Robertson et al. 1995).

RhoB also plays a role in cytoskeletal organization but the mechanism how Rho achieves cytoskeletal control is varied (Ridley & Hall 1992). RhoB has been shown to activated kinases which in turn activate myosin binding to actin and hence affect actomyosin interaction (Kimura et al. 1996; Amano et al. 1996). As well, Rho regulates phosphatidyl-inositol 4-phosphate 5-kinase which controls numerous actin-binding proteins (Chong et al. 1994). Activation of RhoB produced a slight reversion of statin-induced rounding of cells suggesting that RhoB is responsible for maintaining proper cell structure (Koch et al. 1997).
g) Rho signaling

Rho can activate multiple signaling pathways including phospholipase D, phosphatidylinositol-3-kinase (PI3K), PI5K, myosin phosphatase, MAPKs (Renshaw et al. 1996), and the transcription factors Jun, serum response factor, and NF-κB which can activate cell death or growth (Gomez et al. 1998). In addition, Rho has been implicated in modulating gene expression (Morissette et al. 2000), MAPK activation, cell cycle progression, transformation, and smooth muscle contraction (Renshaw et al. 1996; Sahai et al. 1999). However, Rho does not regulate MAPK activity in cardiac myocytes (Sah et al. 1996). A protein kinase that is also activated by Rho, known as Rho kinase (ROK), regulates Rho-dependent cytoskeletal rearrangement (Amano et al. 1997). RhoA in cardiac myocytes has been reported to be involved in the formation of myofibrils from actin (Aoki et al. 1998).

h) Rho and apoptosis

Rho enhances apoptosis in murine fibroblasts and erythroleukemia cells after serum deprivation by a p53-independent mechanism (Jiminez et al. 1995). However, apoptosis induction by Rho needs complementary signals, such as the apoptotic inducer ceramide (Esteve et al. 1995; Jiminez et al. 1995), to induce cell death. RhoGDI has been observed in the cytosol to translocate to the nucleus during DNA fragmentation associated with apoptosis (Krieser & Eastman 1999).

i) Rho and heart disease

RhoA has been reported to mediate hypertrophic growth and atrial natriuretic factor gene expression in neonatal rat ventricular myocytes (Morissette et al. 2000). A role for Rho in myofibrillar organization and gene expression associated with myocardial cell hypertrophy has been repeatedly demonstrated (Aoki et al. 1998; Kuwahara et al. 1999; Sah et al. 1996).
IV. FATTY ACIDS

A. FATTY ACIDS AND THE HEART

In normal mammalian hearts, the majority of fatty acids are incorporated into various pools: phospholipids, triacylglycerols, cholesteryl esters, and free fatty acids (van der Vusse et al. 1992). These pools allow storage of fatty acids for cardiomyocyte energy metabolism. It is estimated that fatty acid concentrations within the cardiomyocyte cytoplasmic space are 10μM (van der Vusse et al. 1992).

There is a relationship between increased metabolism of free fatty acid in the heart and increased ischemic injury in myocardial infarction. Decreased uptake of free fatty acids results in decreased ischemic injury (Opie et al. 1988). Elevated levels of fatty acids are found in patients with hypertension, diabetes mellitus, and cardiomyopathy (Salomaa et al. 1990; Mazeaud et al. 1992; Factor et al. 1996; Coughlin et al. 1994). The fatty acid palmitate is elevated in the blood of patients with acute myocardial infarction within 4h of acute MI and potentiates myocardial ischemic injury (Oliver et al. 1968; Opie et al. 1977). As well, there was a significant increase in palmitic acid-containing phospholipids in subjects who sustained coronary events compared to controls (Miettinen et al. 1982). Indeed, fatty acid composition of serum lipids was proposed a prediction of ischemic heart disease (Miettinen et al. 1982).

B. FATTY ACID METABOLISM (FIGURE 6)

Fatty acids are a principal source of energy for the heart upon birth. Fatty acid metabolism involves fatty acids binding to carrier binding proteins, being transported across the sarcolemmal membrane and once inside the cell being metabolized to long chain acyl-CoA by acyl-CoA synthetase (for review, see van der Vusse et al. 1992; Lopaschuk et al. 1994a&b).
Figure 6 - Fatty acid and carbohydrate oxidation. From Lopaschuk et al. 1994
Fatty acyl CoA is then transported across the mitochondrial membrane via the carnitine-palmitoyl transferase I & II (CPT 1 & 2) which enables the fatty Co A to be delivered into the inner mitochondrial membrane while using carnitine as a recyclable transporter. Once inside the inner mitochondrial matrix, the fatty acyl Co A undergoes β oxidation, a series of chemical reactions resulting in the production of FADH$_2$ and NADH (to be used in the respiratory chain), acetyl CoA, and a fatty acyl Co A chain that is shortened by 2 carbons. The shortened fatty acyl CoA chain is primed to enter the β oxidation spiral again. In addition, acetyl CoA can enter the citric acid cycle for further energy production. It is the buildup of intermediate products of the β oxidation spiral which can create a “toxic” environment in which fatty acid oxidation cannot continue. Hence, anything that blocks the last stages of fatty acid oxidation can cause the buildup of these “toxic” intermediates, which, in turn, will further inhibit fatty acid metabolism. The fatty acid excess may then be available for signal transduction purposes.

Fatty acid oxidation occurs predominantly in the mitochondria as well in peroxisomes (van der Vusse et al. 1992; Lopaschuk et al. 1994a&b). The only differences in fatty acid oxidation between mitochondria and peroxisomes is that (I) peroxisomes do not contain the CPT transport system whereas mitochondria require CPT, and (ii) the eventual electron recipient of the respiratory chain is oxygen thus forming H$_2$O, which is decomposed by catalase in peroxisomes whereas water is produced at the final step in mitochondrial respiratory chain (van der Vusse et al. 1992). Hence, peroxisomal fatty acid oxidation is the preferred mechanism for metabolism of fatty acid chains exceeding C22 because of the ease of fatty acid transport. Peroxisomal oxidation capacity may account for up to 45% of the metabolism of these very long chain fatty acids (van der Vusse et al. 1992). Estimates of fatty acid β-oxidation in peroxisomes
of the heart vary from 4% (Chu et al. 1994) to 39% (Veerkamp et al. 1986) but likely are closer to 22 to 27% that has been noted in hearts from different species (Piot et al. 1998).

Fatty acid oxidation is subject to control by carbohydrate oxidation (Lopaschuk et al. 1994a&b). Excess acetyl CoA from carbohydrate oxidation (oxidation of glucose, pyruvate, or lactate) is transported out of the mitochondria and is converted to malonyl CoA in the cytosol by acetyl CoA carboxylase (ACC) (Mabrouk et al. 1990). Malonyl CoA can directly inhibit CPT-1 (McGarry & Brown 1977). Malonyl CoA levels can be controlled at two different points: generation of malonyl CoA by ACC activity and by malonyl CoA degradation. Upregulation of glycolysis can occur by glycolytic substrates such as glucose, lactate, or pyruvate. ACC activity is sensitive to hormones such as insulin and glucagon (Makinde et al. 1998). Glycolytic substrates enter the mitochondria as pyruvate and are converted and transported by the pyruvate dehydrogenase complex (PDC) to acetyl CoA. An increase in acetyl-CoA production from PDC will result in a shuttling of acetyl groups via various acetyl CoA forms (eg. acetylcarnitine, citrate) into the cytoplasm producing an increase in malonyl-CoA production, which in turn, will inhibit fatty acid oxidation (for review, see Lopaschuk et al. 1994b).

The main factors controlling the rate of cardiac fatty acid oxidation are i) the supply of fatty acids by the blood; ii) the level of high-energy phosphates; iii) the redox state of the mitochondria; and iv) the availability of CoA in both the mitochondrial compartment and the cytoplasmic space (van der Vusse et al. 1992). A regulatory control point for fatty acid metabolism is CPT-1, which is crucial as it is the rate-limiting step of fatty acid transport into the mitochondria for its oxidation (McGarry et al. 1977). Carnitine is a substrate for and an important regulator of CPT-1. It is a necessary co-factor for fatty acyl CoA translocation, and its intramitochondrial concentration governs the enzyme kinetics of CPT-1 (Cook et al. 1984).
Various agents including oxfenicine (hydroxyphenylglycine) and etomoxir inhibit CPT1
(Madden et al. 1995; Varela et al. 1997).

C. THE FATTY ACID PALMITATE

1. The fatty acid palmitate and the heart

   As mentioned, Oliver et al. (1968) measured and correlated serum free fatty acids and myocardial infarctions, with the maximal fatty acid concentrations occurred at 4 to 8 hours after the MI. The occurrence of abnormally high fatty acid levels was associated with the occurrence of serious arrhythmia and conduction disorders (Oliver et al. 1968). As palmitate, a 16 carbon saturated fatty acid, is the most common fatty acid in blood (van der Vusse et al. 1983), palmitate is an excellent fatty acid to investigate.

2. Free vs. bound fatty acids

   Fatty acids are often bound to carriers during transport in the body. During transport in the blood, fatty acids are bound to albumin whereas fatty acids are bound to fatty acid binding proteins within the cell. Due to this binding, total fatty acid concentration is the sum of bound plus unbound free fatty acids. Richieri and Kleinfeld (1995) established a complex enzymatic equation to determine the amount of free unbound fatty acids in serum, taking into account the numerous binding sites of albumin. They calculated the average unbound fatty acid concentration in normal patient serum to be 7.5 nM. As palmitate constitutes 25% of all serum fatty acids (determined by Richieri & Kleinfeld 1995 by comparing various clinical studies), normal concentrations of unbound palmitate in serum is 1.9 nM.
However, as Richieri and Kleinfeld (1995) point out, patients in a diseased state may not have the same proportion of total fatty acid:albumin as in normal patients and that this proportion may vary between patients. For example, Richieri observed that patients undergoing angioplasty have values that exceed 100 nM. Thus, to calculate the serum concentration of free fatty acid in diseased patients require some assumptions. Patients who have suffered a myocardial infarction, have elevated serum fatty acid levels on average of 1.2 mM (Opie et al. 1977). Using the aforementioned binding constants and assuming that these patients have the same 6 μM (or 0.04%) albumin as in normal patients, I calculate the free fatty acid concentration to be 125 nM, of which 25% of this is palmitate or approximately 30 nM.

The present study routinely used 100 μM of exogenous palmitate to represent serum palmitate concentrations. However, cardiomyocytes were maintained in culture in the presence of 0.18% albumin (818 media with 6% fetal calf serum which contains 3% albumin), an albumin concentration less than what would be seen in serum. Assuming the same albumin binding constants, I calculate the concentration of free palmitate in my cell model to be approximately 20 nM, 10 fold greater than normal palmitate concentrations in serum and similar to concentration in patients with acute myocardial infarction.

3. Palmitate and apoptosis

Palmitate-induced apoptosis has been demonstrated in breast cancer cells (Hardy et al. 2000), murine hematopoietic cell lines (Paumen et al. 1997), embryonic chick astrocytes and neuronal cells (Mangoura et al. 1998; Wiesner et al. 1996), pancreatic beta cells (Shimabukuro et al. 1998), and neonatal rat cardiomyocytes (Sparagna et al. 2000 & 2001, de Vries et al. 1997), and embryonic chick cardiomyocytes (Kong & Rabkin 1999, Kong & Rabkin 2000).
De Vries et al (1997) reported palmitate induced apoptosis in neonatal rat cardiomyocytes by observing DNA fragmentation with TUNEL and electrophoretic demonstration of 180bp DNA fragments. In the same study, apoptosis was inhibitable by the unsaturated fatty acids oleic (C18:1). In DeVries’ study, palmitate was initially prepared in ethanol but then treated in a bovine serum albumin solution to mimic the albumin-fatty acid complex in blood. Staining of treated cells with Oil Red O demonstrated that palmitate treatment increased the number of triacylglycerol pools suggesting that the addition of exogenous fatty acids causes major alterations in endogenous lipid pools. Although the study by DeVries et al. (1997) observed palmitate-induced apoptosis, the mechanism was not elucidated.

It should also be mentioned that fatty acids have been also implicated in the induction of oncrosis (Tanigaki et al. 1998). Furthermore palmitate-induced membrane alterations that are consistent with oncrosis have been also described (Gudbjarnason et al. 1975).

The mechanisms underlying palmitate-induced cell death are incompletely understood. It has been suggested that palmitate is incorporated into de novo synthesis of ceramide, a lipid-signaling molecule implicated in the induction of apoptosis so that excess palmitate may induce cell death through increased intracellular ceramide concentration (Paumen et al. 1997; Shimabukuro et al. 1998). An equally attractive hypothesis is that palmitate metabolism within the mitochondria can induce a pro-apoptotic cascade as interruptions of the β-oxidation chain, such as inhibition of CPT-1, results in accumulation of toxic byproducts. Another hypothesis is that during palmitate metabolism, acetyl CoA is generated which can then stimulate cholesterol synthesis which has been implicated in heart dysfunction.
4. Palmitate in other cellular forms

When not in the process of active metabolism, palmitate exists within the cell in various forms. Palmitate is metabolized to and stored within triglycerides and phospholipids for future metabolism, especially when cardiomyocyte metabolism favors glycolysis such as in ischemia (for review, see van der Vusse 1992). Partially oxidized forms of palmitate, such as palmitoyl CoA and palmitoyl carnitine, exist within the cell and these have been implicated in damaging the cell (van der Vusse 1992). Mu et al. (2001) reported that palmitoyl CoA induced apoptosis in human granulosa cells. Mutomba et al. (2000) report that levels of palmitoylcarnitine increased whereas levels of both carnitine and palmitate decreased in response to Fas-induced apoptosis in Jurkat cells. Haruna et al. (2000) reported a negative relationship between accumulation of palmitoylcarnitine and modulation of K\textsubscript{ATP} channels in guinea pig ventricular myocytes. Taking this into account, the death induced by palmitate may also be due to the accumulation of these palmitoyl forms and that apoptosis may be regulated in part by the balance of carnitine and palmitoylcarnitine.

D. CERAMIDE - AN APOPTOTIC SIGNALING MOLECULE (Figure 7)

Ceramide signaling pathways have been implicated in cell proliferation, differentiation and cell death, by both apoptosis and oncosis (for in depth review, see Mathias et al. 1998), including apoptosis in adult and neonatal rat cardiomyocytes (Andrieu-Abadie et al. 1999; Bielawska et al. 1997). Ceramide is mainly generated in two different ways: \textit{de novo} synthesis from palmitoyl CoA and by sphingomyelinase (SMase) cleavage of sphingomyelin (for review, see Mathias et al. 1998). Once generated, ceramide may transiently accumulate or be converted
Figure 7 - Summary of de novo synthesis of ceramide. SM=sphingomyelin; S1P=sphingosine-1-phosphate. From Mathias et al. 1998
into various metabolites. Phosphorylation, deacylation, glycosylation and other modifications of ceramide allow ceramide derivatives to serve as effector molecules. Also, ceramide can also be converted back to sphingomyelin by SM synthase.

1. Ceramide generation
   
   a) *De novo* ceramide synthesis

   Ceramide is synthesized *de novo* by condensation of serine and palmitoyl-CoA to form 3-oxosphinganine, which is then reduced to dihydrosphingosine, acylated by ceramide synthase to yield dihydroceramide, and finally oxidized to ceramide (Merrill *et al.* 1997). This pathway takes several hours to generate detectable levels of ceramide (Bose *et al.* 1995). *De novo* ceramide synthesis has been observed to occur during danorubicin-induced apoptosis in HL-60 and U937 cells and ionizing radiation over a period of hours (Bose *et al.* 1995; Boland *et al.* 1997; Merrill *et al.* 1996).

   b) Sphingomyelinase degradation

   Ceramide can also be generated rapidly by sphingomyelin (SM) degradation (Verheij *et al.* 1996; Cuvillier *et al.* 1996). Hydrolysis of sphingomyelin at the level of the plasma membrane or intracellular in lysosomal membrane, is facilitated by various sphingomyelinases (SMases). There are several isoforms of SMase, distinguished by different pH optima and therefore referred to as acid, neutral or alkaline SMases. Both neutral and acid SMases rapidly and transiently produce ceramide in response to diverse exogenous stimuli in a time frame of seconds to minutes. Ceramide generation by SMase degradation has been observed in response to ionizing radiation (Pena *et al.* 1997), nerve growth factor (Hannun *et al.* 1996; Spiegel *et al.* 1996; Ballou *et al.* 1996), and ligand stimulation of the CD95 (Genestier *et al.* 1998) or TNFα receptors (Dbaibo *et al.* 1997). In addition, this ceramide generation correlated with observed
hallmarks of apoptosis (increased cell death, DNA fragmentation, and membrane blebbing).

Hence ceramide generation is associated with apoptosis.

2. Ceramide targets

The pleiotropic nature of ceramide signaling observed in previous studies is dependent on several things (for review, see Mathias et al. 1998). Ceramide has variable effects dependent on cell type, which receptors are present (eg. TNF), which downstream effector ceramide engages, the cellular pH (Monney et al. 1998), and whether there is concomitant activation of other mechanisms that may convert ceramide into another metabolite (e.g. sphingosine-1-phosphate)(Spiegel 1998). For example, ceramide can induce an inflammatory response by activating ceramide-activated protein kinase, which in turn activates the Raf/ERK mitogenic pathway (Yao 1995; Muller 1998). In addition, ceramide has been shown to induce apoptosis by initiating SAPK/JNK (for review, see Basu & Kolesnick 1998) and p38 (Jain 1998; Tavarini 2000; Hida 1999), and activating caspases (Monney 1998; Tepper 1999). Ceramide can also induce an anti-apoptotic response by activating ceramide-activated protein phosphatases (CAPP) (Reyes 1996). Ceramide also induces both activation of PKCζ, which leads to the control of the transcriptional apparatus (Bertolaso 1998), and translocation of PKCδ and ε during apoptosis (Sawai 1997). Ceramide has also been shown to have a direct inhibitory effect on mitochondrial complex III resulting in physical changes to the mitochondria (Gudz 1997).

3. Ceramide and the heart

In the cardiovascular system, ceramide has been observed to regulate apoptosis. The cytokine TNFα has been implicated as a potential pathogenetic mechanism for cardiac disease
states (for review, see Ceconi 1998; Meldrum 1998). Ceramide has been observed to be produced in response to TNFα, leading to apoptosis (Krown 1996; Oral 1997). Ceramide has also been implicated in the pathogenesis of ischemic heart disease as there was an increase in ceramide production in response to myocardial ischemia in rats (Bielawska 1997). In addition, the analogue C2-ceramide induced apoptosis in neonatal rat cardiomyocytes in vitro indicating that all components necessary for ceramide-initiated apoptotic pathway are present in this cell culture. Hence, ceramide is an excellent model to investigate as it has been implicated to induce apoptosis in heart and is related to fatty acid synthesis.

D. CHOLESTEROL SYNTHESIS/MEVALONIC PATHWAY AND CARDIAC DISEASE

1. Cholesterol and heart disease

Cholesterol synthesis has been widely investigated for its role in cardiovascular disease. High serum cholesterol or hypercholesterolemia, is considered a risk factor for atherosclerosis and coronary heart disease, as found in several studies such as the Framingham study (for review, see Betteridge & Morrell 1998). A diet of high cholesterol and fat can yield a high circulating concentration of cholesterol/fat attached to low density lipoproteins, LDLs. The circulating LDLs form lesions in the arterial wall by entering the subendothelial space. Here, the LDL is oxidatively modified by reactive oxygen species and this attracts monocytes to adhere to the arterial endothelium. Later, monocytes penetrate the endothelium, accumulate in the subendothelial space and acquire the characteristics of macrophages, which engulf lipid and become lipid-laden foam cells (for review, see Betteridge & Morrell 1998). This process is toxic to the endothelium, which is disrupted. This allows platelet adhesion and aggregation with
release of potent growth factors that stimulate smooth muscle proliferation and connective tissue accumulation, with consequent development of the mature atherosclerotic plaque (for review, see Betteridge & Morrell 1998). An atherosclerotic plaque within a coronary artery leads to coronary artery disease (for review, see Betteridge and Morrell 1998).

2. Cholesterol biosynthesis (Figure 8)

The cholesterol requirements for cells are substantial, particularly for membrane synthesis in cellular hypertrophy or cell division, and most cells meet this requirement primarily through endogenous cholesterol biosynthesis (Dietschy 1993). Endogenous cholesterol biosynthesis occurs within all cells. Cholesterol is synthesized through a complex series of reactions in which a trimer of acetyl CoA molecules generated from fatty acid oxidation, known as 3-hydroxy-3-methylglutaryl CoA or HMG-CoA, is converted by HMG-CoA reductase into the key intermediate, mevalonate (Mathews & van Holde 1990). Mevalonate is then converted to an isoprenoid unit, six of which condense to form squalene (Mathews & van Holde 1990). Cyclization of squalene forms lanosterol, which is converted to cholesterol. Importantly, this pathway leads to the synthesis of numerous nonsterol compounds that have roles in electron transport, glycoprotein biosynthesis and cell growth. Cell growth effects are mediated through isoprenylation, or attachment of cholesterol biosynthesis byproducts. Isoprenylation of several key cellular proteins, such as small GTPs, lead to cell growth (Goldstein and Brown 1990; Russell et al. 1992).
3. Cholesterol biosynthesis in embryonic/neonatal heart

Cholesterol biosynthesis figures prominently during the embryonic and neonatal stages. The embryonic growth rate is unparalleled by that during any other stage of life. To maintain its rapid
Figure 8: Overview of cholesterol biosynthesis. ER = endoplasmic reticulum. From Mathews & van Holde 1990.
growth rate, the embryo requires a significant amount of cholesterol and fatty acids for structural purposes and as precursors for various steroidogenic hormones that are critical to normal development. (for review, see Woolet 2001) Recently, brain abnormalities have implicated deficiencies in cholesterol during fetal development (Kelley 2000)

4. Acetyl CoA in apoptosis

To my knowledge, there has been no previous work implicating acetyl CoA directly to apoptosis. However, there is a previous report of acetyl CoA being used as a source of non-enzymatic acetylation of sphinganine, a precursor of ceramide (Kashiwagi et al. 1997). However, the authors of this study concluded that the acetylation of sphinganine would produce a form of dihydro-ceramide which is inconvertible to apoptotic ceramide.

5. HMG-CoA Reductase

A rate limiting enzyme in cholesterol biosynthesis is the enzyme HMG-CoA reductase which converts HMG-CoA to mevalonate (Goldstein and Brown 1990; Russell et al. 1992). HMG CoA reductase is a 97kDa endoplasmic reticulum protein that consists of a membrane spanning domain and a soluble catalytic domain (Goldstein and Brown 1990; Russell et al. 1992). HMG CoA reductase is present in cardiomyocytes (Schmeela et al. 1994).

6. Inhibitors of cholesterol synthesis - Statins

Statins, or HMG-CoA reductase inhibitors, are currently used to treat hypercholesteremia and atherosclerosis (Bustos et al. 1998; Bellosta et al. 1998) by the inhibition of cholesterol
biosynthesis at HMG CoA reductase. Some common statin drugs are lovastatin, simvastatin, pravastatin, fluvasatin, cerivastatin, and atorvastatin. They are produced in different forms. For example, lovastatin and simvastatin are lactones whereas pravastatin, fluvasatin, cerivastatin and atorvastatin are in the open acid form. The open acid part of the statin molecule has a striking structural similarity to HMG-CoA, the trimer of acetyl-CoA molecules, which are the substrate for HMG-CoA reductase (Mathews & van Holde 1990). Hence, statins are specific, competitive inhibitors of the HMG CoA reductase (Betteridge and Morrell 1998). In vivo, statins can inhibit approximately 40% of HMG-CoA reductase activity (Betteridge and Morrell 1998).

7. Prenylation

Prenylation is a general term for the post-translational modification of a protein by the addition of a lipid group. Attachments of fatty acids, such as palmitic acid (C16) and myristic acid (C14), are also known as acylation whereas attachment of much larger lipid groups, such as farnesyl (C15) and geranylgeranyl (C20), are termed isoprenylation. (Mathews & van Holde 1990). The fatty acids needed for acylation are most likely gleaned from the fatty acids meant for metabolism whereas the isoprenoids are derived from the basic pathway of cholesterol biosynthesis. The main purpose for prenylation is for targeting proteins to various intracellular location and to the membrane in particular (for reviews, see Milligan and Grassie 1997; Levis et al. 1992; Robinson et al. 1995). For a number of membrane-bound proteins, extra lipids are attached to the membrane-associated side of the protein to enhance membrane docking. Extensive work with G-proteins demonstrate that G-proteins are inserted into the membrane, close to the GPCR, because of the acylated N-terminal of their α-subunit and isoprenylated γ-subunit C-terminus (for review, see Milligan and Grassie 1997). For example, activation of Gα
enhances its translocation from the plasma membrane to other cellular loci including the cytosol. Previous studies have shown that Gαs can be depalmitoylated and dissociated from the plasma membrane to enable translocation and that this dissociation from the membrane results in an enhanced rate of degradation of the protein (Levis et al. 1992). Palmitoylation of endothelial nitric oxide synthase allows it to be associated with its membrane association (Robinson et al. 1995). As previously mentioned, prenylated versions of RhoA and RhoB are responsible for its activity and cellular localization (see previous section on G-proteins).

The choice of farnesyl or geranylgeranyl is based primarily on the nature of the amino acid motif Cys-A-A-X, where A is an aliphatic amino acid and X is any amino acid (Maltese et al. 1990). When methionine is in the place of A, farnesylation occurs whereas leucine is in the first place geranylgeranylation occurs (for review, see Milligan and Grassie 1997). Likewise, the choice of fatty acids is dependent on the Met-Gly-X-X-X-Ser-X-X motif. (for review, see Milligan and Grassie 1997). Interactions of these lipid modifications also act to stabilize the tertiary structure of the protein. For example, myristoylation of the α-subunit and isoprenylation of the γ-subunit of G-proteins stabilize the G-protein heterotrimeric complex. (for review, see Milligan and Grassie 1997)

8. Statins and apoptosis

Although statins are beneficial to the individual hypercholesteremic patient by inhibition of cholesterol biosynthesis, interruption of the biosynthesis pathway may have serious deleterious effects. Statins such as lovastatin, simvastatin and atorvastatin induce apoptosis in various cell types including vascular smooth muscle cells (Guijarro et al. 1998), HL60 cells (Perez-Salsa et al 1994), macrophages (Rogers et al 1996), prostate cells (Padayatty et al 1997;
Marcelli et al. 1998), osteoclasts (Luckman et al. 1998), and mouse proximal tubular cells (Iimura et al. 1997). Statin-induced apoptosis is dependent on protein synthesis (Coxon et al. 1998).

9. Other deleterious effects of statins

   a) Effects on actin

   In addition to inducing apoptosis, statins have also been reported to affect the cytoskeleton. Lovastatin was observed to depolymerize F-actin observed in HL60 cells (Levee et al. 1996), renal proximal tubule cells (van De Water et al. 1996), renal carcinoma cells (Fenton et al. 1992) and NIH323 cells (Koch et al. 1997). Koch et al. (1997) reported that lovastatin induced a loss of F-actin in fibroblasts with no change to monomeric G-actin, as assessed by F-actin quantitative (NBD-phallacidin binding & fluorescence) and G-actin qualitative determinations (chromatography & DNAse activity). Lovastatin also induced an increase in cytosolic Rho with a decrease in membrane Rho suggesting a translocation of this G-protein (Koch et al. 1997). Lovastatin’s effect on actin culminated in a rounding of cells, which was inhibitable by inhibition of protein synthesis with cycloheximide (Koch et al. 1997).

   b) Effects on prenylation

   By inhibiting cholesterol biosynthesis, statins also inhibit the synthesis of other important biological compounds, the ubiquinones and dolichols and prenyl groups, formed from mevalonate (Mathews & van Holde 1990). Dolichol is required for glycoprotein synthesis and ubiquinones are important in mitochondrial electron transport (Maltese et al. 1990). Prenyl groups, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate, are necessary for
post-translational modification of proteins and will be discussed in the following section. As well, statins affect steroid hormone synthesis, as cholesterol is necessary for steroid synthesis.

10. Lovastatin and the heart

The HMG-CoA reductase inhibitor lovastatin has been previously studied in the heart. Kashiwagi et al (1998) report that lovastatin prevented the farnesylation of Ras which is critical for Ras's membrane localization and function. As a result, Ras was not membrane anchored and was unable to transmit the signal from receptor activation to activate MAPK, which eventually led to stretch-induced cardiac hypertrophy (Kashiwagi et al. 1998). Repko and Maltese reported that lovastatin causes a rapid depletion of isoprenoid groups in murine erythroleukemia cells and that mevalonate prevents this depletion (Repko and Maltese et al. 1989). These studies suggest that lovastatin has effects on the heart and can be used to investigate contribution of mevalonic pathway in this thesis.

V. CELL SURVIVAL FROM FATTY ACID APOPTOSIS

A. FATTY ACID OXIDATION IN PEROXISOMES

As apoptosis has been documented to have mitochondrial effects and that the majority of fatty acid metabolism occurs in the mitochondria, a change in location of fatty acid metabolism may prove beneficial to the dying cell. As mentioned previously, fatty acid metabolism occurs, in part, in the peroxisomes. Previous studies observed that certain agents, known as fibric acids, stimulated the proliferation of peroxisomes, which led to the discovery of the peroxisomal proliferator-activated receptor (PPAR) (for reviews, see Betteridge & Morrell 1998; Packard 1998). Fibric acid drugs such as fenofibrate, clofibrate, gemfibrozil, bezagibrate, and
Cipropfibrate are peroxisome proliferators. Cardiac peroxisomal \( \beta \)-oxidation capacity was increased 2.5-fold by clofibrate (Norseth et al. 1983) and the activity of \( \beta \)-oxidation enzymes such as acyl CoA oxidase was increased up to 30 fold by fibrates in liver (Orton et al. 1982).

1. PPARs

In addition to the beneficial effects of fibric acid on peroxisomal metabolism, these peroxisome proliferators were found to activate a family of nuclear receptors known as peroxisome proliferator activated receptors, PPAR. PPARs are identified as members of the intracellular nuclear receptor super family (Issemann et al. 1990). Three PPAR subtypes (\( \alpha \), \( \beta \) or \( \delta \), and \( \gamma \)) have distinct tissue distribution (Amri et al. 1995). For example, PPAR\( \alpha \) is expressed in tissues actively metabolizing fatty acids such as the heart (Beck et al. 1992). When PPARs are induced by fibric acids, PPARs are capable of dimerizing with the 9-cis retinoic acid receptor. This complex can bind to response elements in the genome (PPRE) leading to the regulation of transcription of the pivotal enzymes that regulate mitochondrial and peroxisomal fatty acid metabolism (Veerkamp et al. 1986; Staels et al. 1998) such as acyl-CoA synthetase (Schoonjans et al. 1996), acyl-CoA oxidase (Keller et al. 1993), and medium chain acyl-CoA dehydrogenase (Gulick et al. 1984). As well, putative fatty acid transporter genes are regulated by PPAR\( \alpha \) and \( \gamma \) (Motojima et al. 1998). A role for PPAR has been proposed for various disease states including atherosclerosis, diabetes mellitus, obesity, and cancer (Vamecq and Latruffe 1999).

VI) PROS AND CONS OF USING TWO CELL MODELS, CHICK AND MOUSE, IN CARDIOLOGY RESEARCH

The use of in vitro cell cultures allows study of the biochemistry of the cardiomyocyte without the influence of systemic vasculature and circulating hormones. In addition, use of
cardiomyocyte culture eliminates the potential action of stimulation of cell surface receptors on the coronary and systemic vasculature. Furthermore, the use of a cell culture permits observation of apoptosis without presence of other circulating modulator of apoptosis.

Fatty acids are the principal source of energy for the heart at birth. However, in some embryonic hearts, energy production from fatty acids is negligible, as glycolysis is the major contributor to energy production (Fisher et al. 1980; Rolph et al. 1983; Werner et al. 1987). This may be attributed to fewer and smaller mitochondria, with a lower density of cristae, lighter matrices, and more variable inner membrane configurations in the fetal heart than in the adult heart (Sordahl et al. 1972). However, this is not the case in embryonic chick heart. Pugh & Sidbury (1971) reported that myocardial fatty acid oxidation in chick embryos conforms with the mechanism shown in adult mammalian tissue and oxidize palmitate to acetyl CoA. They also reported that the rate of oxidation rose to adult levels prior to hatching, approximately after 14 days of embryonic age. Warshaw (1972) observed similar results and theorized that the difference between embryonic chick heart and embryonic/fetal heart is due to the dependence of the chick embryo on the lipid laden yolk in contrast to the greater dependence of fetal rats on carbohydrate as an energy source. Thus, the embryonic chick heart is an ideal model in which to test the hypothesis that regulatory control of fatty acid metabolism by other factors such as glycolysis modulates palmitate-induced cell toxicity. Conversely, neonatal mouse cardiomyocytes will prefer fatty acids as their principal source of energy.

An advantage to the concomitant study of both chick and mouse models is that the basic research can be done on more efficient and cost-effective chick culture whereas relevance to human is achieved by the mammalian mouse model.
VI. RATIONALE, HYPOTHESIS, AND OBJECTIVES

A. HYPOTHESIS (FIGURE 9)

The hypothesis to be tested is briefly as follows:

The fatty acid palmitate induces apoptosis in cardiomyocytes through its oxidation or metabolism in mitochondria. Increased production of palmitate metabolites damage mitochondria producing cell death. Palmitate-induced death does not occur by its incorporation into ceramide, a known apoptotic inducer. Rather, ceramide induces apoptosis utilizing a different mechanism than palmitate, transmitting its death signal by the MAPK pathways. Palmitate-induced apoptosis does not occur through increased cholesterol biosynthesis. Rather, inhibition of cholesterol biosynthesis will induce death itself and this mechanism is in part due to the prevention of cholesterol byproduct production that prevent caspase-2 and caspase-3 activation. I hypothesize that common signaling molecules are responsible for both changes to morphology and the induction of apoptosis, in particular RhoB and caspase-2.

B. RATIONALE

Some heart diseases involve cell death of cardiomyocytes. Apoptosis is being investigated as it is a form of cell death that is controlled and can occur repeatedly, over days or even hours. Loss of cardiomyocytes has clear implications for the function of the heart as cardiac cells can not regenerate. Elevated fatty acid levels occur with myocardial infarction and correlate with the extent and severity of myocardial injury (Opie et al. 1977). Palmitic acid is one of the most common fatty acids found circulating in the blood and is found in elevated levels in patients after myocardial infarction.
Figure 9 - Hypothesis.
(Miettinen et al 1982). Hence, it is logical to investigate the role of fatty acids in heart disease and palmitate is an ideal candidate for investigation.

Palmitate is metabolized predominantly within the mitochondria to generate the ATP necessary for proper heart function. Excessive palmitate presented to the heart results in increased palmitate oxidation within the mitochondria. It may be hypothesized that the effects of this increased oxidation will yield byproducts such as acetyl Co A or physically damage the mitochondria or its enzymes. Damaged mitochondria would be unable to produce ATP which would eventually lead to cell death. As well, increased palmitate oxidation may result in increased activity of oxidation enzymes and increased β-oxidation products and may be the means by which palmitate induces apoptosis. The enzyme carnitine palmitoyl transferase 1 is an ideal enzyme to study to observe as it is the rate limiting step for fatty acid oxidation.

Although palmitate oxidation occurs predominantly within the mitochondria, it is also oxidized within the peroxisomes but to a lesser extent. If palmitate’s apoptotic mechanism is located within the mitochondria, especially if it involves enzymes that can only be found in mitochondria, the stimulation of peroxisomal palmitate oxidation may result in a diminished apoptotic response because the mitochondrial mechanism will no longer be involved. The peroxisome proliferator fenofibrate will be used to increase peroxisomal oxidation of palmitate and compared to the another peroxisome proliferator and PPARα agonist WY 14643.

Palmitate may also induce apoptosis via its incorporation into de novo ceramide, a known apoptotic inducer. An inhibition of de novo ceramide synthesis with fumonisin B1 will determine whether palmitate executes its apoptotic mechanism via ceramide synthesis. As well, if palmitate-induced apoptosis is mediated by ceramide, then it is logical to assume that the same mitochondrial effects observed in palmitate-induced apoptosis will be observed during ceramide
treatment. If ceramide induces similar mitochondrial changes to palmitate, then it is likely that palmitate exerts its apoptotic mechanism via ceramide. If ceramide induces a different response, it would suggest that there is another mechanism by which ceramide is inducing apoptosis.

Fatty acid metabolism also produces acetyl CoA which is the starting substrate for cholesterol biosynthesis. If palmitate induces apoptosis by its increased metabolism, then one can assume that there will be an increase in acetyl CoA produced which will stimulate cholesterol biosynthesis. It is possible that palmitate may induce its death mechanism by increased cholesterol biosynthesis. Thus, inhibition of cholesterol biosynthesis with HMG-CoA reductase inhibitor lovastatin concomitant with palmitate may reduce palmitate-induced apoptosis. Hence, HMG-CoA reductase inhibition with lovastatin treatment concomitant with palmitate will be a good model to determine the role of cholesterol biosynthesis in palmitate-induced apoptosis.

Lovastatin treatment will not only inhibit HMG-CoA reductase hence preventing the processing of HMG-CoA, but it will also inhibit cholesterol biosynthesis and all cholesterol-related products and processes. Thus it is necessary to investigate the effect of HMG-CoA reductase inhibition but with a functional cholesterol biosynthesis. This can be achieved with mevalonic acid, a cholesterol metabolite downstream of HMG-CoA reductase inhibition. Hence, co-treatment of mevalonic acid and lovastatin will be an ideal model for the effects of acetyl CoA accumulation and HMG-CoA reductase inhibition but with a functional cholesterol pathway.

As apoptosis has been documented to involve caspase signaling, caspase-2 and caspase-3 signaling will be examined. Caspase-2 will be examined as it is an initiator caspase whereas caspase-3 is an executor caspase. The roles of these caspases during apoptosis via cholesterol
biosynthesis will be determined by using the specific caspase-2 inhibitor zVDVAD and caspase-3 inhibitor AcDEVD and antibodies specific for active caspase-2 and -3.

Several cellular changes occur in response to one apoptotic signal. It is possible that one signaling complex is responsible for more than one apoptotic effect. Hence it is logical to determine whether such a common signaling complex exists. The small G-protein RhoB is an ideal candidate for such a complex as it is involved in cytoskeleton maintenance and has some signaling abilities. In addition, RhoB is isoprenylated which suggests RhoB will be affected by changes to cholesterol biosynthesis during apoptosis. Caspase-2 is an ideal candidate for this complex as it is a known apoptotic signaling molecule and it is anchored to the membrane where cytoskeletal maintenance by RhoB occurs. Hence, an association between RhoB and caspase-2 will be determined.

C. OBJECTIVES

1. To determine that palmitate induces apoptosis in cardiomyocytes.

Cardiomyocytes, from embryonic chick and neonatal mice, will be cultured and treated with palmitate and the resulting apoptotic effects will be observed. Apoptosis has been documented to yield various changes in cell organelles. In particular, i) nuclei with low DNA content but with an enrichment of 180bp fragments; ii) loss of mitochondrial respiration; ii) increased mitochondrial permeability; iii) loss of $\Delta\Psi_m$; and iv) disassembly and reassembly of F-actin during changes in the cytoskeleton and hence changes in overall cell morphology.

DNA fragmentation will be observed in two separate mechanisms: i) DNA laddering – DNA will be isolated and run on an agarose gel to observe DNA fragments of 180bp or multiples; and ii) nuclear hypoploidy – as apoptotic DNA is being degraded, the nucleus will exhibit
copy DNA (for cells in G0/G1), 2 copy DNA (for cells in DNA synthesis, mitosis, or G2), or less than one copy DNA (for degraded, apoptotic DNA). Both methods will be validated in embryonic chick cardiomyocytes and whichever method is more specific, sensitive, and efficient will be used for this thesis. Apoptotic effects on mitochondria and cytoskeleton will be described in the forthcoming objectives.

2. To determine that palmitate-induced cell death involves a mitochondrial pathway because palmitate oxidation occurs within the mitochondria.

   Because an increase in mitochondrial permeability has been documented during apoptotic development, whether palmitate induces an increase in mitochondrial permeability and the mechanisms involved need to be established. An increase in permeability will be evidenced by the presence of mitochondrial cyt C in the cytosol. As cyclosporin A is an established mitochondrial transition pore blocker, cyclosporin A will determine the presence of MTPs which may be the cause of the increase in mitochondrial permeability leading to the leakage of mitochondrial cyt C.

3. To determine whether inhibition of palmitate transport into the mitochondria can affect apoptosis by investigating the rate-limiting enzyme carnitine-palmitoyltransferase 1 (CPT-1).

   To assess whether palmitate induces apoptosis by its own metabolism within the mitochondria, I will examine the effects of enhanced palmitate transport into the mitochondria by CPT-1 with the exogenous addition of carnitine, the cofactor needed for palmitate's transport into the mitochondria. I will also examine the effect of CPT-1 inhibition with oxfencinice on palmitate-induced apoptosis. CPT-1 inhibition will also be produced by stimulating glycolysis with the exogenous addition of glucose, insulin, or lactic acid.
4. To determine whether fenofibrate, which affects peroxisomal oxidation rather than mitochondrial metabolism, will alter palmitate-induced apoptosis.

   Palmitate metabolism occurs predominantly within mitochondria in which apoptotic effects culminate in the destruction of mitochondrial activity. Hence, it is logical to determine whether palmitate’s death effects are influenced by the site of its metabolism. Because palmitate metabolism can also occur in peroxisomes, peroxisomal palmitate metabolism will be promoted with fenofibrate, an established agent used for proliferating peroxisomes to increase fatty acid oxidation in peroxisomes. Because fenofibrate affects peroxisomal fatty acid oxidation, it will not involve CPT-1 and the role of CPT-1 in palmitate-induced apoptosis can be further elucidated.

5. To determine whether palmitate induces apoptosis via its incorporation into the de novo synthesis of the apoptotic inducer ceramide.

   It has been established that palmitate is involved in the de novo synthesis of ceramide. To assess whether palmitate’s death effects are due to ceramide rather than palmitate, the ceramide synthase inhibitor fumonisin B1 will be used to inhibit de novo ceramide synthesis. Hence, any changes in palmitate-induced cell death with fumonisin B1 pretreatment may suggest the involvement of ceramide.

6. To establish whether palmitate’s mechanism is via ceramide action on mitochondria by comparing palmitate and exogenous C2-ceramide addition.
As ceramide is a known apoptotic inducer and palmitate is a component of ceramide biosynthesis, I will compare the apoptotic effects of ceramide to palmitate. If palmitate is inducing apoptosis via ceramide, ceramide-induced changes to apoptotic DNA fragmentation, loss of mitochondrial enzyme activity, loss of ΔΨₘ, and release of cyt C will be exactly the same with ceramide as with palmitate. Dyes specific for intramitochondrial “negative sink” as an indirect measure of mitochondrial respiration (CMX-Ros) and ΔΨₘ (DePsipher™) will be used with flow cytometry whereas cyt C release will be observed with immunoblotting.

7. To determine whether MAPK pathways, particularly p38 MAPK, SAPK/JNK, and MEK/ERK, are involved in ceramide-induced apoptosis.

As the MEK/ERK, SAPK, and p38 MAPK pathways have been documented to be involved in apoptosis in other cellular models, it will be determined which MAPK is involved in ceramide-induced apoptosis. The established MEK/ERK inhibitor (PD 98059) and p38 MAPK inhibitor (SB 202190) will be used to determine the role of these MAPK pathways in ceramide-induced apoptosis. Use of antibodies for the phosphorylated forms of SAPK, p38, and ERK will also be used to determine ceramide-induced MAPK activity.

8. To determine whether palmitate-induced apoptosis involves increased downstream cholesterol biosynthesis

To assess the effects of palmitate-induced increase in fatty acid oxidation, I will determine whether cholesterol biosynthesis is part of this death mechanism. Lovastatin inhibits cholesterol biosynthesis at the rate limiting step HMG-CoA reductase. Co-treatment of palmitate and lovastatin will theoretically reduce palmitate-induced apoptosis if cholesterol biosynthesis is
the part of the apoptotic mechanism. However, if lovastatin plus palmitate induce death, I will
determine why HMG-CoA reductase inhibition complicated palmitate-induced apoptosis.

9. To determine whether apoptosis induced by HMG-CoA reductase inhibition via lovastatin
operates via caspase-2 and caspase-3.

Caspase activation has been established in apoptosis, including caspase-2 and caspase-3.
These particular caspases are being investigated to represent the stages of the caspase cascade.
Caspase-2 is an initiator caspase as it transmits its signal from the cell membrane to the cytosol
whereas caspase 3 is an effector caspase as it receives its activation within the cytosol and
transmits its signal into the nucleus. zVDVAD and AcDEVD are established inhibitors of
caspase-2 and -3, respectively, and will be used to establish caspase roles.

10. To determine whether reactivation of cholesterol biosynthesis during HMG-CoA reductase
inhibition with mevalonic acid will prevent lovastatin-induced cell death

Interruption of cholesterol biosynthesis by lovastatin will also prevent prenylation of
several cellular components. The reinstatement of prenylation with mevalonate will allow
prenylation to occur with no effect on HMG-CoA reductase inhibition.

11. To determine whether lovastatin induces changes to cellular morphology involve changes to
cellular F-actin

One of the eventual targets of effector caspases is actin, a central component of the
cytoskeleton responsible for cell morphology. Changes in cell morphology involve the
disassembly and reassembly of F-actin. Changes to F-actin will be observed with the use of
NBD-Phallacidin, a fluorescent dye specific for F-actin. The roles of caspase-2 and -3 in apoptotic cytoskeleton will be established with the use of the aforementioned caspase inhibitors.

12. To determine whether there are any associations between caspases and a signalling molecule which are responsible for changes to cellular morphology and apoptosis during inhibition of cholesterol biosynthesis.

A potential mechanism by which HMG-CoA reductase inhibition induces its cellular morphology and apoptotic effects is by a common complex which initiates each pathway. A physical link between caspase-2 and RhoB will be determined. Reciprocal immunoprecipitation and immunoblots with RhoB and caspase-2 will be performed to determine such an association.
CHAPTER II
MATERIALS AND METHODS

I. CELL CULTURE

A. EMBRYONIC CHICK CARDIOMYOCYTES

Chick embryonic ventricular cells were cultured from 7-day chick embryos from white
Leghorn eggs using previously described methods (Rabkin et al. 1987; Rabkin et al. 1993). The
protocol was approved by the University committee on use of animals for research and conforms
with the guide for the Care and Use of Laboratory Animals published by the US National
Institutes of Health (NIH Publication No. 85-23, revised 1985). Myocytes were maintained in
culture for 72 h prior to the experiment in medium 818A which is composed of 73% DBSK
(NaCl 116 mM, MgSO\textsubscript{4} 0.8 mM, Na\textsubscript{2}HPO\textsubscript{4} 0.9 mM, dextrose 5.5 mM, CaCl\textsubscript{2} 1.8 mM, NaHCO\textsubscript{3} 26 mM), 20% Medium 199, 6% fetal calf serum, and 1% antibiotic-antimycotic (10,000 mg/ml
streptomycin sulfate, 10,000 U/mL penicillin G sodium and 25 μg/ml amphotericin B). The
proportion of myocytes at this time was over 90% as verified by the frequency of cells showing
spontaneous contraction or displaying muscle specific markers (myosin) on immunohistologic
examination.

B. NEONATAL MOUSE CARDIOMYOCYTES

Neonatal mouse cardiomyocytes were cultured from CD1 mice, less than 1 day old that
were euthanized by decapitation. The heart was removed and transferred to a petri dish
containing Dulbecco modified Eagles medium, DMEM, with 10% FCS and 1% antibiotic-
antimycotic (i.e. 10,000 mg/ml streptomycin sulfate and 25 mg/ml amphotericin B). The
ventricles were minced with collagenase mix (0.4% collagenase, 0.08% BSA). Tissue was
digested with more collagenase by mixing for 7 min at 37°C. The digestate was discarded.
Digestion was repeated three times with a collagenase mix (0.01% trypsin, 0.2% BSA, 1.1 mg
DNAse A) in a modified saline buffer (0.68% NaCl, 0.006% NaH$_2$PO$_4$, 0.027% Na$_2$HPO$_4$,
0.04% KCl). The digestion was stopped by dilution in DMEM with 10% FCS. Digestates were
collected and then centrifuged at 1000g for 4 min. to pellet the cells. The cell pellet was
resuspended in 10% DMEM and incubated at 37°C for 72 h.

II) MICROSCOPY

A. WRIGHT-GIEMSA

To examine cell morphology, cardiomyocytes, grown on cover slips, were treated with
agent or its diluent, ethanol. Cardiomyocytes grown on coverslips cells were stained according to
the Wright-Giemsa method (Sigma, St.Louis, USA), washed, mounted and examined
microscopically with a Zeiss (Standard 16) microscope.

B. ACRIDINE ORANGE

For acridine orange staining, the medium was removed and cells stained with 10 µg/ml
acridine orange for 5 min. at room temperature and examined under fluorescent light with a Zeiss
(IM35) microscope.

C. IMMUNOCYTOCHEMISTRY

To examine for the particular proteins, cardiomyocytes were grown on coverslips for 72 h
before drug treatment. Cells were washed briefly with PBS, fixed in 3.7% paraformaldehyde for
1 min., and treated with 1% triton X-100 diluted in 1x stabilization buffer (0.9g imidazol, 0.93g KCl, 0.002g MgCl2, 0.1g EGTA, 0.01g EDTA, 92g glycerol, and 17 μL mercaptoethanol in 250 ml deionized water). Coverslips were then incubated in 25% glutaraldehyde (10 min.), washed in 1mg/ml NaBH₄ (3 x 4 min. washes), washed in PBS (3 x 4 min. washes), and then reacted with the primary antibody (1/20 dilution in PBS) for 1 h. After PBS washing (3 x 4 min. washes), a secondary antibody linked to FITC (1/15 dilution in PBS) was incubated for 1 h to detect the primary antibody. Cells were washed in PBS (3 x 4 min. washes) before examination with a Zeiss (Standard 16) microscope using a fluorescent light source.

**D. NBD-PHALACIDIN**

To examine for the presence of cytoskeletal actin, neonatal mouse cardiomyocytes grown on coverslips for 72 h were treated with agent or diluent for 24 h. Cells were washed briefly with PBS, fixed in 3.7% paraformaldehyde, and stained with 100 nM NBD Phallacidin for 1 hour. Cells were examined with a Zeiss (Standard 16) microscope using a fluorescent light source, as outlined previously (Rabkin & Sunga 1987).

**E. DEPSIPHER™ (a.k.a. 5,5',6,6',tetrachloro-1,1',3,3'-tetaethylbenzimidazolyl carbocyaniniodide or JC-1)**

To examine for ΔΨₘ, cardiomyocytes grown on coverslips for 72h before use in the experiment. Cells were washed briefly with PBS and then allowed to incubate in 0.1% DePsipher™ reaction mix with stabilizer at 37°C for 20 min. Cells were washed with prewarmed reaction mix before microscopic examination.
F. TERMINAL DUTP-FLUORESCEIN NICK-END LABELING (TUNEL)

To examine for apoptotic DNA cleavage, cardiomyocytes grown on coverslips for 72h before use in the experiment. Cells were washed briefly with PBS and then treated with 50 µL of TUNEL reaction mixture (terminal transferase, CoCl$_2$, BSA, cacodylate, dATP and dUTP-fluorescein) (Roche, Laval, Que). The reaction occurred in a dark humidified chamber at 37°C for 1 h. Cells were then washed thrice with PBS and analyzed under a fluorescence microscope.

G. ANNEXIN V

To examine for apoptotic exposure of phosphatidylserine on the cell’s outer surface, cardiomyocytes were grown on coverslips for 72 h before use in the experiment. Cells were washed briefly with PBS and treated with 250 µL of annexin V-FITC staining solution (Annexin V-FITC, Tris, HEPES, CaCl$_2$, NaCl, EDTA, sodium azide, and BSA) at room temperature, in the dark, for 30 min. Cells were then washed briefly with PBS and analyzed under a fluorescence microscope.

III. FLOW CYTOMETRY

Cardiomyocytes were exposed to drug for a predetermined time. The incubation was stopped by the removal of media from the cells followed by trypsin (0.01% in DMSO) digestion to suspend the adherent culture. Trypsinization was stopped by dilution with 818A containing 6% fetal calf serum. Cardiomyocytes, approximately 10 000, were aspirated into the flow cytometer (Model Epics XL MCL, Coulter Electronics, Burlington, Canada) and examined for cell size and fluorescence.
A. APOPTOTIC NUCLEI BASED ON NUCLEAR CONTENT

Cardiomyocytes were removed from the culture as outlined above. The suspended cardiomyocytes were gently spun down and washed with phosphate buffered saline. Cells were then permeabilized with 70% ethanol for 30 min at room temperature, spun down and the ethanol removed. The resulting permeabilized cells were then stained with propidium iodide staining mix (Triton X-100, EDTA, RNAse A, propidium iodide) to detect the nuclei. Cells were examined on a flow cytometer on the fluorescence channel 3 (FL3) to detect the wavelength of propidium iodide (PI). The resulting histogram is a measure of PI staining, which is indicative of the amount of DNA in the nucleus to which the PI is bound. Hence, the histogram of PI staining is a reflection of chromatin content and cell cycle.

B. NUCLEAR SIZE

Cells were examined by flow cytometry using forward light scatter (FS) which measures cell size. The resulting FS histogram is a measure of cell size in a population of 10 000 cells. The median for each histogram is the FS channel in which most of the cells share the same cell size. A similar histogram of “DNA Check Beads” which are 10 μm in size was used as a reference for nuclear size.

C. APOPTOSIS VS. ONCOSIS BASED ON DUAL STAINING WITH FDA AND PI

Cardiomyocytes, at day three of culture, were exposed to palmitate and/or other agents for 24 h. The incubation was stopped by the removal of media followed by brief exposure to a trypsin (0.01% in DMS8) to suspend the adherent cells. Trypsinization was stopped by dilution with 818A media containing 6% fetal calf serum. Suspended cardiomyocytes were gently spun...
down and washed with phosphate buffered saline (PBS). Cardiomyocytes were incubated with (0.02%) fluorescein diacetate (FDA) that is converted by esterases to the fluorochrome fluorescein which becomes entrapped in live cells but not necrotic cells. Cells were then resuspended in 0.5 μg/mL PI for 30 min at room temperature, washed twice and then resuspended in PBS for flow cytometric analysis. Cardiomyocytes, approximately 10,000, were aspirated into the flow cytometer (Model Epics XL MCL, Coulter Electronics, Burlington, Canada) and examined for fluorescence on separate channels for FDA (FL1) and PI (FL3).

D. CELL STRUCTURE BASED ON F-ACTIN CONTENT STAINED BY NBD-PHALLACIDIN

Cells were fixed in 3.7% paraformaldehyde for 60 min at room temperature, washed with PBS, and stained with 0.5% Triton X-100 with 0.16 μM NBD-phallacidin for a further 60 min at room temperature in the dark. Cells were then examined by flow cytometry using the fluorescence channel 1 (FL1) to detect phallacidin (excitation at 488 nM & emission at 530 nm). The resulting histogram is a measure of NBD-Phallacidin staining, which is indicative of the amount of F-actin to which phallacidin is bound and reflects the abundance of F-actin. (Levee et al. 1996).

E. MITOCHONDRIAL ENZYME ACTIVITY BASED ON CHLOROMETHYL-X-ROSAMINE STAINING

Three day old cells were treated with palmitate and/or cyclosporin A for 24 h. Chloromethyl-X-Rosamine (CMX-Ros), 500 nM, was added to the cell culture 16 h before the incubation was stopped or 8 h after the initiation of treatment. The incubation was stopped and
cardiomyocytes were prepared for analysis by flow cytometry in the same manner as outlined for FDA staining. Cells examined by flow cytometry and the resulting histograms are representative of CMX-Ros fluorescence (FL3).

F. MITOCHONDRIAL POTENTIAL $\Delta \psi_m$ BASED ON DEPSIPHER™

Cardiomyocytes were removed from the culture as outlined above. DePsipher™ (0.1%) was added and allowed to incubate at 37°C for 20 min. Cells were washed twice with PBS before flow cytometry. Cells examined by flow cytometry were analyzed for green monomeric DePsipher™ (FL1) and red aggregates of DePsipher™ (FL3).

G. PHOSPHATIDYLSERINE EXPOSURE BASED ON ANNEXIN V-FITC BINDING

Cardiomyocytes were removed from the culture as outlined above. Cells were washed briefly with PBS and treated with 500 μL of annexin V-FITC staining solution (Annexin V-FITC, Tris, HEPES, CaCl$_2$, NaCl, EDTA, sodium azide, and BSA) (BD Pharmingen, Mississauga, Canada) at room temperature, in the dark, for 20 min. Cells examined by flow cytometry for cells exhibiting FITC fluorescence (i.e.-annexin V-bound) and the resulting histograms are representative of annexin V-FITC fluorescence (FL1).

IV. DNA LADDERING

Cardiomyocytes were grown in petri dishes at 5 x 10$^6$ cells/dish, for 72 h prior to the experiment. Medium was removed and centrifuged at 200 x g for 10 min. The pellet was resuspended in lysis buffer consisting of 0.6% SDS, 10 mM EDTA, pH 8.0 and transferred to eppendorff tubes. Lysis buffer was added to the dish with adherent cells after the medium had
been removed. Cells were scraped and transferred into eppendorff tubes containing the cells recovered from the medium. Samples were incubated with 5 M NaCl for 16 h at 4°C and centrifuged at 16 000 x g for 20 min. Supernatants were removed and incubated for 20 min at 37°C with RNAase A (Roche Molecular Biochemicals, Montreal, Canada). Protein was extracted with phenol-chloroform-isoamyl alcohol (25:24:1). After mixing and centrifugation, the aqueous phase was incubated with 3M sodium acetate and 100% ethanol for 10 min at -20°C. The DNA was pelleted by centrifugation, washed with 70% ethanol, resuspended and combined with gel loading buffer. Samples were subjected to agarose gel electrophoresis with 2 % agarose and resolved at 90 V for 2 h. Gels were stained with ethidium bromide and visualized with UV light to ensure staining. They were destained with distilled water for 90 min.

V. BIOCHEMICAL TECHNIQUES

A. CELL FRACTIONATION

1. Whole cell lysate

   Cells were lysed with RIPA buffer (150 mM NaCl, 1% Triton, 0.5% Na-deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, 1 mM DTT, 1 mM PMSF, 100 µg/ml aprotinin, 40 µg/ml leupeptin, 1 µM pepstatin) and homogenized by passage through a 26 g. needle.

2. Nuclear and cytosol

   Cells were lysed with RIPA buffer and the lysates were spun at 600 x g to separate nuclear fraction (pellet) from cytosolic (supernatant) (Reid and Williamson 1974). The purity of nuclear and cytosolic fractions by this method was confirmed previously by organelle markers and enzyme activities (Aronson et al. 1974; Gletsu et al. 1999; Stromhaug et al. 1998). Nuclear
preparation was also validated by immunoblotting with the nuclear marker histone-3 (see Chapter III - Section I).

3. Mitochondria

The method was modified from Yang and Cortopassi 1998. Cells were lysed with hypotonic buffer (10 mM Tris pH 7.4, 25 mM NaF, 2 mM Na$_3$VO$_4$, 1 mM ZnCl$_2$, 10 mM β-glycerol-P, 10 mM NaPP$_i$, 1 mM PMSF, 40 µg/ml leupeptin, 100 U/ml aprotinin) passaged through a 26 G. needle 10 times, and spun at 600 x g for 10 min. to remove nuclei (pellet). Lysates were then spun at 15 000g for 5min to separate mitochondria (pellet) from cytosol (nuclei). Mitochondrial preparations were validated with immunoblotting for the mitochondrial marker cytochrome C.

4. Membrane fractions

To prepare membrane fractions from cardiomyocytes, cells were washed twice in PBS and lysed in hypotonic sonication buffer (2 mM EDTA, 2 mM PMSF, 1 µM pepstatin). Lysates were sonicated for 10 sec and then spun at 50 000g on a Beckman TL 1000 ultracentrifuge for 30 min. Supernatants were discarded and the isolated pellet (membrane) was resuspended in resuspension buffer (100 mM Tris, 300 mM NaCl, 1% Triton, 0.1% SDS, 2 mM EDTA, 2 mM PMSF, 1 µM pepstatin) (Guijarro et al. 1998). Membrane fractions were validated by the membrane marker focal adhesion kinase.
B. IMMUNOPRECIPITATION

Cells were lysed and nuclear, cytosolic, and membrane fractions were isolated. To each sample, 2 µg of antibody, either caspase-2 (mouse) or Rho B (rabbit), was added and rocked for 1 h at 4°C. Either Protein G (for mouse antibodies) or Protein A (for rabbit antibodies) Sepharose was added to precipitate the immuno-lysates. Incubations were allowed to rock for 1 h for 4°C. Samples were then centrifuged at 600 x g to pellet the immunoprecipitates which were then washed four times with cold RIPA buffer. After boiling with gel loading buffer, samples were loaded onto a 12% PAGE gel for separation of proteins before western blotting (immunoblotting).

C. IMMUNOBLOTS

Cells were lysed and sub-cellular fractions prepared. Equivalent amounts of membrane, cytosolic and nuclear protein, assessed by Bradford protein assay, were loaded on 12% polyacrylamide gels for electrophoresis. Gels were then transferred to nitrocellulose membranes and blocked with 5% skim milk powder or BSA (for phospho-antibodies) in Tris-buffered saline (TBS) overnight at 4°C. Membranes were then washed with 0.3% TBS-Tween 20 (TBST) and incubated with primary antibodies followed by HRP-linked secondary antibody. Signals were detected using Renaissance chemiluminescence reagents (NEN Life Sciences, Massachusetts, USA) and chemiluminescent film (Kodak, Rochester, USA).
VI. DENSITOMETRIC ANALYSIS

Denistometric analysis of immunoblots were performed using Fluorchem 2000 program from Alpha Innotech Corp. (San Leandro, CA, USA).

VII. MATERIALS

A. ANIMALS

1. Embryonic Chick - White leghorn chicken eggs were purchased from Coastline Chicks, Abbotsford, B.C.

2. Neonatal Mice - Neonatal CD1 mice were purchased from the UBC Animal Centre, UBC campus.

B. CELL CULTURE MATERIALS

Culture media, fetal calf serum, antibiotics and antimycotics were obtained from GIBCO (Burlington, Ontario, Canada).

C. BIOCHEMICALS

Antimycin A, camptothecin, carnitine (both L- and DL-), glucose, palmitic acid, oleic acid, capric acid, carnitine, fenofibrate, WY-14643, Wright-Giemsa stain, lactate, mevalonic acid, oxfenicine, acridine orange, propidium iodide, and fluorescein diacetate were from Sigma Chemical Co. (St. Louis, Mo). Ceramide (both C2 and C2-dihydro), cyclosporin A, fumonisin B1, PD 98059, SB 202190, staurosporine, rotenone, zVDVAD, and AcDEVD were from Calbiochem (LaJolla, CA, USA) Angiotensin II was from Peninsula Laboratories (Belmont, USA). Annexin
V-FITC was from BD Pharmingen (Mississauga, Canada). Components for the TUNEL assay were from Roche Biochemicals (Laval, Canada). Lovastatin was kindly given by Merck & Co., Inc. (Rahway, NJ) and purchased from A.G. Scientific Ltd. (San Diego, CA) Palmitate was dissolved in either hot 100% ethanol (chick) or methanol (mouse) and added to warm medium from which it was aliquoted for experimental use.

- Function of inhibitors:
  2. Caspase-2 inhibitor zVDVAD-fmk - irreversibly inactivates caspases by forming a thiomethyl-ketone adduct with the active site cysteine (Thornberry et al 1994)
  3. Caspase-3 inhibitor AcDEVD-CHO – an inhibitor of caspase-3 (Thornberry et al. 1994)
  4. PD 98059 – an inhibitor of MEK by non-ATP competitive binding to the inactive form of MEK.
  5. SB 202190 – a cell-permeable pyridinyl imidazole inhibitor of p38 MAPK with an IC\textsubscript{50} of 350 nM in cells. This inhibitor has no effect on the activity of the ERK or SAPK/JNK (Singh et al. 1999). This inhibitor was chosen over the other p38 inhibitor, SB 203580, because it selectivly inhibits p38 whereas SB 203580 inhibits both p38 (with an IC\textsubscript{50} of 600 nM in cells) and MAPKAP kinase-2 (Iwasaki et al 1999).
  6. Oxfenicine - an inhibitor of CPT-1 with structural similarities to malonyl CoA
  7. Cyclosporin A - an inhibitor of cyclophilin D, a component of the mitochondrial transition pore, and calcineurin
  8. Fenofibrate - a peroxisome proliferator and PPAR\textsubscript{\alpha} agonist
  9. WY 14643 - a peroxisome proliferator and PPAR\textsubscript{\alpha} agonist
10) Lovastatin - an inhibitor of HMG-CoA reductase with an IC50 of 80 µM (Rao et al. 1999)

11) Rotenone - an inhibitor of the mitochondrial respiratory chain

12) Antimycin A - an inhibitor of the mitochondrial respiratory chain

D. ANTIBODIES

Secondary antibodies, Protein A- and Protein G-Sepharose, and antibodies against Rho B, troponin I-C, and actin (α, β, and γ isoforms) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against active and native caspase-2, active caspase-3, and cyt C were from Pharmingen (Mississauga, Ont.). Antibodies against phospho-p38, phospho-SAPK, and phospho-ERK, as well as the native forms of p38, SAPK, and ERK, and control C6 glioma cell lysates were from Cell Signaling Technology (Mississauga, Ont.). The antibody for PPARα was from Calbiochem (La Jolla, CA, USA). The antibody specific for histone-3 was from Upstate Biotechnologies Inc. (Lake Placid, NY, USA)

E. FLOW CYTOMETRY

DNA Check 10 µm beads were from Coulter. All other chemicals for flow cytometry were from VWR (Mississauga, Canada).

F. FLUORESCENT DYES

NBD-Phallacidin and chloromethyl-X-rosamine (CMX-Ros) were purchased from Molecular Probes (Portland, Ore.) under the name of Mitotracker Red®. DePsipher™ was purchased from Trevigen Inc. (Gaithersburg, MD)
VIII. STATISTICAL DATA ANALYSIS

The data are presented as the mean ± one SEM. Hypothesis testing used one-way analysis of variance. The null hypothesis was rejected if the probability of a Type I error was less than 5% (p<0.05).
CHAPTER III

RESULTS


SUMMARY

The objective of this chapter was to validate the methods I used to observe apoptosis and to provide both negative and positive controls for the forthcoming data involving palmitate-induced cell death. Known apoptotic inducers, camptothecin and staurosporine, were used to compare the flow cytometric analysis of apoptotic DNA to the “gold standards” in detection of apoptosis, DNA laddering and Annexin V. It was observed that flow cytometric analysis of apoptotic DNA degradation is comparable to DNA laddering but is also a quantitative method. Annexin V staining was not optimal for assessing apoptosis in cardiomyocyte cell culture as there was a significant amount of annexin V staining in control embryonic chick cardiomyocytes. Flow cytometric assessment of intracellular enzyme activity and membrane integrity was performed using dual staining with fluorescein diacetate (FDA) and propidium iodide (PI). This method quantitated the amount of apoptotic and onotic death in response to camptothecin and staurosporine. To assess whether apoptosis can be observed in this cell model, serum deprivation was used as a non-pharmacological method of inducing apoptosis. Simultaneous observations of apoptosis were achieved by flow cytometric analysis of apoptotic DNA, FDA/PI dual staining,
and annexin V staining. Serum deprivation induced a significant increase in apoptotic DNA, loss of intracellular esterase activity, and loss of membrane integrity. However, serum deprivation did not produce a significant increase in annexin V staining in embryonic chick cardiomyocytes. To assess whether the effects of palmitate are due to random and nonspecific action of fatty acids, cardiomyocytes were treated with the fatty acid oleic acid. Oleic acid is a C18 unsaturated fatty acid which differs from palmitic acid a C16 saturated fatty acid by one unsaturated bond one chain length. Angiotensin was used as a control for the effects of the addition of an exogenous drug. Angiotensin is an ideal choice as it is a circulating hormone with multiple effects on the heart. There was no evidence of apoptosis induced by either oleic acid or angiotensin II. This contrasts the significant amount of apoptosis induced by serum deprivation, as ascertained by flow cytometric analysis of apoptotic DNA and dual FDA/PI stains.

I conclude that flow cytometric analysis of apoptosis in cardiomyocytes using low DNA content and FDA/PI dual stains is a valid and appropriate method that are comparable to the “gold standards” of DNA laddering and annexin V, respectively. In addition, palmitate induced apoptosis in a specific manner.

INTRODUCTION

The purpose of the work in this chapter was to validate the methods to detect apoptosis that will be used extensively throughout this thesis. As well, this chapter will establish that palmitate-induced effects are specific and not due to a random or nonspecific action of a fatty acid. Currently, the “gold standards” for apoptotic assessment is DNA laddering and annexin V staining. However, flow cytometry with various probes is becoming acceptable as a reliable means of more rapidly observing apoptosis in a quantitative manner (for review, see Sgonc and
Apoptosis and oncosis are discernable based on several characteristics associated with each form of cell death. Cells undergoing apoptosis display classic characteristics such as nuclear fragmentation into 180bp or multiples, cell shrinkage, membrane bleb formation, loss of intracellular enzyme activity, and activation of pro-apoptotic kinases known as caspases (for review, see Saraste and Pulkki 2000, Feuerstein et al. 2000, Green and Reed 1998, Fiers et al. 1999). In contrast, cells undergoing oncosis display a non-specific degradation of DNA, loss of membrane integrity, and loss of intracellular enzyme activity (for review, see Allen et al. 1997).

Apoptosis is associated with fragmented DNA of 180 bp. The enrichment of mono- and oligonucleosomes in the cytoplasm of apoptotic cells occurs because of activation of endonucleases that cleave DNA except those protected by histones leaving DNA fragments typically 180 bp in length (Schwartzman & Cidlowski 1993; Wyllie et al. 1992). These fragments are separable by gel electrophoresis thus exhibiting a distinct “DNA ladder” (Schwartzman & Cidlowski 1993; Wyllie et al. 1992). Similarly, as nuclear DNA is being degraded, the DNA content is reduced.

PI staining of permeabilized nuclei for DNA content

Flow cytometry is a rapid and quantitative method of analyzing cell size, granularity, and fluorescent labeling. Cells are examined, one at a time, by a series of lasers in the flow cytometer. Cell size is interpolated based on the amount of light scatter produced by forward light scatter laser (FS) whereas granularity is similarly measured by the side scatter laser (SS). Flow cytometers can also analyze fluorochromes from 400-600 nm, or red to green spectrum of light. Thus, flow cytometric analysis can provide quantitative data on cell size, granularity, and
binding characteristics of fluorochromes linked to antibodies or specific for various cell organelles (for in depth review of flow cytometry, see Cotter and Martin 1996).

PI staining of permeabilized nuclei allows PI to bind to the DNA and hence, permitting quantitation of the amount of DNA that is present as flow cytometric analysis of these PI stained nuclei quantifies the loss of DNA during apoptosis (Darzynkiewicz et al 1992). One (1N) or two copies (2N) of DNA express greater PI fluorescence than apoptotic cells which are hypoploid (<1N) cells (Darzynkiewicz et al 1992). As the majority of cells are in quiescence (G0/G1), the largest peak (i.e. - frequency) on the PI histogram is designated as G0/G1. The second most distinguishable peak with greater PI fluorescence than the G0/G1 was designated as 2N DNA which occurs during DNA synthesis and mitosis (S and G2/M). Likewise, all PI fluorescence less than 1N DNA was designated as hypoploid (<1N) cells. Hence, flow cytometric analysis of DNA content also provide data on cell cycle as 1N and 2N nuclei can also be quantitated. Studies confirm the utility of flow cytometric analysis of PI stained DNA content to observe apoptotic DNA fragmentation (Tepper et al. 1999, Susin et al. 1997, Diez et al. 1997).

FDA + PI dual staining

Intracellular enzyme activity and membrane integrity were both observed by flow cytometric analysis of the dual stains FDA and PI. FDA is converted into its fluorescent form by active esterases whereas PI is excluded from cells with intact membranes (Frey et al. 1997). Hence, early apoptotic cells exhibit no active esterases but good membrane integrity (low FDA and low PI) whereas oncotic cells exhibit no active esterases and a loss of membrane activity (low FDA and high PI). This dual staining is an established method of assessing viable, apoptotic, and oncotic populations in various cells such as CNS cells (Katsube et al 1999, Ikonomovic et al 1997), HL-60 (Barkowiak et al 1999), eosinophils (Atsuta et al 1995),
fibroblasts (Kulkarni and McCulloch 1994), human lymphoblastoid cells (Morris et al. 1994), and hepatoma cells (Tolleson et al 1996).

**Annexin V staining**

Another means of assessing apoptosis is observing the phosphatidylserine (PS) content on the extracellular membrane with the specific PS marker Annexin V. During apoptosis, PS is flipped within the cell membrane from intracellular to extracellular location (Van den Eijnden 1997). The PS asymmetry of the plasma membrane is generated and maintained by an aminophospholipid translocase, which selectively transports aminophospholipids such as PS (Bratton et al. 1997). Cell surface exposure of PS has a functional significance in the removal of apoptotic cells from the tissue or, in the case of the reticuloendothelial system, removal from circulation (Schroit et al. 1985). PS exposure on the cell surface precedes nuclear changes during apoptosis (Stuart et al 1998). The exposure of PS does not depend on the involvement of the nucleus, but requires the activation of caspase-3 and a Ca^{2+} flux across the plasma membrane (Bratton et al. 1997). PS enrichment of the membrane can be detected using the phospholipid binding agent Annexin V which can be linked to the fluorochrome FITC to allow for quantitative observation by microscopy or quantitation by flow cytometry (for review, see van Heerde et al. 2000).

It is possible that the effects of palmitate are due to a nonspecific action of fatty acids. To determine the possibility of a nonspecific fatty acid effect, I will investigate the fatty acid oleic acid (C18:1) which is an unsaturated fatty acid slightly greater in length than palmitate. To determine whether these cardiomyocytes may spontaneously undergo apoptosis, I first will establish that apoptosis can be selectively induced and then determine that this selective induction is not due to the addition of any agent. To achieve this, I will induce apoptosis in
these cardiomyocytes in a non-pharmacological method, serum deprivation. Serum deprivation is an established method of inducing cell death in many cell lines (Yamada et al. 1996) and is an ideal choice for a positive control because it induces apoptosis in a non-pharmacological manner. To observe whether spontaneous apoptosis occurs due to the addition of any agent, angiotensin II (ang II) was chosen as a potential negative control as it is an important hormone for the heart. (Kusarah et al. 1998; Ishida et al. 1998; Schmitz et al. 1998; Nakamura et al. 1999).

RESULTS

Both camptothecin and staurosporine are known inducers of apoptosis in various cell lines (Onishi et al. 1993, Hotz et al. 1994) including embryonic chick cardiomyocytes (Kong & Rabkin 1999; Kong & Rabkin 2000a). Figure 1 depicts apoptotic effects on DNA due to either camptothecin 10 μM or staurosporine 1 μM for 6 h as evidenced by both DNA laddering (Panel A) and flow cytometry (Panels B & C). Panel A shows a representative agarose gel of fragmented DNA from cells treated with camptothecin 10 μM (Cam), staurosporine 1 μM (ST), or diluent DMSO (C) for 6 h. Both camptothecin and staurosporine yield distinct small molecular weight DNA of 180bp and multiples. Flow cytometric analysis of these treated cells yield a quantitative measurement of the number of cells with low DNA content associated with PI staining of the degraded apoptotic DNA (Panel B). Camptothecin 10 μM induced a significant (p<0.01) increase in the number of cells with low DNA content from control 942±146 to 1601±120 (N=15), or 1.7±0.1 fold increase. Similarly, staurosporine 1 μM induced a significant (p<0.01) increase in the number of cells with low DNA content from control 1153±109 to 3920±1002 (N=3) for 6 h induced a significant (p<0.01) and 3.4±1.3 fold increase in the number.
Figure A1 - Comparison of apoptotic DNA fragmentation in embryonic chick cardiomyocytes: agarose gel vs. flow cytometry - Apoptotic DNA fragmentation and degradation was observed by either separation of fragments on an agarose gel (Panel A) or by flow cytometry (Panels B & C). Panel A - Representative agarose gels of cells treated with either camptothecin 10 μM (Cam) or staurosporine 1 μM (ST) or diluent DMSO (C) for 6 h are shown. Lanes unaccounted for are results to an unrelated experiment. bp = base pairs Panel B - Cardiomyocytes were treated with either camptothecin 10 μM or staurosporine 1 μM for 6 h and the amount of DNA content assessed by flow cytometry. A representative histogram of PI fluorescence is shown for each. Apoptotic DNA was identified on the histogram as having FL3 fluorescence less than 10^3, denoted by the marker M1. Panel C - The relative amount of apoptosis as measured by the number of cells with low apoptotic DNA content compared to control for camptothecin-treated (N=15) or staurosporine-treated (N=3).
CAMPTOTHECIN

A

C Cam

bp

2036
1636
1018
506

STAUROSPORINE

C ST

bp

2036
1636
1018
506

B

Control Camptothecin Control Staurosporine

C

Relative amount of cells with low DNA content compared to control

Control Camptothecin

Control Staurosporine

**
of cells with low DNA content, respectively (Panel C). Thus induction of apoptosis produces DNA laddering associated with an increase in the number of cells with low DNA content.

To validate the flow cytometric analysis of apoptotic DNA content, both flow cytometry and annexin V, another “gold standard” of measuring apoptosis, was performed. Cardiomyocytes were treated with oleic acid 100 μM (N=3) or palmitate 100 (N=14) and 300 μM (N=3) for 24h. At the end of the treatment, cells were split into two. One set was stained with annexin V-FITC and the other was subjected to ethanol permeabilization followed by PI staining. Although both palmitate 100 and 300 μM significantly (p<0.01) increased the amount of cells with apoptotic low DNA content 2.0±0.1 and 3.7±1.8 fold respectively, annexin V binding was not increased as great a magnitude (Figure 2). Palmitate 100 μM yielded a significant (p<0.01) 1.3±0.1 (N=8) fold increase whereas 300 μM yielded a significant (p<0.05) 2.0±0.3 (N=3) fold increase in annexin V binding. In contrast oleic acid did not yield any significant increases in annexin V staining or low DNA content. These results suggest that palmitate’s effects are specific as oleic acid failed at inducing apoptosis. They also suggest that the DNA content approach is more sensitive than annexin V staining for detection of apoptosis.

To further explore the use of annexin V in this cell model, cardiomyocytes were stained with annexin V-FITC and visualized on a fluorescent microscope. Cells were treated with oleic acid 100 μM, palmitate 100 or 300 μM, or the diluent ethanol for 24 h and then stained with annexin V-FITC (Figure 3). As annexin V-FITC will selectively bind to PS and PS is only visible on the outside of the plasma membrane during apoptosis, annexin V-FITC fluorescence seen bound to the cells is a measure of the PS exposure on the membrane implying apoptosis. Hence, the strong fluorescence suggests significant apoptosis. In both flow cytometric analysis (Panel A) and microscopic observation (Panel B), control cells surprisingly exhibited some
Figure A2 - Comparison of Annexin V binding vs. apoptotic DNA content by flow cytometric analysis - Cardiomyocytes were treated with oleic acid (C18:1) (N=3), or 100 or 300 μM palmitate (C16:0) (N=14 and 3, respectively). At the end of the treatment, cells were either stained with annexin V-FITC or fixed and then stained with PI before flow cytometric analysis. Data are represented as apoptotic index, or relative amount FITC/PI fluorescence compared to control. *p<0.05, **p<0.01 compared to control.
Figure A3 - Annexin V binding to embryonic chick cardiomyocytes. Cardiomyocytes were grown for 72 h and treated with palmitate 100 or 300 μM, oleic acid 100 μM, or diluent (ethanol) for 24 h. Cells were then stained with annexin V-FITC and either analyzed by flow cytometry (Panel A) or observed on a Zeiss IM 35 fluorescent microscope (Panel B). Pictures are 200x magnification.
annexin V-FITC staining in that green fluorescence was seen on control cells which should theoretically not be exposing PS. Quantitated by flow cytometry, control cells also exhibited annexin V-FITC fluorescence >10², which was designated as positive annexin V staining, in approximately 30% of cells. Palmitate induced a stronger annexin V fluorescence, 300 µM being stronger than 100 µM, suggesting that palmitate 300 µM induced a stronger apoptotic response. In contrast, oleic acid yielded annexin V fluorescence equal to that of control suggesting that oleic acid was not inducing apoptosis (greater than that observed in control).

To validate another method of observing apoptosis, cells were dually stained with FDA and PI and examined by flow cytometry. FDA reflects cellular activity as this dye readily penetrates the cell and is converted by active esterases into its fluorescent form. An active, intact cell will retain the fluorescence whereas in a damaged cell FDA will leak out (Frey et al. 1997; Darzynkiewicz et al. 1997). PI is a fluorochrome that only penetrates cells with damaged membranes but does not penetrate viable cells with an intact membrane. (Darzynkiewicz et al. 1997). This dual staining permits cell to be identified as viable cells by FDA staining and further classifies dead cells by PI staining according to the type of cell death. Dead cells which lack membrane integrity (low FDA and high PI) are classified as oncostic cells, while dead cells with little PI staining are considered early apoptotic cells (low FDA, low PI) as they continue to possess membrane integrity (Wyllie 1992). Cardiomyocytes were treated with either camptothecin 10 µM or staurosporine 1 µM for 24 h, dually stained with FDA and PI, and then analyzed by flow cytometry (Figure 4). The majority of the 10 000 cells sampled show FDA uptake with fluorescent values over 10² thereby defining the normal population. Two small populations of cells were identified with lower FDA uptake but distinguished by PI fluorescence. The population with low FDA and low PI uptake (fluorescence between 10⁰ to 10¹) were
Figure A4 - Changes in intracellular enzyme activity and membrane integrity distinguish apoptotic and oncocytic death with dual staining with FDA/PI. Cardiomyocytes were treated with either camptothecin 10 μM (N=4) or staurosporine 1 μM (N=11). Panel A shows a representative dotplot. Apoptotic populations were identified as having low FDA and low PI whereas oncocytic populations were considered to have low FDA and high PI. Panel B - Quantitation of cells exhibiting apoptosis or oncosis in population of 10 000 cells is shown. Data are represented as relative amounts of apoptotic/oncocytic cells compared to control (**p<0.01).
identified as the apoptotic population whereas the population with low FDA and PI fluorescence $10^2$ to $10^3$ were identified as the oncosis population. Both camptothecin and staurosporine induced a significant (p<0.01) 1.3±0.3 (N=4) and 3.1±0.5 (N=11) fold increase in apoptosis. Interestingly, camptothecin induced a significant (p<0.01) 1.5±0.3 (N=4) fold increase in the oncosis population but staurosporine induced a significant (p<0.01) decrease in oncosis to 0.6±0.2 (N=11) that of control.

To determine whether the apoptosis observed during agent addition was not due to the actual addition of agent, the cardiomyocytes were serum starved for 24h as a positive control for apoptosis (Figure 5). Serum-starved-induced apoptosis were concomitantly observed by apoptotic DNA content (Panel A), dual stain with FDA and PI (Panel B), and annexin V (Panel C). Figure 5 is a representation of one experiment when all three flow cytometric methods were done simultaneously. Although serum starvation induced an increase in apoptotic low DNA content, apoptosis and oncosis, serum starvation did not induce an increase in annexin V binding.

To further evaluate the response of these cardiomyocytes to apoptosis and recovery from apoptosis, cardiomyocytes were serum deprived for 24 h but then treated with angiotensin II (ang II), a hormone normally active in cardiomyocytes. After 24 h of serum deprivation with or without ang II 1 μM, cardiomyocytes were permeabilized and analyzed for apoptotic low DNA content (<$10^2$ FL3 fluorescence) by flow cytometry (Figure 6 - Panel A). There was a significant (p<0.05) increase in the population of apoptotic nuclei (1.5±0.2, N=3) compared to cells not serum starved in control (Panel B). Ang II significantly (p<0.05) reduced serum-induced increases in cells with apoptotic nuclei (0.9±0.4, N=3) whereas ang II alone remained near control levels (1.1±0.1, N=5).
Figure A5 - Serum deprivation as a positive control for apoptosis in embryonic chick cardiomyocytes. Cardiomyocytes were grown for 72 h in 6% FCS and then either in 6% (Control) or 0% FCS (Serum deprivation) for the final 24h. Cells were then prepared for flow cytometric analysis for apoptotic DNA content (DNA) (Panel A), dual FDA and PI staining (FDA/PI) (Panel B), and annexin V (Annexin) (Panel C). This is a representative experiment of control and serum deprived samples analyzed by these three methods simultaneously.
CONTROL

SERUM DEPRIVATION

Apoptotic 5.75%
G0/G1
M0

Apoptotic 9.74%
M4

M1

M2
G2/M

M3

Annexin V

41.55% positive

Annexin V

28.48% positive

Annexin V
Figure A6 - Angiotensin addition does not induce apoptosis and blocks serum deprivation-induced apoptosis. Cardiomyocytes maintained in culture for 72 h before changing the media to the same medium without FCS-serum free medium (0% FCS) (N=5) or medium with the usual amount of FCS (6%) (Control) (N=5) or medium without FCS but with ang II 1 μM (0% + Ang II) (N=5) or medium with the usual amount of FCS + ang II 1μM (Ang II) (N=5). After 24 h cells were fixed with ethanol and then stained with propidium iodide and assessed by flow cytometry. Panel A is a representative histogram of PI staining from 5 separate experiments. Panel B- The data are expressed as the number of cells exhibiting low DNA content relative to controls done simultaneously. The data are presented as the mean + SEM (* p<0.05 compared to control; Δ<0.05 compared to 0% FCS).
DISCUSSION

In this chapter, I examined four different methods for detection of apoptosis: DNA laddering, low DNA content as defined by low PI staining of permeabilized nuclei, annexin V, and dual staining with FDA and PI. Concomitant comparisons between methods demonstrated that flow cytometric analysis of apoptotic low DNA correlated with DNA laddering. As well, low DNA content detected a greater magnitude of the apoptotic response compared to annexin V staining. FDA and PI dual stain permitted the identification and quantitation of apoptotic and oncotic populations.

Although both DNA laddering and DNA content by flow cytometry are established methods, there are several advantages and disadvantages to each. DNA laddering is the most established and frequently used method, having been developed over 20 years ago (Wyllie 1980). This method produces very clean, protein-free fragments of apoptotic DNA and is selective for apoptosis because oncosis is associated with randomly digested DNA that runs faster than 180 bp apoptotic fragment on an agarose gel. There are several problems with DNA laddering method. First, it only shows the presence or absence of apoptotic DNA fragmentation in a population of cells. Second, DNA isolation is time-consuming. Third, it requires >5 x 10⁶ cells per sample. Fourth, DNA laddering requires the use of hazardous materials such as phenol, chloroform, and ethidium bromide. Fifth, quantitation of DNA laddering by radioactive labeling of the DNA breaks is possible but is undesirable due to the necessity for radioactive contamination of all equipment necessary for DNA isolation and separation by agarose.

In contrast, assessment of DNA content and cell cycle by flow cytometry is faster, requires only 5 x 10⁵ cells per sample, and is a less biohazardous procedure than DNA laddering. Nicoletti et al. (1991) and Telford et al. (1991) echoed these advantages and stated that DNA
staining with PI was the easiest and most rapid method for measuring apoptosis, especially for large scale in vitro studies. Darzynkiewicz et al. (1992 and 1996) were one of the first groups to develop this method and establish its use in detection of apoptosis. DNA content allows the quantitation of apoptotic cells within a population of non-apoptotic cells. The degree of apoptotic death is represented as an apoptotic index, determined as relative amounts of cells with low DNA content compared to non-apoptotic control cells. In contrast, DNA laddering shows only the cumulative presence or absence of DNA fragments in a population of cells. Flow cytometric analysis of DNA content also provides data on cell cycle in a population of cells, useful data if the population is undergoing DNA synthesis or mitosis. Assessment of DNA content by flow cytometry also allows for the exclusion of oncotic degradation of DNA as a discriminator of cell size is applied during flow cytometric analysis to exclude cells that are no longer intact (Darzynkiewicz et al. 1992).

This chapter is not the first to compare DNA laddering with flow cytometry. Both Moore et al. (1998) and Sgonc and Gruber (1998) did an extensive literature review comparing the various methods to observe apoptotic DNA degradation, including DNA laddering and flow cytometry. Both studies concluded that flow cytometry was the more efficient method of observing apoptotic DNA cleavage. Elasser et al. (2000) and Van Heerde et al. (2000) did a vast literature research regarding cardiomyocytes and methods of apoptotic observation. As with the results from this chapter, the overall consensus of these discussions was that both DNA laddering and flow cytometry were valid as qualitative (DNA laddering) and quantitative (flow cytometry) measures of apoptotic DNA fragmentation. Bedner et al. (1999) performed a comparative study of flow cytometric analysis of nuclear DNA content with microscopic observation of apoptosis. The authors reported that flow cytometric analysis of apoptotic DNA
content in HL-60 cells treated with camptothecin correlated with apoptotic morphology and that flow cytometry analysis allowed the quantitation of apoptosis in a population (Bedner et al. 1999).

However, assessment of low DNA content in apoptotic cells is not 100% specific. Apoptosis is determined as the “sub-G1 peak”, or any DNA with less than one copy. This sub-G1 peak may include cells that are mechanically damaged, cells with lower DNA content (if the cell preparation is not purely one cell type), or cells with different chromatin structure in which the accessibility of DNA to PI is diminished (Bedner et al. 1997). However, when performing flow cytometric analysis, I have excluded cellular debris and oncotic DNA by discriminating against size. As well, embryonic chick cardiomyocyte culture has been previously shown to be >90% pure cardiomyocytes (Sunga and Rabkin 1987) hence eliminating the question of a mixed cell population with varying nuclear contents. Thus, I feel that analysis of low DNA content is an appropriate method of observing apoptotic cells with low DNA content.

The present thesis also determined the efficiency of FDA/PI staining in cardiomyocytes by comparing FDA/PI staining results with DNA laddering using the known apoptotic inducers camptothecin and staurosporine. This dual staining is an established method of assessing viable, apoptotic, and oncotic populations in various cells (Katsube et al 1999, Ikonomovic et al 1997, Bartowiak et al 1999, Atsuta et al 1995, Kulkarni and McCulloch 1994, Morris et al. 1994, Tolleson et al 1996). A comparative study between FDA/PI staining and Annexin V with HL-60 cells reported that both methods are equally good to detect apoptosis (Bartowiak et al 1999). Bartowiak et al. (1999) observed and quantitated apoptosis induced in HL-60 cells by FDA/PI and Annexin V. Bartowiak et al. (1999) concluded that both FDA/PI and annexin V are equally suitable for detecting apoptosis.
This chapter demonstrated increased annexin V staining of cardiomyocytes undergoing apoptosis as observed by flow cytometry and microscopy. However, it is worth noting that both microscopic and flow cytometric analysis of annexin V binding demonstrated annexin V background in untreated control cells, thus raising concerns that annexin V binding is not specific in this cell model. Approximately 30% of control cells exhibited positive annexin V binding, as assessed by flow cytometry (Figure 3A). As the annexin V data is represented as relative amounts compared to control (Fig 2), death inducers such as palmitate do express a significant (p<0.01) increase in positive annexin V binding suggesting that the method used for annexin V staining is correct. It should be noted that annexin V staining method has been extensively used with continuous cell lines (eg. HL-60), peripheral blood mononuclear cells, and neutrophils (for review, see pp 110-111, Cotter & Martin 1994). There have been reports of the use of annexin V in adult rodent hearts (van Heerde et al 2000, Ravassa et al 2000, Dumont et al 2000, Wang et al 2001, Otani et al 2000, Taimor et al 2000). None of these authors reported false annexin V positive staining in controls. To my knowledge, there have been no reports of annexin V use in embryonic or neonatal cardiomyocytes. It is not known whether the background binding seen with the embryonic chick cardiomyocytes is due to an increased PS expression compared to other cells or that annexin V is interacting with some other membrane component seen in embryonic but not adult cardiomyocytes. As annexin V binding is stronger in control than in serum deprived cells (Figure 5), I speculate that annexin V is interacting with some other membrane component present in these cells. Endogenous annexin V is present in the heart which is maximal at the embryonic/neonate level (Jans et al 1998). It is likely that the endogenous annexin V is interacting with the exogenous annexin V-FITC resulting in the high background. Regardless of the cause, the high annexin V background observed in chick
embryonic cardiomyocytes raised concerns as to the specificity of this method for this cell model. Since FDA/PI was determined to be as good a method for apoptosis determination as annexin V, annexin V was not used extensively in this thesis. A comparison of methods to detect apoptosis is shown in Table 1.

Serum deprivation induced a significant (p<0.05) level of apoptosis, validating this cell model as one that can undergo apoptosis in a specific manner. These results also suggest that ang II has anti-apoptotic properties in cardiomyocytes which is consistent with data in vascular smooth muscle cells subjected to serum deprivation (Kusarah et al. 1998; Ishida et al 1998; Schmitz et al. 1998).

In this chapter, palmitate’s effects on these cardiomyocytes were proven to be specific to this fatty acid as oleic acid did not induce cell death. Oleic acid, an unsaturated fatty acid with similar chain length of palmitate, did not induce apoptotic low DNA content or positive annexin V binding. These findings with oleic acid agree with previous reports in neonatal rat ventricular myocytes (De Vries et al. 1997) and breast cancer cells (Hardy et al 2000) which demonstrated oleic acid to be antiapoptotic. Interestingly, levels of oleic acid and other polyunsaturated fatty acids were reduced while saturated fatty acids like palmitate were increased in cellular membrane content in rat models of hypertension that are associated with heart failure (Vasquez et al. 2001).

Although apoptotic cell death is the focus of this thesis, it is necessary to acknowledge the significant amount of oncotic death induced by palmitate. Apoptotic cells will undergo secondary oncasis/necrosis once the cell has been eliminated by blebbing and the individual apoptotic bodies (blebs) are degraded via secondary oncasis/necrosis. Thus, the amount of oncotic death visualized is a culmination of rapid death (oncotic) in response to palmitate addition as well as apoptotic death that has entered secondary necrosis by the 24 h observation
time point.

In conclusion, the major findings in this chapter are:

- Cell death by both apoptosis and oncosis was observable in embryonic chick cardiomyocytes.
- Low DNA content assessed by PI staining of nuclei and analyzed by flow cytometry correlates with DNA laddering.
- Low DNA content is indicative of apoptosis as shown by the classic apoptotic inducers, camptothecin and staurosporine. Contrary to DNA laddering, this method can be quantitated and can provide information on cell cycle.
- Dual staining with FDA and PI detects apoptosis and oncosis as shown by the classic apoptotic inducers camptothecin and staurosporine.
- Annexin V staining shows apoptosis in embryonic chick cardiomyocytes, but it is not an ideal method. Annexin V has low sensitivity, stains control cells are not undergoing apoptosis, and does not appreciably stain greater than control upon apoptotic induction by serum deprivation.
- Palmitate-induced apoptosis is not by a non-specific effect of fatty acids.
- Not all factors added to cardiomyocytes will induce apoptosis as the addition of angiotensin did not yield any cell death.
Table A1. Comparison of apoptotic methodologies used in this thesis.

<table>
<thead>
<tr>
<th>Method</th>
<th>Specific</th>
<th>Sensi­tive</th>
<th>Pros</th>
<th>Cons</th>
<th>Overall rating</th>
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| DNA ladder        | Yes      | No         | Visualize 180bp DNA fragments                  | • Time-consuming
• Use hazardous reagents
• Require $>5\times10^6$ cells/sample
• Cannot be quantitated                                             | Fair           |
| DNA content by FC | Yes      | Yes        | • Rapid and quantitative analysis
• Require $5\times10^5$ cells/sample                | Hypoploid population may not be purely apoptotic                    | Good           |
| Annexin V microscopy | No    | No         | Visualization of increased PS surface expression | High annexin V pos. staining in control cells                          | Poor for embryonic cells |
| Annexin V FC      | No       | No         | • Rapid and quantitative analysis
• Require $5\times10^5$ cells/sample                  | High annexin V pos. staining in control cells                        | Poor for embryonic cells |
| FDA+PI by FC      | Yes      | Yes        | • Rapid and quantitative analysis
• Require $5\times10^5$ cells/sample
• Allows for separation of viable, apoptotic, and oncotic populations | Only early apoptotic cells are quantitated. Late apoptotic cells would be lumped in with oncotic cells | Good           |
| TUNEL             | No       | No         | Visualize cleaved DNA in a qualitatively (microscope) and quantitatively (FC) | False positives                                                       | Poor           |

bp = base pairs
FC = flow cytometry
FDA = fluorescein diacetate
PI = propidium iodide
Pos = positive
PS = phosphatidylserine
TUNEL = terminal dUTP nick-end labeling

**SUMMARY**

Palmitate is a saturated fatty acid present in high circulating concentrations in patients with myocardial infarctions or with conditions associated with a high probability of developing cardiomyopathy. The objective of this chapter was to examine the hypothesis that the alterations of cardiac nuclei, that have been noted in some cardiomyopathies, can be produced by palmitate. Cardiomyocytes isolated from embryonic chick ventricles were maintained in culture for 72 hours and treated with palmitate 100 μM for 24h. Microscopic changes were observed with the stains acridine orange (for cell morphology), Wright-Giemsa (for visualization of nuclei), and terminal dUTP nick end label TUNEL (for apoptotic DNA cleavage) and examined microscopically. Cardiomyocytes treated with palmitate displayed changes in nuclear appearance as nuclei were larger, relative to cell size, with more intense acridine orange staining in a peripheral location. Nucleoli were often disrupted. To verify that the palmitate-induced low DNA content presented in the previous chapter was due to apoptotic degradation, cardiomyocytes were stained with TUNEL. Palmitate-treated cells exhibited a strong TUNEL fluorescence compared to control suggesting that the nuclei are undergoing apoptotic degradation as well as being enlarged. Flow cytometric analysis of palmitate-treated cardiomyocytes showed changes to cell and nuclear size, examined by forward light scatter. Palmitate was observed to produce a 17% (p<0.001) increase in the size of nuclei compared to untreated cells. There were no significant changes in overall cell size. However, the ratio of nuclear to whole cell size was
significantly (p<0.01) increased compared to control cells. Flow cytometric analysis of propidium iodide-stained nuclei demonstrated that the nuclear enlargement was not due to induction of mitosis as the proportion of nuclei in G0/G1 was not changed by palmitate. In summary, these data identify that palmitate can induce structural abnormalities of cardiomyocyte nuclei that included changes due to apoptosis plus increased nuclear size and nucleolar destruction.

INTRODUCTION

As discussed previously in the Introduction, various abnormalities in nuclei have been identified in patients with cardiac disease, specifically cardiomyopathy (Scholz et al 1994; Baandrup et al. 1981; Popovich et al 1995; Maron et al. 1975; Noda et al. 1980; Unverferth et al. 1983). Increased nuclear size may be an adaptation to the loss of viable myocytes due to cardiomyopathy or may be an integral part of pathogenesis. Alternatively, cellular signaling pathways, activated during excess cardiac stretch as the heart dilates to adjust to the loss of cardiomyocytes (Yamazaki et al. 1995), may lead to nuclear enlargement (Cluzeaud et al. 1984). Another possible cause for enlarged nuclei is that molecules circulating in high concentrations in conditions associated with cardiomyopathy may play a pathophysiologic role in altering nuclear structure. The present thesis postulated that palmitate is a likely candidate molecule. It has been calculated that the normal fatty acid concentration within the cardiomyocyte is approximately 10 µM, assuming that all fatty acids are localized in the aqueous cytoplasmic compartment (Van der Vusse et al 1982). Opie et al (1977) had reported high concentration of circulating free fatty acids correlated with infarct size, with an average concentration of 2 mM, in patients 12 h after large myocardial infarctions. As well, these fatty acids contain a high proportion of palmitic and oleic acid, serving as potential substrates for mitochondrial oxidative energy production (Van der
Vusse et al. 1982). The objective of this section was to examine the potential role of palmitate to alter cardiomyocyte nuclear size and structure similar to some features associated with cardiomyopathy.

RESULTS

The dose at which to treat cardiomyocytes with palmitate was previously established by this laboratory using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cell viability assay. Palmitate 100 μM and greater concentrations significantly increased the loss of cell viability (Figure 1). Palmitate 100μM was chosen for further experiments as it is a concentration that induces a significant loss of MTT absorbance at 570nm, indicative of cell death: from control 0.40±0.01 (N=54) to palmitate 0.26±0.04 (N=54) indicating a 42.3±3.2 % (N=54) death but not as extreme as the 70.6±5.7% (N=3) death observed with 150 μM of palmitate.

Cardiomyocytes stained with acridine orange showed the cytosol as well as nuclei and nucleolar structure (Figure 2). Acridine orange is a pH sensitive fluorescent dye which is fluorescent green at neutral pH and red at an acidic pH (approx. pH 5) and allows for observation of overall cell morphology (Nairn and Rolland 1980). Compared to control cells (Panel A), the nuclei of palmitate-treated cardiomyocytes were more elongated and appeared to be larger relative to the size of the cell (Panel B). Nuclei were also more homogenous and more intensely stained compared to untreated cardiomyocytes, suggesting chromatin condensation in a location around the periphery of the nucleus. Nucleoli were not readily visible. The cytoplasm appeared more granular and red compared to control cells. There were no other cytoplasmic and no membrane changes apparent in palmitate-treated cells.

To focus more intensively on nuclear changes, cardiomyocytes were stained with a modified
Figure B1 - Cardiomyocytes were treated with diluent ethanol (0) (N=54), or 10 (N=5), 100 (N=54), 150 (N=4) or 200 μM (N=3) palmitate for 24h and cell viability was assessed by the MTT assay. As cells lose cell viability, the A_{570} decreases. Inset - Compared to the control A_{570}, absorbance readings from palmitate treated cells are represented as a % loss of cell viability. ** p<0.01 compared to control.
Figure B2 - Microscopic analysis of cardiomyocytes treated with diluent (Panel A) or palmitate 100 μM 24h (Panel B) and then stained with acridine orange. Acridine orange stains the entire cytoplasm but highlights the nucleus (Kasten 1967).
Wright-Giemsa stain (Figure 3). The Wright-Giemsa stain allows visualization of cellular organelles, particularly the nucleus. The nuclei and nucleoli were more clearly seen in control cells (Panel B) than palmitate treated cells (Panel A). Palmitate treatment produced an enlarged nucleus with disruption of nucleolar structure. In addition, the nuclear membrane in palmitate-treated cells were less defined (fuzzy) compared to control cells. The increased cytoplasmic granularity produced by palmitate was also evident.

Nuclear size was examined by flow cytometry analyzing forward light scatter (Figure 4). The resulting histogram, representing the different FS readings of a population of 10 000 cells, shows the median FS channel, where most of the cells share the same size. Treatment with 100 μM palmitate, increased the majority of the population to exhibit a larger than control nuclear size, evidenced by the positive rightward shift in the FS median for nuclear size. In addition, there was also a small population of smaller than control nuclei (near the y-axis) which may be apoptotic fragmented DNA. DNA Check beads of 10 μm size were analyzed to permit size comparison.

To validate that the palmitate-induced changes to DNA content (apoptotic fragmented DNA) are due to apoptotic DNA strand breaks, cardiomyocytes treated with palmitate 100 μM for 24h and stained with the terminal dUTP nick-end labeling with fluorescein (TUNEL) method (Figure 5). Compared to the little fluorescent TUNEL labeling in control, palmitate induced a striking fluorescent nuclei. This result is a representation of several palmitate-treated TUNEL-positive cells grown on a coverslip, and not a quantitation of TUNEL positivity. These results suggest that palmitate induced apoptotic DNA cleavage that was detected by TUNEL labeling.

Utilizing 16 different cell cultures, flow cytometric analysis of 10 000 cardiomyocytes each demonstrated that palmitate produced a 1.2± 0.3 fold increase in nuclear size that was significantly (p<0.001) greater than control (Figure 6). In contrast there was no significant
Figure B3 - Microscopic analysis of cardiomyocytes treated with diluent (Panel B) or palmitate 100 μM 24h (Panel A) and then stained with Wright-Giemsa approach. This method preferentially stains nuclei (Wittekind 1983)
Figure B4 - Representative histograms of forward light scatter (FS) analysis of nuclei from permeabilized cardiomyocytes stained with propidium iodide (PI). As PI preferentially binds to nuclei, the FS readings reflect nuclear size. Control or diluent treated cells are shown on the left and palmitate, 100 μM for 24h, treated cells are in the center. Median denotes the FS channel where most nuclei share the same FS reading, hence size, 10 μm “DNA Check” beads are shown as a reference (right panel).
Figure B5 - Microscopic analysis of cardiomyocytes treated diluent (Control) or palmitate 100 μM 24h and then stained with the TUNEL method with fluorescein as the fluorochrome.
change in cell size (p=0.39). The nucleus/cell size ratio reflects the magnitude of the effect of palmitate on nuclear size in relation to the total cell size. Palmitate produced a significant (p<0.01) 1.4±0.1 fold increase in the nuclear/cell size ratio.

To explore potential explanations for the change in nuclear size, the characteristics of DNA staining with PI were examined to determine whether nuclear polyploidy was present (Figure 7). Palmitate did not alter the proportion of cells in different phases of the cell cycle, i.e. rest/growth (Go/G1), DNA synthesis (S) or growth/mitosis (G2/M) (Panel A). There were no significant differences in the proportion of cell nuclei in these three phases, thus excluding the possibility that nuclei were enlarging because of nuclear division (Panel B).

DISCUSSION

The data from this analysis showed that palmitate simultaneously induces cell death and increases the size of cardiomyocyte nuclei which is not attributable to changes in the cell cycle - i.e. there were no significant increases in DNA synthesis nor mitosis. Morphologic examination of embryonic cardiomyocytes revealed that palmitate treatment induced nuclei to appear larger with more granularity and with damaged nucleoli, manifesting in cleaved apoptotic DNA that was detectable with TUNEL. It has been previously demonstrated that forward light scatter can distinguish isolated cardiomyocytes of normal hearts compared with cardiomyocytes of hypertrophied hearts (Sunga & Rabkin 1994). Using this semiquantitative assay - flow cytometry analysis of the forward light scatter of a large population of cardiomyocytes treated with palmitate - a significant increase in nuclear size compared to control cells was observed.

These findings are consistent with observations that palmitate produces cell death (Paumen et al. 1997; Mangoura and Dawson 1998; Wiesner and Dawson 1996; Shimabukuro et al. 1998) and does so in cardiomyocytes (de Vries et al. 1997). The present results suggest that
Figure B6 - The effect of palmitate on nuclear and whole cell size as well as the ratio of nuclei: whole cell size is shown from flow cytometry analysis of 10,000 cells from each of 14 to 16 separate experiments. Whole cell size was determined by flow cytometry using forward light scatter of non-permeabilized cardiomyocytes from the same culture treated with palmitate at the same time. The data is expressed relative to control (mean±SEM). ** p<0.01 compared to control.
Figure B7 - The effect of palmitate on nuclear DNA is shown from FACS analysis of propidium iodide stained with nuclei from 10,000 cells each from 17 separate experiments. Panel A - Representative histogram of propidium iodide binding (FL3 LOG) to DNA within the nuclei. More propidium iodide binding is equivalent to more DNA which can reflect changes in nuclear size during the cell cycle. Go/G1 = quiescent/growth; S = DNA synthesis; M = mitosis were determined based on previous cell cycle histograms (Tepper et al 1999, Susin et al 1997, Diez et al 1997).

Panel B - Ratio of DNA content of different stages of cellular cycle, reflecting Go/G1, S, and M phases, in cardiomyocytes treated with diluent (control) or palmitate 100 µM 24 h. The data are expressed as number of cells exhibiting a cell cycle stage relative to control (mean±SEM).
palmitate induces significant cell death as well as TUNEL-positive, fragmented DNA. As discussed in the Introduction chapter, apoptosis is a form of cell death which involves DNA fragmentation that is detectable by TUNEL (Hermann 1995). Hence, I conclude that palmitate induces apoptosis in cardiomyocytes.

Although TUNEL was one of the first methods to observe apoptosis, it has recently criticized for its lack of specificity (Jerome et al 2000, Saraste and Pulkki 2000, Grasl-Kraupp et al. 1995), especially in cardiomyocytes (Freude et al 1998). TUNEL staining was found to be non-specific as some TUNEL positive cells were found to be artifacts caused by inadequate fixation, over-exposure of the TUNEL reaction, and cellular damage during cell/tissue preparation (Saraste and Pulkki 2000). As well, TUNEL is also criticized to be unable to distinguish DNA damage during apoptosis, necrosis, DNA repair, or post-mortem autolysis (Saraste and Pulkki 2000). Hence, TUNEL was not used extensively in this thesis. The following chapters will validate palmitate-induced apoptosis in other experimental methods.

These findings also demonstrate that palmitate alters the size and structure of cardiomyocyte nuclei. Morphologic examination of embryonic cardiomyocytes revealed that nuclei appeared larger with more granularity and had damaged nucleoli. These observations are consistent with the suggestion of an altered appearance of nuclei in mouse fibroblasts treated with palmitate (Gordon et al. 1977). However, to my knowledge, these palmitate-induced nuclear changes have not been previously described in cardiomyocytes. There have been previous reports of increased cardiac nuclear size in various kinds of cardiomyopathy, hypothetically due to an increase in DNA content (Maron et al 1975, Noda et al. 1980; Baandrup et al 1981; Popovich et al 1995; Scholz et al 1994). Therefore, these results are novel in that this study is the first to observe in cardiomyocytes that palmitate increased nuclear size, an observation also seen clinically in patients with cardiomyopathies, but that this increase in nuclear size was not due to
increased DNA content.

Also reminiscent of some cardiomyopathies (Scholz et al. 1994), palmitate-treated cardiomyocytes exhibited fuzzy nuclear membranes with disrupted nucleoli. Nuclear enlargement may involve nuclear envelope breakdown, and this may explain the observation of a less defined nuclear membrane in palmitate-treated cardiomyocytes. In mammalian cells, nuclear envelope breakdown involves stepwise lamina disassembly and microtubule-driven deformation of the nuclear membrane (Georgatos et al. 1997). Nucleolar disintegration, such as was observed here, was also noted in various cardiomyopathies in association with increased nuclear size (Unveferth et al. 1983).

Flow cytometric analysis of nuclear polyploidy demonstrated that palmitate-induced increase in nuclear size was not due to cell growth or division. If a significant proportion of the cell population under study was undergoing growth and division, flow cytometric analysis would show a significant increase in DNA synthesis or mitosis phase. In contrast, palmitate did not significantly alter the frequencies of cells in the G0/G1, S, or G2/M, suggesting that the observed increase in nuclear size is not due to palmitate-induced alterations in cell cycle. Nuclear polyploidy and hyperplasia of myocytes may represent an adaptive response to myocardial injury, perhaps as a cellular (compensatory) response for the loss of viable myocytes (Herget et al. 1997). However, there was no evidence of nuclear polyploidy in palmitate-treated cells. The nuclear changes appear to represent the direct effect of palmitate.

In summary, palmitate induced nuclear changes consistent with apoptosis. Further, palmitate produced an increase in nuclear size without altering cell size or cell cycle of cardiomyocytes. These results suggest palmitate may be a potential causal factor of altered cardiomyocyte structure consistent with cardiomyopathy.
SECTION C - PALMITATE-INDUCED APOPTOSIS IN CARDIOMYOCYTES IS MEDIATED THROUGH ALTERATIONS IN MITOCHONDRIA: PREVENTION BY CYCLOSPORIN (Kong JY and Rabkin SW. Biochim et Biophys Acta 1485 (2000): 45-55)

SUMMARY

Palmitate was reported in the previous chapters to induce apoptotic cell death in embryonic chick cardiomyocytes. Dual staining with PI and FDA and subsequent analysis of 10,000 cardiomyocytes by flow cytometry established that palmitate-induced cell death was predominantly due to oncosis whereas apoptosis occurred in 13% of all dead cells. This low proportion of palmitate-induced apoptosis was confirmed by evaluation of the DNA content or PI fluorescent staining of the DNA of permeabilized cardiomyocytes. These data demonstrate, for the first time, a critical role for mitochondria in the pathogenesis of palmitate-induced cell death based on (i) palmitate-induced reduction of mitochondrial activity as assessed by the mitochondrial-selective dye chloromethyl-X-rosamine (ii) more of the mitochondrial marker cyt C in the cytosol of palmitate-treated cardiomyocytes than control cells (iii) the finding that cyclosporin A, which inhibits the development of mitochondrial transition pores, blocked palmitate-induced alteration in mitochondrial function and palmitate-induced cell death. These data demonstrate the nature of palmitate-induced cell death in cardiomyocytes (both apoptotic and necrotic), suggest a mitochondrial basis for its pathogenesis, and show that cyclosporin A prevents palmitate-induced apoptotic cardiomyocyte cell death.

INTRODUCTION

As discussed previously in the Introduction chapter, fatty acids such as palmitate, are present in high levels in the blood of patients following acute myocardial infarction (Oliver et al.
Recently, palmitate-induced apoptosis has been demonstrated in murine hematopoietic cell lines (Paumen et al. 1997), embryonic chick astrocytes, neuronal cells (Mangoura et al. 1998; Weisner et al. 1996), pancreatic beta cells (Shimabukuro et al. 1998), and neonatal rat cardiomyocytes (de Vries et al. 1997). Yet fatty acids has been also implicated in the induction of oncosis (Tanigaki et al. 1998; Gudbjarnason et al. 1975). The nature of palmitate-induced cardiomyocyte death, however, has not been well characterized.

The central role of mitochondria in palmitate metabolism raises the possibility that mitochondria may play a critical role in palmitate-induced cardiomyocyte cell death (Kroemer et al. 1998). Mitochondrial responses during apoptosis include a decrease in mitochondrial respiration and ATP generation (Di Lisa et al. 1998), altered mitochondrial permeability by the formation of mitochondrial transition pores (Tatton et al. 1999, Budd et al. 1998), mitochondrial calcium sequestration (Murphy 1999), generation of reactive oxygen species (Cai et al. 1998), and the release of activators of the caspase cascade such as cyt C (for review, see Lemasters 1998a,b).

The proximity of mitochondria to various caspases further links mitochondria to apoptosis (Krajewski et al. 1998; Samali et al. 1999). Previous studies have observed that lowering of the mitochondrial membrane potential, the driving force of the cell’s energy generation results in decreased ATP production. Formation of mitochondrial transition pores (MTP) complicate the loss of membrane potential. MTPs, which are formed at the contact site between the inner and outer mitochondrial membranes, enhance mitochondrial membrane permeability thus allowing the release of mitochondrial elements, such as cyt C, into the cytosol.

The role of mitochondrial permeability in cell death was examined using of cyclosporin A. Cyclosporin A inhibits the breakdown of inner mitochondrial transmembrane potential by
affecting cyclophilin D, a component of the MTP (Petronilli et al. 1994). Cyclosporin has been previously shown to have anti-apoptotic effects in lymphocytes due to its ability to prevent disruption of mitochondrial transmembrane potential (Zamzani et al. 1996).

The objective of this section was to determine the role of mitochondria in palmitate metabolism and cell death. Palmitate-induced death via the formation of MTPs leading to the loss of $\Delta \Psi_m$ and death by apoptosis was examined. I also examined whether preventing the formation of these transition pores by pretreatment with cyclosporin A, would abolish palmitate-induced apoptosis in cardiomyocytes.

RESULTS

Palmitate induces both oncosis and early apoptosis

Cardiomyocytes treated with palmitate (100 $\mu$M for 24h) were examined by flow cytometry using the dual staining abilities of the fluorochromes FDA and PI (Figure 1). The time and dose of palmitate was previously established by this laboratory. FDA is a substrate for esterases, ubiquitous enzymes present in all cell types. Uncharged FDA easily penetrates live cells where esterases will convert it to its charged, hydrolyzed fluorescein product. The charged FDA becomes trapped inside the live cell, but can escape from necrotic cells. Hence, FDA labeled cells are a measure of live and early apoptotic cells (Hamori et al. 1980). PI measures the loss of plasma membrane integrity, which occurs early during cell oncosis although it is also a late event of apoptosis. Thus cells can be divided into three populations-viable cells or necrotic cells (low FDA, high PI) and apoptotic cells (low FDA, low PI)(Hamori et al. 1980).

Cardiomyocytes treated with 100$\mu$M palmitate demonstrate an increase in the apoptotic and necrotic populations. Palmitate induced a significant (p<0.01) increase in apoptotic cell death (from control 448±66 to palmitate 929±215 cells per population of 10 000) that was 2.0±0.2
FIGURE C1 - Palmitate induces apoptosis and oncosis as assessed by FDA & PI staining. - Panel A - A representative dotplot of 19 separate experiments. Cardiomyocytes were treated with 100 μM palmitate or ethanol (control) for 24 h, dually stained with fluorescein diacetate (FDA) (y-axis) and propidium iodide (PI) (x-axis), analyzed on the FACS. Apoptotic and necrotic populations were determined by the difference in FDA and PI staining intensities. Panel B - Number of cells exhibiting apoptosis and necrosis. Palmitate induced a significant (** p<0.01) population of apoptosis and oncosis (N=19).
(N=19) fold greater than control (N=19) (Fig. 1). Palmitate also induced a significant (p<0.01) increase in the amount of necrotic death (from control 2 453±231 to palmitate 6 008±287 cells per population of 10,000) that was 3.0±0.3 fold greater than control (N=17). The amount of death, both apoptotic and oncotic, seen in control cells are representative of the amount of cell death induced by palmitate’s diluent, ethanol, and by cellular damage acquired in lifting these adherent cells from the dish.

**Palmitate induces a loss of DNA content**

To further examine the kind of cell death and confirm that palmitate induces apoptosis, another approach to the study of apoptosis was undertaken. The late apoptotic event DNA fragmentation leading to loss of intact DNA content can be demonstrated by flow cytometry of permeabilized cardiomyocytes stained with propidium iodide (PI) which intercalates in the DNA of the nuclei. Hence, PI fluorescence is a measure of DNA content. Palmitate induced an increase in proportion of the population with low DNA content (PI fluorescence <10^2) (Fig. 2a). The proportion of the population with this characteristic increased from 1267±88 (N=27) in control to 1765±203 (N=27) in palmitate treated cells per population of 10,000 cells. Indeed there was a significant (p<0.01) and 1.5±0.1 fold increase in nuclei with apoptotic low DNA content (Fig2b).

**Palmitate altered mitochondrial activity**

To determine whether palmitate affected mitochondrial function, palmitate was added with or without cyclosporin pre-treatment to cardiomyocytes and the dye CMX-Ros used to evaluate for active mitochondrial respiratory function. CMX-Ros is a lipophilic and cationic mitochondrial selective dye, which readily enters the mitochondria in an uncharged form (Metivier et al 1998). Uptake of cationic CMX-Ros by mitochondria is related to the presence of a “negative sink” inside mitochondria created by proton pumps during respiration (Smiley et al. 1991).
Figure C2 - Palmitate induces apoptotic nuclei with low DNA content – Panel A - Representative histogram of 27 separate experiments; each of 10 000 cardiomyocytes treated with 100 μM palmitate (right panel) or ethanol (control) (left panel) for 24 h were stained with propidium iodide (PI), and analyzed by flow cytometry. Resulting histograms is an indication of nuclear size and DNA content. G0/G1 = Quiescence/Growth, S = Synthesis, M = Mitosis/Growth, Apo = apoptotic DNA with low DNA content. Panel B - Number of cells with low DNA content (Apo) in a population of 10 000 cells. Cardiomyocytes treated with 100 μM palmitate (N=27) for 24 h showed a significant (**p<0.01) increase in cells with low DNA content.
CMX-Ros readily enters mitochondria and is oxidized to a fluorescent form. Within the mitochondria, oxidized CMX-Ros binds to proteins within the mitochondria preventing its loss from mitochondria (Poot et al 1996). Hence, an increase in CMX-Ros fluorescence denotes an increase in the mitochondrial oxidation of the fluorochrome and is therefore an indirect measure of mitochondrial metabolism. After treatment with palmitate and/or cyclosporin A, cardiomyocytes were stained with CMX-Ros for 16h and then analyzed by flow cytometry. A representative histogram shows the distribution of CMX-Ros fluorescence that was shifted to the left, compared to control, after palmitate treatment. The change in CMX-Ros fluorescence was blocked by cyclosporin (Fig 3a). Palmitate treated cells exhibited significantly (p<0.05) less CMX-Ros fluorescence that was 0.73±0.08 times that of control whereas cyclosporin A had a slightly increased CMX-Ros fluorescence, (1.32±0.11 fold) compared to control (Figure 3b). Cyclosporin A pre-treatment before palmitate resulted in a significant (p<0.05) increase in CMX-Ros fluorescence compared to palmitate so that the combination of palmitate plus cyclosporin A was not different from control (1.09±0.04 times control).

Palmitate induces mitochondrial leakage of cyt C into the cytoplasm

To further explore the consequences of palmitate on mitochondrial permeability, I investigated whether palmitate would induce the release of the mitochondrial marker cyt C into the cytoplasm. Cyt C has been implicated in initiating the caspase cascade leading to apoptotic cell death (Green & Reed 1998). Cardiomyocytes were treated with palmitate 100 μM, with or without cyclosporin A 1 μM pre-treatment, for 24 h and cyt C western blotting was performed on the cytosolic fraction of these lysed samples (Figure 4). A definite increase in cytosolic cyt C was observed in palmitate-treated cells.

Cyclosporin A pre-treatment blocks palmitate-induced apoptosis but not oncosis

To define the kind of palmitate-induced cell death affected by cyclosporin, cell death was
Figure C3 - *Palmitate affects CMX-Ros fluorescence*. Panel A - A representative population distribution, from 3 separate experiments, of cardiomyocytes treated with diluent (control), palmitate 100 μM, cyclosporin A 1 μM, or the combination for 24h. CMX-Ros (500 nM) was added for the final 16 h of incubation and the cells examined by flow cytometry. M1 denotes the median in control cells. Panel B - The relative change in CMX-Ros fluorescence compared to control. Data are presented as median channel number compared to control median channel. The data are presented as mean±SEM for control (N=19), 100 μM palmitate (N=19), 1 μM cyclosporin A (Cyclo) (N=3) and the combination (N=3). Data analysis notes a significant decrease in median channel number compared to control (* p<0.05) and a significant change compared to palmitate (△ p<0.05).
Figure C4 - Palmitate treated cardiomyocytes demonstrate more cytochrome C in the cytosol-
Cardiomyocytes, grown in culture for 72h were treated with palmitate (100 μM), Cyclosporin A (Cyclo) 1 μM, the combination or the diluent (control) for 24 h. Cells were then lysed with RIPA buffer, subjected to differential ultracentrifugation to separate mitochondria and cytosol. Equivalent amounts of cytosolic protein, assessed by Bradford protein assay, were loaded on a 15% acrylamide gel for electrophoresis. The membrane was reacted with a monoclonal anti-cytochrome C and detected with a HRP-linked secondary antibody. Signals were detected using chemiluminescence methodology. A representative gel is shown from one of three different experiments.
characterized by FDA and PI dual staining followed by flow cytometric analysis. Cyclosporin A pretreatment beginning 30 min before palmitate treatment reduced the amount of apoptosis but did not affect the amount of necrotic cell death (Figures 5&6). Data analysis demonstrated that cyclosporin A (1µM) significantly (p<0.05) blocked palmitate-induced early apoptosis: palmitate induced 929 cells to undergo early apoptosis whereas with cyclosporin A pretreatment this was reduced to 453 cells (comparable to control’s 448 cells) in a population of 10 000 cells (Figure 5). Cyclosporin A significantly (P<0.05) blocked palmitate induced apoptosis reducing the relative increase from a 2.0±0.2 {N=18} fold greater than control to levels similar to control (1.0±0.2 {N=3} fold greater than control). In contrast, cyclosporin A pre-treatment had not effect on palmitate-induced oncosis (Figure 6).

To ensure that the effects of cyclosporin were reproducible with another methodology to assess apoptosis, the DNA content of cardiomyocytes was examined. Similar results were obtained. Cyclosporin A pre-treatment blunted the palmitate-induced formation of nuclei with low DNA content which occurs at late apoptotic development (Figure 7). Cyclosporin A pre-treatment was associated with only 1345 ±110 (N=11) cells exhibiting nuclei with low DNA content whereas palmitate-induced cell alterations indicative of apoptotic nuclei were demonstrated in 1756 ±160 (N=35) cells per 10 000 cells (Figure 7).

DISCUSSION

The work in this chapter presents a novel explanation for palmitate-induced apoptosis, namely palmitate-induced loss of mitochondrial function. The mitochondrial-selective dye CMX-Ros was used to observe mitochondrial respiration. As CMX-Ros can not reach its fluorescent state without oxidation within an active, respiring, mitochondria, CMX-Ros is a useful tool for identifying not only the mitochondrial activity within a cell, but also the loss of
Figure C5- **Cyclosporin A pretreatment significantly blocks apoptosis induced by palmitate** - Cardiomyocytes treated with 100μM palmitate, with or without 1μM cyclosporin A pretreatment, for 24h were dually stained with FDA and PI and examined by flow cytometry. The data are presented as mean±SEM for control (N=19), 100 μM palmitate (Palm) (N=19), 1 μM cyclosporin A (Cyclo)(N=3) and the combination (N=3). Data analysis notes a significant increase in apoptotic population compared to control (* p<0.05) and a significant difference compared to palmitate (Δ p<0.05). Inset - Data presented as the number of cells in the apoptotic population relative to the number of apoptotic cells in control.
Figure C6 - *Cyclosporin A* pretreatment does not affect palmitate-induced oncosis - Cardiomyocytes treated with 100 μM palmitate, with or without 1 μM cyclosporin A pretreatment, for 24h were dually stained with FDA and PI and examined by flow cytometry. The data are presented as mean±SEM for control (N=19), 100 μM palmitate (N=19), 1 μM cyclosporin A (N=3) and the combination (N=3). Data analysis notes a significant increase in oncotic population compared to control (** p<0.01). *Inset* - Data presented as the number of cells in the oncotic population relative to the number of oncotic cells in control.
Figure C7 - *Cyclosporin A* pretreatment blunts the development of apoptotic nuclei induced by palmitate. Cardiomyocytes treated with control (N=11), 100 μM palmitate (N=11), 1 μM cyclosporin A (N=11) and the combination (N=11) for 24h were stained with PI and examined by flow cytometry. The data are presented as mean±SEM. Data analysis notes a significant increase in low DNA content nuclei with palmitate treatment compared to control (*p<0.05). Inset - Data presented as population of low DNA content relative to control. Data analysis notes a significant increase in the population containing low DNA content nuclei in palmitate treatment compared to control (**p<0.01).
active mitochondrial function (Metivier et al. 1998; Poot et al. 1996). It was demonstrated that palmitate-treated cardiomyocytes had less CMX-Ros fluorescence, hence less mitochondrial activity, than control. It has been previously documented that cells undergoing apoptosis exhibit a decrease in mitochondrial transmembrane potential which precedes nuclear signs of apoptosis (Petit et al. 1995; Cossarrizza et al. 1995).

In addition to loss of mitochondrial activity, palmitate-treated cardiomyocytes had an increased amount of the mitochondrial marker cyt C in the cytosol. This observation implies that there is an increase in mitochondrial permeability upon palmitate treatment. Loss of cyt C from the mitochondria into the cytoplasm can lead to the induction of caspase-3 and to apoptosis (Green & Reed 1998). This suggests that palmitate may act through two mechanisms to induce apoptosis: by creating mitochondrial permeability pores which allow the loss of $\Delta \psi_m$ and hence mitochondrial respiration and by triggering a cyt C-induced caspase activation in the cytosol. These two mechanisms, however, may act sequentially with loss of mitochondrial function and loss of mitochondrial respiration preceding cyt C loss from mitochondria that in turn magnifies the effect of mitochondrial damage.

Another piece of evidence implying that palmitate induces a loss of mitochondrial permeability is cyclosporin A pretreatment. Cyclosporin A binds to and inhibits the cyclophilin D component of the mitochondrial permeability transition pore (Broekemeir et al. 1989; Szabo and Zoratti et al. 1991; Petronilli et al. 1994). In these experiments, cyclosporin A reduced palmitate-induced apoptosis with no effect on oncosis. Although cyclosporin inhibits the development of apoptosis in other cell types with other apoptotic stimuli (Lemasters et al. 1998a&b; Zamzani et al. 1996), this has not been previously demonstrated in the heart. Importantly it indicates a selectivity of cyclosporin for apoptosis and highlights that apoptosis and oncosis, at least in cardiomyocytes, are subject to different kinds of modification.
Cyclosporin A also causes mitochondrial membrane hyperpolarization (Tanner et al. 1993) and resealing or reclosing of already open transition pores to allow for the reestablishment of an intact electrochemical gradient (Petronilli et al. 1994). This may explain the observation of increased CMX-Ros fluorescence in cyclosporin-treated cardiomyocytes compared to control. Cyclosporin A may have resealed transition pores in normal cardiomyocytes. As well, it prevented the formation of new transition pores during palmitate treatment.

It is also worth noting that cyclosporin also has other non-mitochondrial effects. Cyclosporin A is clinically used as an immunosuppressive drug. It also is a known inhibitor of phosphatases, especially calcineurin. Cyclosporin’s effects on these phosphatases may have ramifications in any phosphorylation/cell signaling that palmitate may induce.

As mentioned, palmitate has been observed to induce apoptosis in cardiomyocytes previous to this thesis (de Vries et al. 1997). De Vries et al (1997) was the first to report that the fatty acid palmitate induced apoptosis in cardiomyocytes and reported apoptotic DNA fragmentation. Although de Vries eliminated palmitate’s death mechanism from a non-specific detergent action by the delivery of palmitate with albumin, de Vries did not report the mechanism by which apoptosis is manifested. De Vries speculated that as the addition of exogenous fatty acids significantly alter endogenous lipid pools including membrane phospholipids, palmitate induces death by affecting the melting temperature of the phospholipid membrane. Hence, the present thesis is novel as it begins to elucidate the complicated signaling mechanism of palmitate-induced apoptosis.

In summary, this thesis is the first to identify an intervention, specifically cyclosporin A, that prevents cardiomyocyte cell death induced by palmitate. These data further highlight the importance of the mitochondria in the pathogenesis of apoptosis in the heart.
SECTION D - PALMITATE-INDUCED APOPTOSIS IN EMBRYONIC CARDIOMYOCYTES IS MEDIATED THROUGH CPT-1, DEPENDENT ON CARNITINE AVAILABILITY (Kong JY and Rabkin SW – In Press - American Journal of Physiology)

SUMMARY

To understand the fundamental mechanism of palmitate-induced cell death, the objective of this chapter was to determine whether transport of palmitate, through carnitine palmitoyl transferase 1 (CPT1), into the mitochondria for oxidation was involved in the pathway of palmitate-mediated cell death. In embryonic chick cardiomyocytes, both carnitine 30mM, an important cofactor needed for the transport of fatty acids into mitochondria via CPT1, and palmitate 100μM significantly (p<0.01) increased the number of cells with apoptotic low DNA content to 3331±564 (N=10) and 1461±204 (N=10), respectively, compared to control 844±88 (N=10). In combination, palmitate + carnitine co-incubation significantly (p<0.01) increased the number of cells exhibiting low DNA content to 5954±1042 (N=10), greater than the combined apoptosis of carnitine and palmitate. Inhibition of CPT-1 with oxfenicine 10mM significantly (p<0.05) blocked apoptotic low DNA content induced by the combination of palmitate + carnitine to 3997±700 (N=8). Oxfenicine also blocked apoptosis induced by carnitine alone. However, oxfenicine did not affect palmitate-induced apoptosis. These results suggest that palmitate-mediated cell death via CPT-1 is dependent on carnitine availability. To further investigate the role of CPT-1 in palmitate-induced cell death, a shorter fatty acid, capric acid (C10:0), was used as it does not require CPT-1 for transport into the mitochondria for oxidation. Capric acid alone did not induce any significant apoptotic changes similar to palmitate, suggesting that oxidation of fatty acids per se is not involved in palmitate-induced apoptosis. To
further investigate the role of CPT-1, simultaneous glycolysis stimulation and fatty acid oxidation deactivation was achieved with glucose, insulin and lactate treatments which inhibit fatty acid oxidation and promote glycolysis by indirectly inhibiting CPT-1 with malonyl-CoA. These data showed that 100 nM insulin + 2 g/L glucose had no effect on palmitate-induced apoptosis. These findings are consonant with the finding with lactate 1mM which also had no effect on palmitate-induced cell death. These results suggest that fatty acid oxidation of palmitate through CPT-1 is dependent on carnitine availability. If sufficient carnitine is available for transport of the excess palmitate, CPT-1 is involved in palmitate-induced apoptosis. If insufficient carnitine is available, CPT-1 does not appear to play a role in palmitate-induced apoptosis, as shown by the ineffectiveness of CPT-1 inhibition with oxfenicine, glucose+insulin, or lactate on palmitate-induced apoptosis.

INTRODUCTION

As discussed earlier in the Introduction, fatty acids such as palmitate are a principal source of energy for the heart and are oxidized in adult, neonatal and embryonic hearts (for review, see van der Vusse et al. 1992; Lopaschuk et al. 1994a&b). Several hypotheses have been advanced to explain palmitate-induced cell death and have been covered in the Introduction. This section will examine carnitine palmitoyltransferase 1 (CPT-1), the mitochondrial enzyme important in fatty acid metabolism because it transports long chain fatty acyl moieties, such as palmitate into the mitochondria, making CPT-1 a control point for fatty acid oxidation (McGarry et al. 1997).

The role of CPT-1 in palmitate-induced cell death will be examined by two approaches: increased availability of substrates for CPT-1, namely carnitine and palmitate, and inhibition of CPT-1 with oxfenicine and stimulation of glycolysis. Increased CPT-1 activity can be achieved
by adding exogenous carnitine, a necessary co-factor for fatty acyl CoA translocation. It has been established that intramitochondrial concentration of carnitine governs the enzyme kinetics of CPT-1 (Cook et al. 1984). In this chapter, carnitine will be combined with palmitate to test the hypothesis that increasing exogenous carnitine, which translocates more palmitoyl CoA into the mitochondria so as to enhance fatty acid oxidation, will augment palmitate-induced cell death.

It should be noted that Lopaschuck et al. (1993) reported that carnitine addition inhibits fatty acid oxidation and stimulates carbohydrate metabolism. However, Lopaschuck et al. (1994) then later established that this inhibition was secondary to the changes in [Acetyl CoA]/[CoA] and NADH₂/NAD⁺ ratios due to fatty acid metabolism.

CPT-1 activity can be inhibited by the drug oxfenicine and by carbohydrate metabolism. Oxfenicine (hydroxyphenylglycine) has been reported to specifically inhibit CPT-1 and has been previously tested in other cell models (Madden et al. 1995; Varela et al. 1997). Oxfenicine mediates its effect via its metabolite 4-hydroxyphenyl glyoxylate which has structural similarities with malonyl CoA (Bielefeld et al 1985). Carbohydrate oxidation, a pathway that catabolizes monosaccharide sugars to pyruvate, produces acetyl CoA. This acetyl CoA, along with the acetyl CoA produced from the citric acid cycle, can also inhibit CPT-1 indirectly via the generation of malonyl CoA. CPT-1 activity is negatively regulated by intracellular malonyl CoA, a molecule generated by acetyl CoA carboxylase (ACC) from the excess acetyl CoA produced by carbohydrate and fatty acid oxidation (Lopaschuk et al. 1994a&b, Mabrouk et al. 1990). Malonyl CoA directly inhibits CPT-1 (Bianchi et al. 1990). Hence, there are 2 points of control of fatty acid oxidation: generation of malonyl CoA and ACC activity. Upregulation of carbohydrate metabolism can occur by the exogenous addition of glucose which produce the acetyl CoA necessary to yield malonyl CoA and hence inhibit CPT-1. Similarly, insulin can inhibit CPT-1 by directly affecting ACC activity to produce more malonyl CoA (Makinde et al. 1990).
Hence, the combination of glucose and insulin shunts energy production of the embryonic cardiomyocyte primarily to glycolysis (Ruderman et al. 1998; Depre et al. 1998). Another approach to examine the same mechanism is through the ability of lactate, another oxidizable carbohydrate, to inhibit palmitate oxidation in cardiomyocytes (Awan et al. 1993). The advantage of administration of exogenous lactate rather than glucose is that lactate is readily converted to pyruvate without the energy-requiring steps of converting glucose to pyruvate. The pyruvate derived from lactate is then converted by the pyruvate dehydrogenase complex (PDC) to acetyl CoA within the mitochondria. An increase in acetyl-CoA production from PDC will result in a shuttling of acetyl groups into the cytoplasm producing an increase in malonyl-CoA production, which in turn, will inhibit fatty acid oxidation (for review, see Lopaschuk et al. 1994b).

Only long chain fatty acids with chain lengths of 16 and greater require transport into the mitochondria via CPT-1 (for review, see van der Vusse et al. 1992). Shorter fatty acids can readily enter mitochondria for β-oxidation and have been shown to be non-lethal (Abdel-Aleem et al. 1996). Capric acid, a saturated C10:0 fatty acid will be used as a negative control to ascertain whether CPT-1 is involved in fatty acid apoptosis.

RESULTS

Palmitate induces apoptosis

To examine the kind of cell death and confirm that palmitate produces apoptosis, cellular DNA content was examined. Apoptosis is associated with patterned DNA fragmentation, hence loss of intact DNA content can be demonstrated by flow cytometry of permeabilized cardiomyocytes stained with propidium iodide (PI) which intercalates in the DNA of the nuclei. Palmitate induced an increased frequency of cells with low DNA content (Fig. 1). Indeed there
Figure D1 - Palmitate induces an increase in the population of cells with low DNA content
Panel A. Representative histograms of 10,000 cardiomyocytes treated with 100 µM palmitate (upper right panel) or diluent (control) (upper left panel) for 24h, stained with propidium iodide (PI), and analyzed by flow cytometry. Resulting histograms are an indication of nuclear size and DNA content. G0/G1 = Quiescence/Growth, S = Synthesis, M = Mitosis, Apo = apoptotic DNA with low DNA content. Apoptotic cells with low DNA content are those with PI fluorescence <10^2.
Panel B. Palmitate induced a significant (**) p<0.01) increase in the number of cells with nuclei with low DNA content (N=15) compared to control (N=15).
was an increase in nuclei with low DNA content (PI fluorescence <10^2) (inset). The proportion of the population with this characteristic increased from 1,002±70 (N=28) in control to 1,505±128 (N=28) in palmitate treated cells per population of 10,000 cells, or a 1.6±0.1 fold increase.

*Palmitate-induced death is a specific, CPT-1 related effect*

To determine whether palmitate-induced apoptosis is a specific effect, related to CPT-1, the short saturated fatty acid capric acid (C10:0) was used as a control. Capric acid does not require CPT1 for transport into the mitochondria for oxidation (for review, see van der Vusse 1992). Cardiomyocytes were treated with either palmitic or capric acid, both at 100 μM for 24h, and analyzed by flow cytometry for either apoptotic DNA content (Panel B) or annexin V binding (Figure 2 - Panel A). Capric acid did not induce any significant changes in low DNA content or annexin V binding compared to control whereas palmitate induced significant (p<0.01) increases in both. This suggests that capric acid is not inducing apoptosis implying that palmitate’s effects are specific for CPT-1 and are not due to a non-specific fatty acid effect.

*Carnitine enhances palmitate-induced apoptotic death*

It was next sought to determine whether carnitine enhanced palmitate-induced formation of apoptotic nuclei with low DNA content. Exogenous carnitine has been reported to be readily transported into the cell across the sarcolemmal membrane (Vary & Neely 1982). The concentrations of carnitine used in this section have been shown to increase intracellular carnitine concentration (Broderick et al. 1992). I speculate that the mechanism of action of carnitine on palmitate-induced cell death is mediated through CPT1 and palmitate metabolism because exogenous L-carnitine increases cardiac CPT-1 activity and palmitate oxidation (Abdel-aleem et al. 1996). Both palmitate 100μM and DL-carnitine 30 mM induced a significant (p<0.01) increase in the number of cells with low DNA content, from control 844±88 (N=1) to
Figure D2 - Capric acid does not induce apoptosis - Cardiomyocytes were treated with either capric acid (N=3) or palmitate (N=14) 100 μM for 24h. Cells were then stained with either annexinV-FITC (upper panel) or permeabilized and the nuclei stained with PI (lower panel) and examined by flow cytometry. Fluorescence of each respective fluorochrome is expressed relative to control fluorescence. ** p<0.01 compared to control.
Figure D3 - *Carnitine enhances palmitate-induced cell death* — Cardiomyocytes were treated with DL-Carnitine 30mM (C30) (N=7), palmitate 100 µM (Palm) (N=12), or the co-incubation of the carnitine+palmitate (C30+P) (N=6) for 24h before FACS analysis of nuclei with low DNA content. **Panel A** - Number of cells exhibiting low DNA content. **p<0.01 compared to control.**

**Panel B** - Relative amount of cells exhibiting low DNA compared to control. **p<0.01** compared to control.
palmitate 1461±204 (N=10), or 1.8±0.2 fold, and carnitine 3331±564 (N=10), or 4.1±0.8 fold (Figure 3). In combination, palmitate + carnitine co-incubation significantly (p<0.01) increased the amount of cells exhibiting low DNA content to 5954±1042 (N=10), or 7.3±1.6 fold. In fact, the combination of carnitine + palmitate was significantly (p<0.05) greater compared to either palmitate or carnitine alone and greater than the combined apoptosis of carnitine and palmitate (i.e. 1.8 fold + 4.1 fold is less than the 7.3 fold observed with the combination).

Oxfenicine blunts carnitine plus palmitate-induced formation of apoptotic nuclei

To further explore the interaction of palmitate and carnitine, the CPT1 inhibitor oxfenicine was used to pretreat the cardiomyocytes 30 min before DL-carnitine (30 mM) + palmitate (100 µM) stimulation. Nuclear DNA content was analyzed by flow cytometry (Figure 4). Whereas oxfenicine 1mM yielded a similar but weaker response, oxfenicine 10 mM pretreatment yielded a significant (p<0.05) decrease in the formation of apoptotic nuclei compared to DL-carnitine 30mM + palmitate 100 µM, from 5254±1042, or 7.3±1.6 fold, to 3997±700, or 4.1±0.6 fold (N=8) compared to control.

Apoptosis induced by carnitine+palmitate is dependent on carnitine availability

To determine why carnitine alone induces greater apoptosis than palmitate alone, I examined the effect of oxfenicine on the effect of carnitine or palmitate. Using flow cytometric analysis of low DNA content, cardiomyocytes were pretreated with oxfenicine 10mM 30 min prior to addition of either DL carnitine 30mM (Figure 5 - Panel A) or palmitate 100µM (Figure 5 - Panel B). CPT-1 prefers the L-carnitine isomer rather than the racemic DL-carnitine mix, L-carnitine 30mM was used to compare the carnitine effects based on isomers (Panel A). Both L-carnitine and DL-carnitine (30mM) induced a significant (p<0.01) increase in apoptosis compared to control with L-carnitine inducing a 5.23±1.4 (N=5) fold increase and DL-carnitine a 4.12±0.76 (N=10) fold increase. Oxfenicine effectively blunted L-carnitine-induced apoptosis to
Figure D4 - Oxfenicine blunted the effect of carnitine and palmitate to induce apoptotic low DNA content. Cardiomyocytes that had been grown in culture for 72 h were treated with either diluent (control) (N=9), palmitate 100μM (P) (N=8), DL carnitine 30mM+ palmitate 100μM (C+P) (N=8), or carnitine 30mM+ palmitate 100μM with oxfenicine pretreatment for 30 min., either 1 (N=4) or 10 mM (N=7) (Ox1+C+P and Ox10+C+P, respectively). Cells were stained with propidium iodide (PI), and analyzed on the FACS. Panel A - The data are presented as the mean±S.E.M. and were analyzed as compared to control (**p<0.01), or palmitate (ΔΔ p<0.01) or carnitine+palmitate (+p<0.05). Panel B The relative change for each treatment group compared to control.
Figure D5 - Apoptosis by CPT-1 is dependent on carnitine, but not palmitate, concentrations - Panel A - Cardiomyocytes were treated with DL-carnitine 30mM (DL-C) (N=10), L-carnitine 30mM (L-C) (N=5) without or with oxenine 10mM pretreatment (Oxf+L-C) (N=3). Cells were examined by flow cytometry for apoptotic low DNA content. ** p<0.01 compared to control, Δ p=0.1 compared to L-carnitine. Panel B - Cardiomyocytes were treated with palmitate 100 μM (P) (N=14), oxenine 10 mM (Oxf) (N=7), or the combination of the two (Oxf+P) (N=8) for 24h. Cells were examined by flow cytometry for apoptotic low DNA content. ** p<0.01 compared to control.
2.06±0.2 (N=3) compared to control (Panel A). In contrast, oxfenicine had no effect on palmitate-induced apoptosis (Panel B). These results suggest that the CPT-1 mediated apoptosis observed during carnitine+palmitate co-treatment is dependent on carnitine availability and not on palmitate availability.

**Stimulation of glycolysis with insulin+glucose had no effect on palmitate induced apoptosis**

Cardiomyocytes were treated with palmitate (100 μM for 24h), with or without glucose (2 g/L) + insulin (100 nM), and examined by flow cytometry for low DNA content. There was no significant change in the number of cells exhibiting low DNA with insulin + glucose pretreatment compared to palmitate alone (Figure 6). Indeed palmitate 100 μM induced a significant (p<0.01) increase in nuclei with low DNA content, 1383±221 (N=8) compared to control 1048±161 (N=8). Insulin + glucose pretreatment had no effect on palmitate-induced loss of DNA content (1309±166 vs. 1383±221).

**Lactate does not affect palmitate-induced apoptosis or loss of DNA content**

To further explore whether another carbohydrate to be oxidized would alter palmitate-induced apoptosis, cardiomyocytes were co-treated with lactate 1 mM and palmitate 100 μM for 24h before analysis by flow cytometry. Although palmitate induced a significant (p<0.01) increase in the apoptotic population, 1764±100 (N=6) compared to control 1257±142 (N=6), this was not significantly altered by lactate (1659±224, N=6) (Figure 7).

**DISCUSSION**

This chapter presents compelling new evidence that, in the presence of sufficient carnitine to transport palmitate into the mitochondria, carnitine palmitoyltransferase-1 (CPT-1) is a crucial step in the pathogenesis of palmitate-induced cardiomyocyte cell death that occurs through increased palmitate metabolism. First, the combination of palmitate with carnitine, both
Figure D6 - **Insulin+glucose pretreatment has no effect on palmitate-induced loss of DNA content** - Cardiomyocytes treated with palmitate (100 μM for 24 h) (N=8), glucose (2g/L)+insulin (100 nM) (N=8), the combination of the two (Gluc+Ins+Palm) (N=8), or diluent (control) (N=8), were examined by flow cytometry using PI fluorescence in permeabilized cells. The data are presented as the mean±SEM number of cells with low DNA content per 10 000 cells. **p<0.01 compared to control.
Figure D7 - Lactate has no effect on palmitate induced loss of DNA content - Cardiomyocytes treated with palmitate (100μM for 24h) (N=6), with or without lactate, 1mM, (N=6), were examined by flow cytometry using PI fluorescence as a measure of DNA content in permeabilized cardiomyocytes. Palmitate induced a significant (**) p<0.01) increase in proportion of the population with low DNA content which was unaffected by lactate.
being substrates for CPT-1, enhanced cell death to levels greater than either palmitate or carnitine alone. In fact, the carnitine enhancement of palmitate-induced apoptosis is an observation that has not been previously published. Second, the CPT-1 inhibitor oxfenicine blocked carnitine+palmitate-induced cell death. Third, apoptosis observed with carnitine+palmitate co-treatment was dependent on carnitine availability as excess carnitine with limited basal palmitate (ie-no exogenous palmitate addition) was inhibitable by oxfenicine whereas palmitate 100 μM with limited basal carnitine (ie - no exogenous carnitine addition) was unaffected by oxfenicine.

The ability of carnitine to enhance palmitate-induced apoptosis implies that palmitate mediates its apoptotic mechanism by its oxidation within the mitochondria. Exogenous carnitine is readily transported into the cell across the sarcolemmal membrane (Vary & Neely 1982) and the concentrations used in this experiment have been documented to increase intracellular carnitine concentration and CPT-1 activity (Broderick et al. 1992; Abdel-Aleem et al. 1996). CPT-1 has a $K_m$ for palmitoyl-CoA of 40 μM (Mills et al 1984). CPT-1 has a $K_m$ for carnitine of 500 μM in neonatal heart (Brown et al. 1995) that is sensitive to the inhibition by malonyl CoA ($K_i$=50nM) (Cook 1984). Transport of fatty acids into the mitochondria via CPT-1 involves a 1:1 interaction between carnitine and fatty acyl CoA. This enzyme shows a high specificity for the L-isomer of carnitine and the transport appears to be driven by carnitine concentration gradient and is independent of metabolic energy (Bieber 1988). It has been established that the intramitochondrial concentration of carnitine regulate the enzyme kinetics of CPT-1 (Cook 1984). Abdel-Aleem et al. (1998) reported that oxidation of palmitate can increase 3 fold in neonatal pig myocytes when co-treated with exogenous 5 mM L-carnitine. Considering those studies, the present results suggest that excess carnitine, be it L- or DL-, induce apoptosis which is inhibited by CPT-1 inhibition with oxfenicine (Figure 5A). To my knowledge, D-carnitine does not affect CPT-1. If sufficient carnitine is available, CPT-1 activity is enhanced thus
transporting the exogenous palmitate into the mitochondria for oxidation which enhances apoptosis.

Previous reports highlight the importance of CPT-1 activity in apoptosis. Mutomba et al. (2000) purported that, apoptosis, in Jurkat cells upon Fas ligation, was induced or inhibited based on the carnitine availability and modification: L-carnitine inhibited apoptosis whereas palmitoylcarnitine, the form by which palmitate is transported into the mitochondria via CPT-1, reversed L-carnitine's effects. As well, L-carnitine inhibited caspases-3, -7, and -8 whereas palmitoylcarnitine stimulated these caspases. Mutomba et al. (2000) reported that during the Fas-mediated apoptosis, levels of carnitine and palmitoyl CoA significantly decreased whereas palmitoylcarnitine formation inside the mitochondria increased suggesting an increase in CPT-1 activity. In addition, fibroblasts deficient in CPT-1 activity were resistant to staurosporine-induced apoptosis. The authors suggested that caspase activity may be regulated in part by the balance of carnitine and palmitoylcarnitine. Their hypothesis is consistent with the present study that with sufficient carnitine available to interact with palmitate, CPT-1 can convert palmitate to palmitoylcarnitine and induce apoptosis.

There are conflicting reports as to the effect elevated fatty acid concentrations have on CPT-1 activity. Cook et al. (1984) reported that fatty acyl-CoA did not alter CPT-1 kinetics whereas Spurway et al (1997) reported that exogenous palmitate concentrations increased CPT-1 activity. McMillin et al. postulated that palmitate decreases CPT-1 activity (Sparagna et al. 2000, Hickson-Bick et al. 2000). The results of this chapter, particularly Figures 5, 6, and 7, indirectly agree with the last two observations: excess palmitate, with no additional carnitine, induces apoptosis by a mechanism perhaps independent of CPT-1 activity. This conclusion was determined by the beneficial effect of oxfenicine during carnitine+palmitate co-treatment (Figure 4), but not achieved with palmitate alone (Figure 5B). As well, CPT-1 inhibition with glycolysis
stimulation (Figures 6 and 7) also did not block palmitate-induced apoptosis. It is possible that even though excess palmitate is available, CPT-1 activity is still limited by the basal levels of carnitine and, as a result, there is no increase in palmitate oxidation. Sparagna et al. (2000) recently observed a palmitate-induced decrease in CPT-1 activity which confirms my postulate of limited carnitine availability. These observations suggest that when additional carnitine is made available, palmitate induces its death action in a manner dependent on CPT-1.

These results agree with the theory presented by Hickson-Bick et al. (2000) and Sparagna et al. (2000). Both papers, originating from the McMillin group, present data for the first time that palmitate induces apoptosis by decreasing the ability of neonatal rat cardiomyocytes to metabolize fatty acids via CPT-1. As a result, there is an accumulation of palmitate and its partially oxidized forms that may be the cause of apoptosis. Sparagna et al. (2000) examined CPT-1 activity upon palmitate treatment and observed a decline at 16 h. The authors speculated that as palmitate induced a decrease in CPT-1 activity, there would be a decrease in the rate of removal of palmitoyl-CoA from the cell making it available for de novo synthesis of ceramide. The results presented in this chapter suggest that exogenous palmitate, given alone, induced apoptosis in a mechanism less dependent on CPT-1 as it was uninhibitable by oxfenicine and glycolysis stimulation. However, the presence of sufficient carnitine to help transport the exogenous palmitate, palmitate induces apoptosis in a CPT-1 dependent mechanism. Perhaps the palmitate-induced insensitivity to mitochondrial β-oxidation suggested by Hickson-Bick (2000) and Sparagna (2000) is due to the cardiomyocyte “running out” of carnitine to transport the excess palmitate via CPT-1.

It is noteworthy to comment on the level of death observed during the combination of carnitine and palmitate. Although the index of apoptosis produced by either carnitine (4.1±0.8) or palmitate (1.8±0.2) alone were significant, the degree of apoptosis achieved with
carnitine+palmitate is greater than the additive effect of carnitine and palmitate. These results suggest that if carnitine+palmitate’s death effects were solely due to the additive effects of each substrate, the maximal death achieved would be \(1.8 + 4.1 = 5.9\). However, as the observed death of the combination is 7.3±1.6 fold greater than control, this emphasizes the contribution of carnitine+palmitate or that another form of death independent of CPT-1 involved.

This thesis is also the first to describe the beneficial effects of CPT-1 inhibition by oxfenicine on the prevention of cardiac cell death, specifically apoptotic cell death. These results echo the beneficial effects of oxfenicine on the heart (Molaparest-Saless et al. 1987; Higgins et al. 1980; Burges et al. 1981; Korb et al. 1984; Vik-Mo et al. 1986). In the ischemic heart, other CPT-1 inhibitors such as etoxomir (Lopaschuk et al. 1990) and POCA (Yamada et al. 1994) demonstrated a beneficial role. It is noteworthy that oxfenicine does not produce complete inhibition of CPT-1 in the heart. In isolated heart mitochondria, the maximum CPT-1 inhibition produced by oxfenicine was 70-80% and less than the 100% achievable by 1 mM oxfenicine in mitochondria (Stephens et al. 1985). Bielefeld et al. (1985) also reported that oxfenicine, 2 mM, reduced fatty acid oxidation by 45% in cardiac muscle of adult rats. Given that the adult heart may have different CPT-1 sensitivity to inhibition with oxfenicine than in chick embryon, this may account for 10 mM oxfenicine to have an effect. This may explain why oxfenicine did not completely prevent the apoptosis induced by the combination of carnitine and palmitate.

There was no apoptosis observed with the short chain fatty acid capric acid (Fig.2). As capric acid is a shorter chain length fatty acid than palmitate and therefore does not require CPT-1, capric acid’s lack of toxic effect suggest that fatty acid oxidation alone is not responsible for fatty acid-induced apoptosis. The results with capric acid also suggest that palmitate’s death effects are specific and not due to a random fatty acid effect. These results agree with Abdel-Aleem et al (1998) who reported the lack of death effect with octanoate, a C8:0 fatty acid.
The present study also observed that oxidation of glucose+insulin or lactate did not affect palmitate-induced cell death. These results are consistent with the previous results with oxfenicine suggesting that inhibition of CPT-1, be it by oxfenicine or glycolysis, is not involved in palmitate-induced apoptotic cell death in cardiomyocytes. The combination of glucose and insulin have been previously reported to shunt energy production of the embryonic cardiomyocyte primarily to glycolysis (Ruderman et al. 1998; Depre et al. 1998) while similar results were also reported with the addition of lactic acid (Awan et al. 1993). Hence, I believe that the addition of glucose+insulin or lactic acid was ineffective in inhibiting CPT-1 as palmitate had already inhibited CPT-1. The results observed may be explained by the absence of a role for CPT-1 in palmitate-induced apoptosis, under some circumstances.

These experiments, and those of Hickson-Bick et al. (2000) and Sparagna et al. (2000), suggest a palmitate-induced decrease in CPT-1 activity. However, this hypothesis is in direct contradiction to the work done by Paumen et al. (1997). Paumen (1997) reported that palmitate induced apoptosis in cardiomyocytes via its incorporation into de novo ceramide synthesis. Paumen found that inhibition of CPT-1 was detrimental to the cell, suggesting that the amount of available palmitate was decreased by CPT-1 activity implying increased CPT-1 activity. In contrast to Paumen, I observed that CPT-1 inhibition with oxfenicine was beneficial to the cell during carnitine+palmitate-induced apoptosis. I believe the differences between Paumen’s studies and my own may relate to the cell type used. I used cardiomyocytes whereas Paumen used murine hematopoietic cell lines. Given that the heart is more reliant on fatty acids as an energy source than hematopoietic cells (fatty acids are preferentially used due to higher ATP production by fatty acid oxidation compared to glycolysis and the high energy demand in heart), it is reasonable to speculate that inhibition of the key enzyme CPT-1 would not cause cell death in cardiomyocytes as CPT-1 is integral to the heart’s energy production.
Inhibition of CPT-1 with oxfenicine or by carbohydrate metabolism had no effect on palmitate-induced apoptosis. Excluding the pre-mentioned hypothesis that carnitine availability is the key to palmitate-induced cell death via CPT-1 and Paumen et al's (1997) speculation on ceramide generation, there are few other theories for palmitate-induced apoptosis via CPT-1. Brandt et al (1998) speculated that control of CPT-1 activity occurs, at least in part, via malonyl-CoA independent mechanisms. Brandt et al (1998) demonstrated that expression of the M-CPT-1 gene, the isoform of CPT-1 found in heart, is regulated in cardiomyocytes at the transcriptional level and that this transcription is activated by long chain fatty acids via peroxisome proliferator-activated receptor α (PPARα). The forthcoming chapter will examine the role of PPARs in palmitate-induced apoptosis.

In summary, these data highlight the importance of CPT-1 as the initial step in the transport of palmitate into the mitochondria by CPT-1. If there is insufficient carnitine available to transport the excess palmitate into the mitochondria for oxidation, palmitate-induced apoptosis does not appear to be dependent on CPT-1. One explanation suggested by Hickson-Bick and Sparagna, palmitate may cause a reduction of CPT-1 activity thus preventing palmitate oxidation. As a result, palmitate and partially metabolized palmitate will accumulate inducing an apoptotic mechanism separate and upstream of CPT-1. However, if sufficient carnitine is available to transport the excessive palmitate into the mitochondria, significant apoptosis occurs as a result of CPT-1 activation and increased palmitate oxidation. This mechanism was shown to be inhibitable by the CPT-1 inhibitor oxfenicine. Taken together, these results suggest that palmitate induces death in at least two different mechanisms: one CPT-1 sensitive and the other CPT-1 independent.
SECTION E - FENOFIBRATE BLOCKS PALMITATE-INDUCED CARDIAC APOPTOSIS (Kong JY and Rabkin SW - Submitted to Journal of Lipid Research)

SUMMARY

The lipid lowering drug fenofibrate increases fatty acid oxidation through multiple mechanisms including binding to peroxisome proliferator activated receptors (PPARs), peroxisome proliferation and enhancement of peroxisomal β-oxidation. Because of these properties, I sought to determine whether fenofibrate might alter palmitate-induced cell death. Cardiomyocytes from neonatal mice and embryonic chicks were treated with palmitate and both oncotic and apoptotic death were observed. Fenofibrate pretreatment, 1 μM, 24 h prior to palmitate, significantly (p<0.05) reduced palmitate-induced apoptosis by 27.8±8.2%. In contrast, fenofibrate had no effect on palmitate-induced apoptosis when fenofibrate treatment was concomitant with palmitate. The protective effect of fenofibrate was restricted to the apoptotic population. The more potent and specific PPARα agonist WY 14643, 1 μM, also reduced palmitate-induced apoptosis but to a smaller extent than fenofibrate. The long pretreatment time, 24 h, was necessary to show fenofibrate's effect on apoptosis, suggesting an increase in gene transcription and protein expression. Indeed, fenofibrate increased PPARα expression that was mainly demonstrated in the nucleus. These data suggest a novel approach to the reduction of palmitate-induced cardiac apoptosis by the chronic treatment with fenofibrate that acts partially through a PPARα mediated mechanism.

INTRODUCTION

A potential strategy to modify palmitate-induced cell death is an intervention that would modify palmitate metabolism. Fatty acid oxidation occurs predominantly in the mitochondria,
but oxidation also occurs in peroxisomes (Lopaschuk et al. 1994). Peroxisomes are membrane-bound organelles that contain enzymes responsible for fatty acid oxidation, the biosynthesis of cholesterol, and other biochemical pathways (Mannaerts & van Veldhoven 1993). Estimates of fatty acid β-oxidation in peroxisomes of the heart vary from 4% (Chu et al. 1994) to 39% (Verrkamp & Moerkerk 1986) but the actual value is closer to 22 to 27% as noted in hearts of different species (Piot et al. 1998). In hepatocytes, peroxisomes preferentially oxidize medium chain fatty acids (C10:0 to C14:0) whereas mitochondria are very active in metabolism of C6:0 to C18:0 acids, including palmitate (Skorin et al. 1992). Palmitate's metabolism within the mitochondria resulted in significant changes within this organelle culminating in apoptotic cell death (Section C or Kong & Rabkin 2000b). Hence, I theorized that an agent that purportedly changes the location of palmitate oxidation may reduce the amount of apoptosis.

Fibric acid drugs such as clofibrate or fenofibrate are peroxisome proliferators which increase the amount of peroxisomal fatty acid oxidation. Cardiac peroxisomal β-oxidation capacity was increased 2.5-fold by clofibrate (Norseth & Thomassen 1983) and the activity of β-oxidation enzymes such as acyl CoA oxidase is increased up to 30 fold by fibrates (Orton & Parker 1982). In hepatocytes, ciprofibrate and bezafibrate increased peroxisomal oxidation, particularly affecting saturated fatty acids with 8 or more carbon atoms (Skorin et al. 1992). As well, this peroxisomal increase was sensitive to a peroxisomal inhibitors (Skorin et al. 1992).

The recognition that certain agents, such as fenofibrate, stimulated the proliferation of peroxisomes, led to the discovery of the peroxisomal proliferator-activated receptors (PPAR) and their identification as members of the intracellular nuclear receptor super family (Isseman & Green 1990). Three PPAR subtypes, PPARα, PPARβ or δ, and PPARγ, have distinct tissue distribution (Amri et al. 1995). PPARα is expressed in tissues actively metabolizing fatty acids such as the heart (Beck et al. 1992). Also, peroxisome proliferators act as ligands for PPARs
whose activation enhances the transcription of enzymes that increase mitochondrial and peroxisomal fatty acid metabolism (Veerkamp & van Moerkerk 1986). PPARs play a key role in the transcription of the pivotal enzymes that regulate fatty acid metabolism (Schoonjans et al. 1996) such as acyl-CoA synthetase (Schoonjans et al. 1996), acyl-CoA oxidase (Keller et al. 1993), and medium chain acyl-CoA dehydrogenase (16). In the heart, PPARα regulates the expression of several key enzymes involved in cardiac fatty acid oxidation (eg. acyl-CoA dehydrogenases) and in peroxisomal β-oxidation (eg. acyl-CoA oxidase) (Martin et al. 1997). As well, putative fatty acid transporter genes are regulated by PPARα and γ (Motojima et al. 1998).

A role for PPAR has been proposed for various disease states including atherosclerosis, diabetes mellitus, obesity, and cancer (Vamecq & Latruffe 1999). Because of the characteristics of fibric acid drugs, such as fenofibrate, that increase the amount of fatty acid oxidation in peroxisomes, the objective of this section was to test the hypothesis that fenofibrate modulates cardiomyocyte cell death induced by palmitate.

RESULTS

Fenofibrate blocked palmitate-induced DNA changes of apoptosis

I sought to determine whether fenofibrate blocked palmitate-induced apoptosis by analyzing DNA content by flow cytometry. Chick cardiomyocytes were used for flow cytometry rather than neonatal cardiomyocytes as the latter require more trypsin to dissociate them and are thus more vulnerable to cellular damage. Palmitate treatment increased the size of the cell population with low PI fluorescence (low DNA content) characteristic of apoptosis (Figure 1A inset). Fenofibrate treatment, commencing 24 h before palmitate, produced a significant (p<0.05) reduction in the number of cardiomyocytes with apoptotic nuclei from 1687±168 (N=13) per 10,000 cells with palmitate to 1255±141 per 10,000 cells (N=13) with fenofibrate.
Figure E1 - The effect of fenofibrate on palmitate-induced DNA signs of apoptosis - After 72 hours in culture, chick cardiomyocytes were treated with diluent (control) (N=13), palmitate 100 μM (N=13), fenofibrate (Feno) 1 μM (N=13), or the combination (N=13). Cells were then permeabilized and nuclei stained with PI. The data is presented as mean±SEM.

Panel A - Fenofibrate was added for 24h before palmitate, followed by an additional 24 h. The number of cells exhibiting low DNA content, based on PI fluorescence, was analyzed by flow cytometry. Data analysis notes a significant increase in population with low DNA content compared to control (*** p<0.01) due to palmitate that is significantly (Δ p<0.05) blocked by fenofibrate pretreatment. Left Inset - Sample histogram from one of 13 separate experiments. Right Inset - Data presented as population of low DNA content relative to control. There is a significant (ΔΔ p<0.01) decrease in cells exhibiting low DNA content due to fenofibrate pretreatment compared to palmitate.

Panel B - Fenofibrate was added concomitantly with palmitate for 24 h. The number of cells exhibiting low DNA content, based on PI fluorescence, was analyzed by flow cytometry. Data analysis notes a significant increase in population with low DNA content compared to control (* p<0.05). Inset - Data presented as population of low DNA content relative to control.
B

Relative number of cells with low DNA content per 10,000 cells

Number of cells with low DNA content/10,000 cells

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Feno</th>
<th>Palmitate</th>
<th>Feno+Palm</th>
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plus palmitate, or a 27.8±8.2% reduction. Fenofibrate did not induce apoptosis in these cardiomyocytes. Concomitant treatment of cardiomyocytes with fenofibrate and palmitate, i.e. both for 24 h, however, did not alter apoptosis (Figure 1B).

_Fenofibrate blunted palmitate-induced apoptosis but not oncosis_

Next, I sought to determine whether the protective effect of fenofibrate extended to palmitate induced apoptosis or oncosis. Embryonic chick cardiomyocytes were treated with palmitate (100 μM for 24 h), with or without fenofibrate 1 μM pretreatment for 24 h and examined by flow cytometry using the dual staining abilities of the fluorochromes FDA and PI (Figure 2A). Fenofibrate pretreatment for 24 h reduced the size of the apoptotic population while the oncotic population was unchanged. Fenofibrate blunted the number of cells exhibiting apoptosis induced by palmitate to 461±72 cells from 567±101 per 10 000 (N=13) with palmitate alone; a difference of 19% less than palmitate (Figure 2B). Fenofibrate had no effect on reducing the number of cells exhibiting oncosis induced by palmitate (Figure 2C).

**Effect of the PPAR agonist WY1463 on palmitate-induced apoptosis**

To determine whether fenofibrate's beneficial effects were due to its effect on PPARα, the more potent PPARα agonist WY 14643 (Isseman _et al._ 1993) was next examined. WY 14643, 1 μM, produced a significant (p<0.05) 15.4±7.6% reduction in apoptosis (Figure 3). In contrast the same concentration of fenofibrate, 1 μM, produced a 27.8± 8.2% reduction in palmitate induced apoptosis.

_Fenofibrate induces an increase in PPARα in the nucleus_

As fenofibrate required 24 h pretreatment to reduce palmitate-induced apoptosis, I hypothesized that fenofibrate was likely inducing an increase in protein expression. As an example target, I chose to examine PPARα itself. As the antibodies against PPARα recognize the mouse sequence and not the chick, I performed the following PPARα antibody-related
Figure E2 - *Fenofibrate pretreatment for 24h prior to palmitate blunts the development of apoptosis but not oncosis induced by palmitate* - After 72 hours in culture, chick cardiomyocytes were treated with diluent (control) (N=13), palmitate 100 μM (N=13), fenofibrate (Feno) 1 μM (N=13), or the combination (N=13), fenofibrate having been added for 24 h before palmitate, followed by an additional 24 h incubation. Cells were then dually stained with FDA and PI and examined by flow cytometry. **Panel A** - A representative dotplot is shown from one of 13 independent experiments. **Panel B** - Palmitate induces a significant (**p<0.01**) amount of apoptosis that is blunted by fenofibrate pretreatment for 24 h. **Panel C** - Palmitate induces a significant (**p<0.01**) amount of oncosis whereas fenofibrate pretreatment had no effect on palmitate-induced oncosis. **Insets** - Relative number of cells exhibiting apoptosis/oncosis compared to control.
Number of cells exhibiting oncasis per 10,000 cells

Control

Feno

Palm

Feno+Pal

7500
5000
2500
0
Figure E3 - *WY 14643 reduces palmitate-induced apoptosis*. Chick cardiomyocytes were treated with diluent (Control), palmitate 100 μM, WY 14643 1 μM, or the combination, WY 14643 having been added for 24 h before palmitate, followed by an additional 24 h incubation (N=6 for all). Cells were then analyzed by flow cytometry for either for PI-stained DNA content. With palmitate-induced low DNA content is expressed as a maximal 100% effect, WY 14643 pretreatment significantly reduced palmitate-induced loss of DNA content. Inset - The number of cells with low DNA content. Δ p<0.05 compared to palmitate; ** p<0.01 compared to control.
experiments on neonatal mouse cardiomyocytes. Mouse cardiomyocytes were grown on coverslips, treated with fenofibrate for 48 h, and were stained with an antibody against PPARα and counterstained with propidium iodide to help visualize the nucleus (Figure 4). As the cardiomyocytes are fixed and permeabilized, both the PPARα antibody and propidium iodide can readily enter inside the cell. Propidium iodide binds to nuclear DNA so that propidium iodide fluorescence, seen as red, is an indicator of the nucleus. No PPARα was readily seen in control with the clearly visible nucleus evident. There was a marked increase in PPARα staining after fenofibrate treatment, which was, localized around the nucleus. The microscopic settings enhanced green fluorescence, which is dominant, so that the red of the nucleus was less prominent than in control.

To further explore this effect of fenofibrate, cardiomyocytes were treated with palmitate, 100 μM for 24 h, with or without pretreatment with either fenofibrate or WY 14643, 1 µM, for 48 h. The nuclear and cytosolic fractions were examined for PPARα by Western blotting. There was a slight increase in PPARα expression in the nuclear fraction in response to fenofibrate but a marked increase in cells that were exposed to the combination of palmitate plus fenofibrate (Fig. 5A&B). The findings were similar with WY 14643 that similarly increased PPAR expression in the nuclear fraction. Densitometric analysis of PPARα confirm the increased presence in response to fenofibrate and WY 14643 (Fig. 5C)

To determine whether increased PPARα upon fenofibrate treatment was due to translocation or synthesis, the nuclear cycle of cardiomyocytes treated with fenofibrate 1 µM for 48 h were analyzed. Using the same procedure for observing apoptotic low DNA content, I determined the G0/G1, S, and G2/M populations of cells treated with fenofibrate (Figure 6). By 48 h, fenofibrate had induced a significant (p<0.01) decrease in DNA synthesis with a
Figure E4 - Effect of fenofibrate on PPARα expression in neonatal mouse cardiomyocytes. Cardiomyocytes from neonatal mice hearts were maintained in culture for 72h then treated with fenofibrate (Panel B) or diluent (control) (Panel A) for 48 h. Cells were permeabilized and stained with monoclonal antibody to PPARα and made visible by the secondary antibody linked to FITC. Cells were also stained with the same PI mix used in flow cytometric analysis of low DNA content to help visualization of the nuclei which is seen in red. This PI staining method is specific for DNA of nucleus.
Figure E5 - Western blotting of cell lysates for PPARα- Neonatal mouse cardiomyocytes were treated as described below, lysed, and subjected to differential centrifugation to separate nuclear and cytosolic fractions. Equivalent amounts of protein were loaded onto a 10% acrylamide gel and transferred to nitrocellulose for immunoblotting. Cells were probed with a polyclonal antibody specific for PPARα and signal detected using a horseradish peroxidase-linked secondary antibody.

Panel A - Cardiomyocytes were treated with diluent (C), palmitate (P) 100 μM for 24h, fenofibrate (F) 1 μM, or the combination (F+P) with 24 h pretreatment before palmitate.

Panel B - Cardiomyocytes were treated with diluent (C), palmitate (P) 100 μM for 24h, WY 14643 (W) 1 μM, or the combination (W+P) with 24 h pretreatment before palmitate.

Panel C - Densitometric analysis of PPAR bands in Panels A & B. Integrated density values (number of pixels/area) is expressed relative to control.
Figure E6 - *Effect of fenofibrate on cardiomyocyte cell cycle*. Cardiomyocytes from embryonic chick were grown for 72h before treatment with fenofibrate 1μM for 48h. Cells were prepared for flow cytometric analysis of PI-stained DNA content. Quiescent cells (G0/G1), cells undergoing DNA synthesis (S), or mitosis (G2/M) were quantitated and compared to control cells to produce the relative DNA content. **p<0.01 compared to control.
significant (p<0.01) increase in mitosis suggesting that by 48 h, cells are well into dividing. This suggests a correlation with increased PPARα expression.

DISCUSSION

Although fatty acids are a principal source of energy for the heart and the metabolism of fatty acids is of fundamental importance for cardiac function (Lopaschuk et al. 1994), considerable evidence has established that high concentrations of fatty acids and specifically palmitate produces cell death (Section C; Kong & Rabkin 2000b; Rabkin et al. 1999, Paumen et al. 1997; Shimabukuro et al 1998; De Vries et al. 1997). The molecular mechanisms underlying palmitate-induced cell death and its prevention, however, have remained relatively unexplored. I provide the first evidence that fenofibrate blunts palmitate-induced cell death in cardiomyocytes.

Fenofibrate selectively inhibits apoptosis and not oncosis

Apoptotic cell death was found in cardiomyocytes after palmitate treatment. The DNA of cardiomyocyte nuclei evaluated by flow cytometry demonstrated palmitate-induced alterations of nuclear structure that were characteristic of apoptosis (Frey 1997). In addition, evaluation of membrane permeability by dual staining with FDA and PI (Frey 1997) also established that palmitate treatment produced apoptosis. The dual staining approach with FDA and PI evaluates membrane integrity and esterase activity of intact cardiomyocytes and demonstrated that palmitate also damaged cellular membrane integrity in a manner consistent with oncotic cell death. Cell death is currently divided into apoptosis or oncosis, with necrosis being the term applied to the end stage of cell death from either apoptosis or oncosis (Levin et al. 1999). Although the FDA/PI technique, like all methodologies to differentiate apoptotic from oncotic cell death, does not have complete accuracy (ie- distinct separate apoptotic and oncotic populations), the absence of an effect of fenofibrate on oncotic cell death provides support for the
method that classifies cell death into two different kinds. The findings with dual staining are consistent with the ability of fenofibrate to prevent palmitate-induced apoptosis ascertained by alterations of DNA content that were characteristic of apoptosis. These data indicate a selectivity of fenofibrate for prevention of palmitate-induced apoptosis and highlights that apoptosis and oncosis in cardiomyocytes are subject to different kinds of modification.

Comparisons of the effects of fenofibrate and palmitate on fatty acid metabolism

Palmitate appears to decreases the ability of cardiomyocytes to metabolize fatty acids in the mitochondria (Sparagna et al. 2000). The accumulation of palmitate and its partially oxidized forms may be responsible, in part, for palmitate-induced apoptosis. In contrast to palmitate, fenofibrate enhanced the oxidative activity of long chain fatty acids in liver of lean Zuker rat (Clouet et al. 1990) and increases peroxisomal fatty acid oxidation in rat liver parenchymal cells from rat (Steinberg et al. 1988). Peroxisomal proliferation is coincident with increases in many peroxisomal enzymes involved in fatty acid oxidation (Reddy and Chu 1996). Thus fibrate treatment may prime cells for more efficient β-oxidation. Such an increase in oxidation enzymes results in greater fatty acid oxidation and clearing of palmitate, preventing palmitate-induced apoptosis from the accumulation of partially oxidized forms. Although fibric acid drugs increase the expression of fatty acid transport proteins (FATP) and acyl-CoA synthase (ACS), these are unlikely mechanisms for fenofibrate induced cardioprotection. Fenofibrate-induced FATP and ACS induction occurs mainly in liver and intestine but not in the heart (Prasad et al. 1988); perhaps because of the already maximal activation of cardiac FATP and ACS promoters in view of the crucial dependence of the heart on fatty acid uptake (Prasad et al. 1988). The effect of fenofibrate may not, however, be only ascribed to increased activity of enzymes involved in fatty acid oxidation as fenofibrate also induces an increase in mitochondrial activity of various
enzymes in liver (Orton & Parker 1982; Clouet et al. 1990) as well as non β-oxidation related enzymes such as catalase (Steinberg et al. 1988).

There has been a previous report of CPT-1 in peroxisomes. Fraser et al. (1999) reported that CPT-1 is expressed in liver peroxisomes and microsomes. Peroxisomal CPT-1 are expressed in the cytosolic side of the membrane and are inhibitable by malonyl CoA (Fraser et al. 1999). Peroxisomal CPT-1 activity contributes about 10% of total cellular CPT activity in liver (Fraser et al. 1999). To my knowledge, there is no data ascertaining whether CPT-1 is present in cardiomyocyte peroxisomes.

Keeping Fraser et al.'s work in mind, it is possible an increase in peroxisomal CPT-1 is induced by peroxisome proliferation with fenofibrate or WY 14643. This is unlikely because peroxisome proliferators should be acting like carnitine addition to increase CPT-1 activity. Fenofibrate and WY 14643 had exactly opposite effects than carnitine - i.e. they prevent apoptosis while carnitine increases apoptosis. Thus, I speculate that CPT-1's role in apoptosis is most likely dependent of mitochondrial CPT-1 activity and that peroxisomal CPT-1 is either not involved or plays a minor role in apoptosis.

Fenofibrate’s mechanism likely involves transcriptional activity

The mechanism of action fenofibrate on palmitate-induced cell death is likely mediated through gene expression rather than a direct action on cardiac mitochondria or enzymes. This suggestion is supported by this data that the effect of fenofibrate requires more than 24 h to become manifested. I previously demonstrated the role of mitochondria in the pathogenesis of palmitate-induced cell death in cardiomyocytes (Section C; Kong & Rabkin 2000b). Alteration of mitochondrial function was apparent within 24 h of palmitate treatment and these adverse effects of palmitate were mitigated within the same time period. The absence of an effect of fenofibrate within the initial 24 h of its treatment argues against the possibility that the effects of
fenofibrate are due to a direct action of fenofibrate on mitochondria that has been previously demonstrated (Zhou & Wallace 1999). It also suggests that other direct actions of fenofibrate on palmitate metabolic pathways are not involved. Instead, the lag time of 24 h or more before fenofibrate protected cardiomyocytes from palmitate-induced apoptosis are consonant with the time frame needed to induce gene expression (Schoonjans et al. 1996). The specific genes operative in the action of fenofibrate remain to be defined.

Role of PPARα stimulation in apoptosis

This section provides data to implicate a role for PPARα in blunting palmitate-induced cardiac apoptosis based on the findings with fenofibrate and the more potent PPARα agonist WY 14643. There is limited previous data that examined a role for PPARα in apoptosis and it has been controversial. The peroxisome proliferator nafenopin inhibited apoptosis in mouse hepatocytes through a mechanism dependent on PPARα (Christensen et al. 1998) consistent with the concept of a protective role for PPARα in hepatic carcinogenesis (James et al. 1998). Inhibition of apoptosis by peroxisome proliferators has been ascribed to PPARα and this beneficial effect could be eliminated by the introduction of a dominant negative effector regulator of PPARα (Roberts et al. 1998). In contrast, activation of PPARα produced apoptosis in macrophages activated with TNFα/IFNγ-l, but not in inactivated differentiated macrophages (Djouadi et al. 1999). Little is known, unfortunately, about the role of PPARα in the cardiac disease.

There have been previous reports of the role of PPARα in the heart using PPARα-deficient mice. PPARα-null mice lack the morphologic and biochemical responses to peroxisome proliferators commonly observed in wild-type mice (Chinetti et al. 1998). As a result of this deficiency, PPAR-null mice have higher levels of serum cholesterol than wild type mice (Peters et al. 1998) and cardiac lipid accumulation (Djouadi et al. 1999). These mice may aid in understanding the role of PPARα in palmitate-induced cell death.
To what extent is the effect of fenofibrate mediated solely through PPARα

While fenofibrate and WY 14643 both protect against palmitate-induced apoptosis it was clear that the more potent PPARα agonist, WY 14643 was less effective than fenofibrate at the same concentration. These data suggest two main possibilities. First, fenofibrate acts through a PPAR-independent mechanism not activated by WY 14643 or second, there are different effects on PPARα induction by these two agonists. Both fenofibrate and WY 14643 are established peroxisome proliferators but differ in chemical structure, and hence, binding locations to PPARs. Fenofibrate, a fibric acid, is the isopropyl ester of 2-[4-(4-chlorobenzoyl)-phenoxy]-2-methyl propanoic acid whereas WY 14643 is 4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid.

Hence, the differing effects of fenofibrate and WY 14643 on palmitate-induced apoptosis may be due to their differing binding characteristics to PPARα. In support of this hypothesis, it is worth noting that there are species-specific differences in response to WY 14643 that are dependent on differences in amino acid residues in the ligand binding domain (Johnson et al. 1997).

Fenofibrate and WY 14643 may differ after interaction with PPARα. Upon stimulation, PPARs form heterodimers with another nuclear retinoid receptor, RXR. This heterodimer then binds to peroxisome proliferator response elements (PPRE) on DNA hence inducing transcription of the gene to which the heterodimers have bound (Marcus et al. 1995). Even after a common PPAR stimulus, there can still be differences in PPAR/RXR binding affinities to PPREs and location of PPRE binding, hence different transcription products. PPARs bind to PPREs with different affinities, depending on the sequence of the PPRE and the PPAR receptor subtype (Juge-Aubry 1997). PPRE-dependent transcriptional activation by PPARα correlated with binding efficiencies of the nuclear receptor heterodimer PPARα/RXRα to the PPRE. Studies reporting differences in peroxisome proliferators have been ascribed to differences in the amino acid residues in the PPAR binding domain (Johnson et al. 1997; James & Roberts 1996). Differences in PPRE...
transcription have been noted between fenofibrate and WY 14643. WY 14643 increased the transcription of plasminogen activator inhibitor (PAI-1) whereas fenofibrate markedly decreased this transcription (Nilsson et al. 1999). Similarly fenofibrate decreased apolipoprotein A-II mRNA whereas WY 14643 increased this mRNA (Berthou et al. 1995). Thus fenofibrate and WY 14643 activate PPARα but there may be differences in activation of PPRE transcription relevant to protection against palmitate-induced apoptosis.

*Increased PPAR expression with fenofibrate and WY 14643*

As both fenofibrate and WY 14643 are known peroxisome proliferators and PPARα agonists, I hypothesized that fenofibrate may increase PPARα expression as well. There was a marked increase in PPARα expression in the nuclear fraction in response to both fenofibrate and WY 14643. This result is consistent with the findings in liver cells after chronic fenofibrate treatment where there is a prominent increase in PPAR protein in both nucleus and cytosol (Gebel et al. 1992). The occurrence in the same time frame as fenofibrate’s and WY 14643’s anti-apoptotic effects suggesting a link between PPAR expression and suppression of apoptosis. However, fatty acids are also PPARα ligands (Keller et al. 1993). In competition binding studies, saturated fatty acids containing between 14C and 16C in length (palmitate is a 16C fatty acid) bind to PPARα predominantly, δ to a lesser extent, and not at all with γ (Xu et al. 1999). This is consistent with the proposal that fatty acids induce PPARα to upregulate enzymes essential for their metabolism. Concomitant treatment with both palmitate plus fenofibrate or WY 14643 produced the greater PPARα expression. The contribution of palmitate to PPAR expression may constitutes a response that is too late to impact PPAR-mediated upregulation of β-oxidation enzymes to abrogate palmitate-induced death.
CONCLUSION

This section demonstrates that the lipid lowering drug fenofibrate can play an important role in cardiac apoptosis by producing a modest but significant 28% reduction in palmitate-induced cardiac apoptosis. These effects are mediated in part through PPARα stimulation and perhaps through a PPAR independent action as well. The prolonged treatment time before palmitate suggests that fenofibrate acts through gene expression. Indeed PPARα protein expression was enhanced by fenofibrate. Considering the difficulty modifying apoptotic cell death, these data suggests a potential new strategy to reduce apoptosis.
SECTION F - THE ROLE OF PALMITATE AND CERAMIDE IN CARDIOMYOCYTE APOPTOSIS - A COMPARISON OF MITOCHONDRIAL EFFECTS (Kong JY and Rabkin SW – Submitted to Journal of Biological Chemistry)

SUMMARY

Palmitate is a precursor for de novo synthesis of ceramide, a known apoptotic inducer. Fumonisin B1, an inhibitor of ceramide synthase, did not alter palmitate-induced changes in apoptosis. This suggests that palmitate, by 48 h, does not induce its death action by its incorporation into ceramide. To determine whether palmitate induces apoptosis through ceramide at times greater than 48 h, cardiomyocytes were treated with the cell permeable ceramide analogue C2-ceramide and the apoptotic effects found with palmitate were compared with those induced by ceramide. Like palmitate, ceramide induced a significant loss of cell viability with attendant changes in DNA content. Effects on mitochondria were first validated by the mitochondrial poisons rotenone and antimycin A. Mitochondrial changes observed were a loss of mitochondrial potential $\Delta \Psi_m$ as assessed by DePsipher™, mitochondrial respiration indirectly assessed by CMX-Ros, and mitochondrial permeability assessed by cyt C release. Ceramide induced unique changes in mitochondria - a loss of $\Delta \Psi_m$ in combination with an increase in mitochondrial respiration. These mitochondrial changes were distinct from the changes produced by palmitate which reduced CMX-Ros fluorescence, or mitochondrial respiration. The ability of ceramide to induce apoptotic cell death was not inhibited by pretreatment with cyclosporin A, contrasting the prevention of palmitate-induced cell death by cyclosporin A. Taken together, these data suggest that palmitate-induced cell death is not primarily mediated by de novo ceramide synthesis. Rather, ceramide induces mitochondrial changes and perhaps a mechanism of cell death that are distinct from palmitate.
INTRODUCTION

Another possible mechanism for palmitate-induced cell death is the hypothesis postulated by Paumen that palmitate induces cell death by its incorporation into ceramide, a known inducer of apoptosis (Paumen 1997). Intracellular ceramide is generated by a number of pathways but the two principle ways are via *de novo* synthesis or sphingomyelin degradation following activation of sphingomyelinases (Merrill & Jones 1990; Mathias *et al.* 1998). Ceramide induces cell death in various cell types (Mathias *et al.* 1998) including the myocyte element of the heart (Andrieu-Abadie *et al.* 1999; Bielawska *et al.* 1997). However the mechanism(s) of ceramide-induced cell death remain incompletely understood.

Alterations in mitochondrial function are seen in the early stages of apoptosis induced by a wide variety of stimuli (for review, see Lemasters *et al.* 1998a&b). Mitochondria may fail at oxidative phosphorylation by decreased enzyme activity, release cyt c, generate reactive oxygen species, and/or undergo permeability pore transition; each of which are observed during apoptosis (Rosser & Gores 1995; De Maria *et al.* 1997). Loss of mitochondrial respiration and increased mitochondrial permeability culminating in the release of cyt C have been demonstrated with palmitate and already discussed in Section C. This method has been established by previous groups concluding changes in mitochondrial permeability by cyt c location (Liu *et al.* 1996; Tepper *et al.* 1999; Ghafourifar *et al.* 1999; Li *et al.* 1998; Halestrap *et al.* 2000). Increased mitochondrial permeability can occur by nonspecific injury to the membrane lipids or by a specific mechanism causing the opening of the mitochondrial transition pore (MTP).

The objective of this section is to determine the impact of ceramide on mitochondria during apoptotic death and to compare the mitochondrial effects of ceramide to those of palmitate. If palmitate is inducing its death effects via its incorporation into ceramide, then I hypothesize that palmitate- and ceramide-induced mitochondrial and death effects will be
similar. While there has been some support for a role for ceramide with regards to signaling to or within the mitochondria (Susin et al. 1997), other data are ambiguous as to a role for mitochondria in ceramide-induced apoptosis (Tepper et al. 1999). Tepper et al. (1999) reported that in Jurkat T cells, ceramide formation depends on initiator caspase activity and cyt C release but is independent of effector caspase activation, thus excluding a role for ceramide in conveying the apoptotic signal to the mitochondria. The potential for involvement of mitochondria in ceramide-induced apoptosis has been proposed (Arora et al. 1997). Ceramide induces cell death, which occurs in part, through the production of reactive oxygen species H$_2$O$_2$ at the respiratory chain implicate the mitochondria in ceramide-induced cell damage (Quillet-Mary et al. 1997; Garcia-Ruiz et al. 1997). Ceramide-induced cell death in isolated hepatocytes was associated with adenosine triphosphate (ATP) depletion and mitochondrial depolarization suggesting that ceramide caused mitochondrial dysfunction (Arora et al. 1997). Also, ceramide altered the permeability transient of mitochondria isolated from rat liver but only when ceramide was combined with Bax, the pro-apoptotic member of the Bcl-2 family (Pastorino et al. 1999). In contrast, in U937 human leukemia cell line, ceramide-induced apoptosis occurred in a time frame before any ceramide-induced changes in mitochondrial membrane potential (Quillet-Mary et al. 1997). Furthermore, two separate groups reported that ceramide did not alter mitochondria permeability of isolated mitochondria from rat liver (Garcia-Ruiz et al. 1997; Pastorino et al. 1996).

As ceramide has been suggested to affect mitochondria, it is necessary to review the importance of the mitochondria to the cell, specifically with regards to energy production. The respiratory chain is the final step in fatty acid/carbohydrate metabolism in which the bulk of ATP is generated (for a review of the respiratory chain, see Nelson and Cox 2000). Reduced electron carriers such as NADH and FADH$_2$, generated upstream of the respiratory chain in metabolic
pathways such as fatty acid oxidation, are re-oxidized with production of ATP. There is a transfer of electrons from reduced carriers in a step-wise manner to several protein electron carriers leading eventually to molecular oxygen to generate water. This step-wise reoxidation involves several protein electron carriers, firmly embedded in the inner membrane of mitochondria, in the sequence of NADH dehydrogenase (Complex I), Coenzyme Q10, succinate dehydrogenase (Complex II), cyt B and C1 (Complex III), cyt C, cyt C oxidase and cyt A and A3 (Complex IV), and ATP synthase (Complex V) to the final oxygen molecule. The overall chain sequence is quite exergonic: one pair of reducing equivalents generated from NADH suffices the drive to the coupled synthesis of 3 moles of ATP by oxidative phosphorylation.

Changes to mitochondrial function, such as an increase in mitochondrial permeability, would eventually result in an impaired respiratory chain and hence, a loss of energy production. As discussed in the Introduction chapter, an increase in mitochondrial permeability would result in the loss of the electrochemical gradient and $\Delta \Psi_m$ necessary for the respiratory chain to function. This gradient is essential for the electron shuttling resulting in ATP generation. As a result, ATP is not generated which will invariably affect the activity of intramitochondrial enzymes. Ceramide-induced changes to mitochondrial permeability, potential, and intramitochondrial enzymes will be investigated in this thesis.

The flow cytometric methods intended to observe mitochondrial changes need to be validated in this cell model. $\Delta \Psi_m$ will be investigated using the mitochondria-specific potentiometric fluorescent dye DePsipher™ whereas the “negative sink” of mitochondria, generated upon active mitochondrial respiration, will be investigated using the mitochondrial-specific dye chloromethyl-X-Rosamine (CMX-Ros). The lipophilic, mitochondrial-specific dye DePsipher™ was used in this thesis to assess $\Delta \Psi_m$. DePsipher™ readily enters the intramitochondrial space where it forms red aggregates, or “J-aggregates”, in the presence of
\( \Delta \Psi_m \), or remains as green monomers in the absence of a potential. DePsipher\textsuperscript{TM}, being a J-aggregate dye, is associated with a large shift of its absorption and fluorescence maximum to a longer wavelength; hence J-aggregates exhibit intense "resonance fluorescence" (Smiley et al. 1991). DePsipher\textsuperscript{TM} has an absorption maximum of 510 nm and a fluorescence maximum of 520 nm for monomers and an absorption and emission maximum of 585 nm for the J-aggregates (Smiley et al. 1991). It has relatively low toxicity, reasonable solubility, and a appropriate fluorescence characteristics convenient for detection by filter systems commonly used by flow cytometry and fluorescent microscopy (Smiley et al. 1991). Uptake of cationic DePsipher\textsuperscript{TM} by mitochondria is related to the presence of a negative sink inside mitochondria created by proton pumps. (Smiley et al. 1991). Examination of the amount of red and green fluorescence, either by fluorescent microscopy or flow cytometry, will determine the overall state of \( \Delta \Psi_m \) in a population of cells. As the mitochondria of a significant portion of the population of cardiomyocytes lose its potential, the population will exhibit an increase in green monomeric DePsipher\textsuperscript{TM} and decrease in red aggregate DePsipher\textsuperscript{TM}. However, there will always be some mitochondria with a loss of potential (green monomers) even though the cell is healthy (predominantly red aggregates) (Jiang et al. 1999). DePsipher\textsuperscript{TM}, or very similar structural analogues such as JC-1, have been used with good results by previous groups to observe changes in \( \Delta \Psi_m \) (Jiang S et al. 1999; Bowser et al. 1998; Zamzani et al. 1995).

Mitochondrial enzyme activity will be measured with the mitochondrial selective dye CMX-Ros. Within the mitochondria, CMX-Ros becomes converted and binds to proteins within the mitochondria thus preventing its loss from mitochondria (Poot et al 1996). Hence, an increase in CMX-Ros fluorescence denotes an increase in the mitochondrial "negative sink" and hence, an indirect measure of respiration. CMX-Ros fluorescence can occur during a loss of mitochondrial membrane potential as it is not dependent on mitochondrial electrochemical
gradient (Gilmore & Wilson 1999). Thus, CMX-Ros is a useful tool to indirectly assess mitochondrial respiration (Jiang S et al 1999).

To validate a specific mitochondrial response with these two dyes, the mitochondrial poisons antimycin A and rotenone will be used to elicit a positive mitochondrial-related death response. Antimycin A inhibits both succinate oxidase and NADH oxidase (Complex II), hence blocking electron flow from cyt b to c1 in the respiratory chain (Wolvetang EJ et al 1994). Similarly, rotenone inhibits electron flow from NADH to CoQ (Complex I) in a reversible and competitive manner upstream of antimycin’s location (Higgins and Greenamyre 1996). Hence, these two poisons inhibit the respiratory chain at two different sites making them ideal positive controls for flow cytometric observation of mitochondrial changes through DePsipher™and CMX-Ros staining.

The objective of this section was to first determine whether palmitate induces cell death by its incorporation into ceramide and to determine whether the mitochondrial effects previously observed with palmitate are due to ceramide.

RESULTS

Fumonisin does not block palmitate-induced apoptotic cell death

To determine whether palmitate-induced apoptotic cell death might be mediated through an increased synthesis of ceramide, cardiomyocytes were treated with palmitate plus the ceramide synthetase inhibitor fumonisin B1 (Merrill et al. 1996), beginning 30 min. before and continuing with palmitate. The concentration of fumonisin B1, 10 μM, was previously established by this lab to inhibit ceramide synthesis in embryonic chick cardiomyocytes. Although palmitate induced a significant (p<0.01) increase in apoptotic DNA, as assessed by PI-
Figure F1 - *Fumonisin did not block palmitate-induced apoptosis*. Cardiomyocytes, grown for 72h, were treated with palmitate (100 μM) for 24 or 48 h (N=3), its diluent (control) (N=3), fumonisin B1, 10 μM, (N=3) or the combination of palmitate with fumonisin B (N=3). Cells were stained with propidium iodide (PI), and analyzed by FACS. The data are presented as the mean±S.E.M. and were analyzed compared to control. Palmitate, with or without fumonisin pretreatment, induced a significant (**p<0.01) increase in the number of cells with low DNA content compared to control.
stained low DNA content, fumonisin 10 μM did not alter palmitate-induced apoptotic cell death, regardless of whether cells were treated with palmitate for 24 or 48 h (Figure 1).

Because the maximum time one can use these relatively pure embryonic chick cardiomyocytes before fibroblast contamination is 96 h, it is impractical to expose cardiomyocytes, which have already been grown for 72 h, to palmitate for greater than 48 h. Hence, further experiments were performed with the cell permeable ceramide, C2-ceramide, to mimic an increased de novo ceramide synthesis potentially seen with palmitate treatment for greater than 48 h. A dose range for ceramide was determined by the MTT cell viability assay previously performed in this lab (Figure 2). Ceramide 100 μM for 24 h significantly (p<0.01) induced 42.9±5.8% cell death. This time and dose were used for further experiments as it was the closest dose to a LD₅₀ response.

Apoptosis was examined by quantitating the number of cells with low DNA content because apoptosis, associated with DNA fragmentation to fragments of 180 bp, is measurable by the number of cells with reduced PI fluorescence (Frey et al. 1997). Based on the results from Figure 2, ceramide 100 μM for 24 h was chosen for further experiments. To test for the specificity of ceramide, cardiomyocytes were treated with C₂-dihydroceramide as a negative control for C₂-ceramide as C₂-dihydroceramide lacks the functionally critical double bond evident in C₂-ceramide (Hannun 1994). Whereas ceramide induced a significant (p<0.01) 3.9±0.6 fold increase in the number of cells exhibiting low DNA content compared to control, C₂-dihydroceramide did not show any significant increase in apoptosis (Fig 3). This suggests that C₂-ceramide’s effects are specific and are not due to the ceramide molecule itself.

To further examine concentration and time dependent effects of ceramide, cardiomyocytes were treated with 100, 200 or 300 μM ceramide for 4, 6, or 24 h and analyzed by flow cytometry for PI fluorescence (Figure 4). The maximal degree of apoptosis (assessed by
Figure F2 - Ceramide induces a loss of cell viability. Cardiomyocytes were treated with C2-ceramide at 10 (N=2), 50 (N=11), 100 (N=13), 150 (N=13) and 200 μM (N=13) for 24h and the cell viability assessed by MTT assay. A loss of MTT absorbance at 570nm signifies a loss of cell viability (inset). The loss of A570 were reported as percentages of loss of cell viability, or alternatively, percentage increases in cell death. This figure was produced previously in this laboratory.
Figure F3 - C2-Ceramide increases the population of cells with low DNA content whereas C2-dihydroceramide does not
Panel A - A representative histogram of C2-ceramide 100 μM for 24 h or diluent (control) treated cells. The apoptotic (Apo) population with low DNA content (PI fluorescence less than 10^3) is indicated by the arrow.
Panel B - Cardiomyocytes were treated with either C2-ceramide (N=16) or C2-dihydroceramide (DHC) (N=4) 100 μM for 24 h. The relative number of cells exhibiting low DNA content of the 10,000 cells analyzed in each experiment. The data are presented as the mean±S.E.M. Statistical analysis shows the difference of ceramide compared to control (**p<0.01).
Figure F4 - Ceramide induces an increase in the population of cells with low DNA content - Chick cardiomyocytes were treated with ceramide (10, 30, 50, 100, or 200 μM for 24 h; 100, 200 300 μM for 4 or 6 h) (N=1-24), and exhibited a significant (**p<0.01) increase in nuclei with low DNA content, indicative of apoptosis. Data expressed as relative amounts of apoptosis compared to control.
relative number of cells exhibiting low DNA content compared to control) was a 5 fold increase compared to control (diluent treated) cells. This was achieved by a low dose for a long time or a high dose for a shorter time - i.e.-the amount of apoptosis observed at ceramide 200μM for 24 h (5.3±0.5 fold greater than control) was similar to 300μM at 6 h (5.2±0.7).

I next sought to determine whether ceramide affected cardiomyocyte mitochondria in a manner similar to palmitate. Cardiomyocytes were treated with both ceramide and palmitate, both 100 μM for 24 h, and stained with DePsipher™. To validate changes to DePsipher™ were attributable to loss of ΔΨₘ, cardiomyocytes were also treated with the known mitochondrial poisons rotenone 100 μM and antimycin A 100 μM (Figure 5). In control cells, DePsipher™ forms red aggregates in the presence of an intact ΔΨₘ. In contrast antimycin A and rotenone exhibit a strong uniform green fluorescence suggesting a loss of ΔΨₘ (Figure 5). As well, both ceramide and palmitate produced a similar picture of green fluorescence suggesting that both agents induce a loss of ΔΨₘ (Figure 5).

Flow cytometric analysis of DePsipher™ treated cardiomyocytes treated with rotenone and antimycin A were performed next to validate this method for observing changes to ΔΨₘ. Cardiomyocytes were treated with 100μM each of rotenone and antimycin A for 24h and exhibited a decrease in DePsipher™ red aggregates (downward) and an increase in DePsipher™ green monomers (to the right), suggesting that these agents induce a loss of ΔΨₘ (Figure 6 - Panel A). Cardiomyocytes treated with rotenone and antimycin A exhibited a significant (p<0.01) loss of red fluorescence (0.82±0.1 and 0.21±0.1 of control, respectively) with a concomitant significant (p<0.01) increase (6.9±1.6 and 6.2±1.5 fold greater than control, respectively) in green fluorescence (Panel B). These results suggest that DePsipher™ staining is a good method to observe loss of ΔΨₘ. To determine whether this mitochondrial effect is cyclosporin A-inhibitable, cardiomyocytes were pretreated with cyclosporin A 10 μM.
Figure F5 - *Loss of mitochondrial potential demonstrated by DePsipher™* - Cardiomyocytes were treated with diluent (control) (A), the mitochondrial poisons rotenone (B) and antimycin A (C), ceramide (D), or palmitate (E), all at 100 μM for 24 h. Cells were stained with DePsipher™ and examined on a fluorescent inverted microscope.
Figure F6 - *Antimycin and rotenone induce a loss of mitochondrial potential* - Cardiomyocytes were treated with diluents (control) or the mitochondrial poisons antimycin A 100 µM or rotenone 100 µM for 24h. Cardiomyocytes were stained with DePsipher™ and analyzed by flow cytometry for red dye aggregates (FL3), denoting an intact mitochondrial potential, or green dye monomers (FL1), denoting a loss of mitochondrial potential. *Panel A* - A representative contour plots of cells stained with DePsipher™ from 4 separate experiments. A downward shift in FL3 and a shift to the right in FL1 suggests a loss of mitochondrial potential.

*Panel B* - Cardiomyocytes were treated with rotenone 100 µM (N=4), antimycin 100 µM (N=4) with or without cyclosporin A 10 µM pretreatment (N=4 for both). Changes in DePsipher™ red aggregate fluorescence (FL3 fluorescence) compared to control fluorescence are shown in the upper panel. Likewise, changes to DePsipher™ green monomer fluorescence (FL 1) are shown in the lower panel. After statistical analysis, overall F- value for red aggregate is 23.2 and green monomer is 3.5.
Cyclosporin A pretreatment did not alter the change in DePsipher™ fluorescence with rotenone or antimycin A treated cells.

Ceramide was next investigated for its effects on $\Delta\Psi_m$. Ceramide-treated cardiomyocytes, 100 and 300 $\mu$M for 24h, were stained with DePsipher™ and analyzed by flow cytometry (Figure 7). The representative contour plot displays the population of 10,000 cardiomyocytes dependent on their mitochondrial state. As the mitochondria of a significant portion of the population of cardiomyocytes lose its potential, the population moves to the bottom right representing an increase in green monomers and decrease in red aggregates. Ceramide shifted the distribution of green monomers compared to control (diluent treated) cells (Panel A). A stronger death response with ceramide 300$\mu$M for 6h (see Fig. 4) compared to 100 $\mu$M for 24 h, produced a more dramatic, significant ($p<0.01$) loss of $\Delta\Psi_m$, as indicated by the 1.8±0.4 fold (N=5) increase in the amount of green fluorescence, and a significant ($p<0.01$) decrease in the amount of red aggregate fluorescence, as indicated by a 0.5±0.2 (N=5) fold decrease in red fluorescence compared to control.

Because the opening of the mitochondrial pores may result in subsequent cell apoptotic cell death and this can be inhibited by cyclosporin A (Kong & Rabkin 2000; Arora 1997), I next sought to determine whether cyclosporin A-inhibitable mitochondrial transition pores (MTP) were formed during ceramide treatment. Cardiomyocyte were treated with ceramide 100 $\mu$M for 24 h, with or without cyclosporin A 1 $\mu$M pretreatment and stained with DePsipher (Figure 8).

Cyclosporin A had no effect in reducing ceramide-induced loss of $\Delta\Psi_m$.

To explore further the nature of the effect of ceramide on mitochondria, I examined the possibility that ceramide might affect mitochondrial "negative sink", hence respiration, using chloromethyl-X-rosamine (CMX-Ros). Again, CMX-Ros method was validated using the mitochondrial poisons rotenone and antimycin A (Figure 9). Antimycin A and rotenone induced
Figure F7 - Ceramide induces a loss of mitochondrial potential.

**Panel A** - Cardiomyocytes were treated with ceramide 100 μM for 24h and stained with DePsipher dye and analyzed by flow cytometry for red dye aggregates (FL3), denoting an intact mitochondrial potential, or green dye monomers (FL1), denoting a loss of mitochondrial potential. A downward shift in FL3 and a shift to the right in FL1 suggests a loss of mitochondrial potential. This panel shows a representative contour plot of 6 separate experiments.

**Panel B** - Cardiomyocytes, treated with diluent (control) (N=15), ceramide 100μM (N=15) for 24h, or ceramide 300μM (N=5) for 6h, were stained with DePsipher and analyzed by flow cytometry. Ceramide induced a significant (** p<0.01) increase in the number of cells with mitochondria that have lost their mitochondrial potential (green monomer) and a decrease in the number of cells with intact mitochondrial potential (red aggregate). Data presented as relative amount of cells with green/red fluorescence relative to control.
Figure F8 - *Cyclosporin A* pretreatment has no effect on ceramide-induced loss of mitochondrial potential - Cardiomyocytes were treated with diluent (control) (N=5), ceramide 100 µM (N=5), with or without cyclosporin A 1µM pretreatment (N=5 for both), for 24 h and then stained with DePsipher. Cells were analyzed by flow cytometry for red aggregates (intact potential) and green monomers (loss of potential) and its fluorescence expressed relatively compared to control. Ceramide significantly (*p<0.05) increased the amount of green monomers which was unaffected by cyclosporin A pretreatment.
a leftward shift in CMX-Ros fluorescence to lower fluorescent levels, suggesting a decrease in mitochondrial respiration (Panel A). Both rotenone 100 μM and antimycin 100 μM induced a significant (p<0.01) decrease to 0.15±0.11 (N=4) and 0.09±0.04 (N=4) that of control in CMX-Ros fluorescence, with antimycin producing a greater reduction than rotenone. However, cyclosporin A 10 μM pretreatment was able to partially reverse this loss of fluorescence as cyclosporin A increased the relative decline of CMX-Ros to 0.69±0.04 (N=4) and 0.69±0.06 (N=4) of control when in combination with rotenone and antimycin, respectively. These results suggest that CMX-Ros is a good method of observing intramitochondrial enzyme mitochondrial effects as Complex I and II inhibitors produced meaningful changes in CMX-Ros fluorescence.

The effects of ceramide on mitochondrial function was next determined using CMX-Ros and flow cytometry. In contrast to antimycin A and rotenone, ceramide induced an increase in CMX-Ros fluorescence (Figure 10 - Panel A). Compared to control, ceramide 100 μM for 24 h and 300 μM for 6h induced a significant (p<0.01) 1.2±0.2 (N=6) and 1.7±0.3 (N=3) fold increase in CMX-Ros fluorescence, respectively, compared to control (Panel B). These results suggest that ceramide is inducing a stronger “negative sink” in mitochondria than control, implicating that ceramide induces mitochondrial respiration.

The mitochondrial effects of ceramide were compared to those of palmitate and ceramide, both 100 μM for 24 h (Figure 11). Palmitate induced a decrease in CMX-Ros fluorescence and a loss of ΔΨm (DePsipher™) (Panel A). In contrast, ceramide produced an increase in CMX-Ros fluorescence as well as the loss in ΔΨm. The differences between palmitate- and ceramide-induced mitochondrial effects are demonstrated in Panel B.

To determine whether ceramide is cyclosporin A-sensitive as seen with palmitate in Section C, cardiomyocytes were treated with ceramide 100 μM and cyclosporin A 1 μM and subjected to flow cytometric analysis to examine DNA content (Figure 12). Although both
Figure F9 - Loss of mitochondrial enzyme activity based on CMX-Ros - Cardiomyocytes were treated with diluent (control) or the mitochondrial poisons antimycin A 100 µM or rotenone 100 µM for 24h. Cardiomyocytes were stained with CMX-Ros and analyzed by flow cytometry for red fluorescence (FL3) denoting a mitochondrial enzyme activity. Panel A shows representative histogram from 4 separate experiments. A leftward shift of FL3 denotes a loss of enzyme activity. Panel B - Cardiomyocytes were treated with antimycin 100 µM (N=4) or rotenone 100 µM (N=4) with or without cyclosporin A 10 µM pretreatment (N=4 for both). Changes in CMX-Ros red fluorescence (FL3 fluorescence) compared to control fluorescence are shown. After statistical analysis, overall F-value is 63.4.
Figure F10 - *Ceramide induces a change in mitochondrial respiration.* Cardiomyocytes are treated with ceramide 100 (N=6) or 300 μM (N=3) for 24h or 6h, respectively. Cells were stained with CMX-Ros and examined by flow cytometry. Panel A - Representative histogram of 3-6 separate experiments. A leftward shift of FL3 denotes a loss of enzyme activity. Panel B - Changes in CMX-Ros red fluorescence (FL3 fluorescence) compared to control fluorescence are shown in relative amounts. After statistical analysis, overall F-value is 3.66.
Figure F11 - *Comparison of mitochondrial effects between palmitate and ceramide*. Cardiomyocytes treated with palmitate 100 μM or C2-ceramide 100 μM for 24 h. Cells were stained with either CMX-Ros or DePsipher. Panel A - Comparison of mitochondrial effects - Control (N=17), palmitate (N=17), and ceramide (N=6) were stained with CMX-Ros and represented as relative fluorescence compared to control. Similar experiments were repeated but stained with DePsipher (Control N=6, Palmitate N=3, Ceramide N=6). ** p<0.01 compared to control. Panel B - The loss of mitochondrial potential, evidenced by the increase in green monomers, are presented on the x-axis whereas the change in mitochondrial respiration (CMX-Ros) is represented on the y-axis.
Figure F12 - Comparison of loss of DNA content and cyclosporin A sensitivity between palmitate and ceramide - Cardiomyocytes were treated with diluent (Control) (N=14), palmitate 100 μM (N=14), cyclosporin A 1 μM + palmitate 100 μM (Cyclo+Palm) (N=11), ceramide 100 μM (N=16), or cyclosporin A 1 μM + ceramide 100 μM (Cyclo+Cer) (N=4) for 24 h. Cells were analyzed for low DNA content by PI staining of nuclei and analysis by flow cytometry. Data for cyclosporin+palmitate was reproduced from previous Section C. ** p<0.01 compared to control; Δ p<0.05 compared to control.
palmitate and ceramide induced significant (p<0.01) 2.0±0.1 and 2.7±0.5 fold increases compared to control, respectively, cyclosporin A pretreatment had no effect on ceramide-induced apoptotic DNA in contrast to palmitate’s effect.

DISCUSSION

This section presents several novel findings. First, these experiments suggest that palmitate-induced cell death was not due to palmitate-induced enhancement of de novo synthesis of ceramide, contrary to the hypothesis put forward by Paumen et al (1997). Second, in intact cardiomyocytes, ceramide induces a loss of mitochondrial membrane potential through a cyclosporin insensitive mechanism. Third, ceramide-induced mitochondrial changes include an increase in CMX-Ros fluorescence that might represent an apparent accentuation of mitochondrial respiration. Fourth, these ceramide-induced effects of ceramide on mitochondria are distinct from the effects of its precursor palmitate suggesting that ceramide and palmitate utilize mitochondrial separate death mechanisms.

Palmitate-induced apoptosis is not through its incorporation into de novo ceramide

Cardiomyocytes were treated with palmitate for sufficient time to permit ceramide synthesis, as evidenced in the LyD9 hematopoietic cell line (Paumen et al. 1997). The use of fumonisin B1, an inhibitor of ceramide synthase, to inhibit de novo ceramide synthesis (Merrill et al. 1996) was a crucial part of the evidence suggesting that palmitate-induced cell death was mediated by ceramide synthesis (Paumen et al. 1997). It was observed that fumonisin B1, at similar concentrations to previous studies (Paumen et al. 1997), did not alter palmitate-induced apoptosis or oncosis in cardiomyocytes. However, the failure of fumonisin B1 to influence apoptosis does not confirm that ceramide synthesis was not increased by palmitate. Rather it suggests that ceramide is not playing a major role in palmitate-induced cell death within the time
frame of these experiments (48 h). The time and dose of fumonisin used was effective as it was shown to antagonize cardiomyocyte-induced cell death from other agents in a 24-48 h time period (Rabkin SW - unpublished observations). The results of this section do not agree with those reported by Paumen et al. (1997). Using hematopoietic cell lines, Paumen et al (1997) reported minimal apoptosis induced by 20 μM palmitate for 48 h. However, Paumen et al. (1997) reported an increase in ceramide levels with 100 μM palmitate within 3 h of treatment. I questioned Paumen’s results since he did not measure ceramide levels at the same time/dose as palmitate-induced apoptosis. My results agree with other studies which also report data contradictory to Paumen et al (1997). In particular, Listenberger observed that palmitate induced apoptosis in CHO cells deficient in de novo ceramide synthesis. (Listenberger et al. 2001).

Justification for use of C2-Ceramide

To examine similarities between palmitate-induced cell death and ceramide at times greater than 48 h, a different model had to be established due to the inability to experimentally use cardiomyocytes for greater than 48h. It was impractical for palmitate treatments greater than 48h in these embryonic cardiomyocytes because the maximum time one can use these relatively pure cells before fibroblast contamination is 96 h. Since, these cardiomyocytes have already been grown for 36 to 72 h before experimental use, it was impractical to treat with palmitate for greater than 48 h before fibroblast contamination. Hence, further experiments were performed with the cell permeable ceramide, C2-ceramide (ceramide), to mimic an increased de novo ceramide synthesis that might occur with palmitate treatment for greater than 48 h.

Ceramide-induced loss of DNA content

As with palmitate, ceramide was shown to induce a significant (p<0.01) loss of cell viability (Fig. 2 - previously produced by this laboratory) and a significant (p<0.01) increase in apoptotic low DNA content (Figs. 3 & 4). Ceramide produced a time-dependent and
concentration-dependent increase in ceramide-induced apoptosis (Fig. 4); however, the effects of ceramide were shown to plateau suggesting that only a fixed proportion of the cell population may be vulnerable to the effects of ceramide (Fig. 4). These results agree with previous reports of ceramide-induced apoptosis and oncosis (for in depth review, see Mathias et al. 1998), including apoptosis in adult and neonatal rat cardiomyocytes (Andrieu-Abadie et al. 1999; Bielawska et al. 1997). This chapter's results are consistent with the postulate that the generation of ceramide in heart may play a role in cardiomyocyte cell death (Bielawska et al. 1997, Andrieu-Abadie 1999). Previous reports suggest that ceramide is generated during ischemia/reperfusion or doxorubicin treatment in heart which correlated with cell death (Bielawska et al. 1997, Andrieu-Abadie 1999). In addition, the results from this chapter show that this C2-ceramide-induced cell death was specific as C2-dihydroceramide, a negative control analogue of C2-ceramide, did not yield any apoptotic low DNA content (Fig 3).

Ceramide-induced mitochondrial changes

This chapter used 2 separate methods to observe changes to mitochondria during apoptosis: i) DePsipher™ as a measure of \( \Delta \Psi_m \), and ii) CMX-Ros as a measure of mitochondrial enzyme activity. Results to immunoblotting of cytosolic cyt c as a measure of mitochondrial permeability will be presented in the next chapter.

i) Results with DePsipher™

As antimycin and rotenone are established mitochondrial-specific inhibitors of the respiratory chain (Wolvetang et al. 1994, Higgins and Greenamyre 1996) and the decrease in DePsipher™ red aggregates and increase in DePsipher™ green monomers agree with previous studies with these agents (Dedov et al. 2001), the DePsipher™ method of observing changes in \( \Delta \Psi_m \) was able to detect the effects of interruptions in the respiratory chain in cardiomyocytes (Figures 5 & 6). I demonstrated a ceramide-induced loss of mitochondrial membrane potential,
both microscopically (Fig 5) and quantitatively (Fig 7) which were similar to antimycin A and rotenone. Like antimycin A and rotenone, ceramide induced a significant (p<0.01) increase in green monomer fluorescence in contrast to control’s red aggregates (Figs. 5&7). There have been limited previous investigations of the ability of ceramide to alter $\Delta \Psi_m$ and the results have been controversial. This chapter’s data are consistent with the findings of Arora et al. (1997) and Ghafourifar et al. (1999). Arora et al. (1997) reported that ceramide-induced a depletion of ATP causing mitochondrial depolarization and dysfunction, using the mitochondrial-potential-sensitive dye tetramethylrhodamine ethyl ester, in isolated hepatocytes in cell culture. Ghafourifar et al (1999) reported that C$_2$-ceramide induced an increase in mitochondrial membrane permeability implicating changes in $\Delta \Psi_m$ of isolated mitochondria from rat liver. In contrast, two studies reported that ceramide did not directly affect $\Delta \Psi_m$. Garcia-Ruiz et al (1997) and Pastorino et al (1999) both concluded that C$_2$-ceramide did not alter membrane potential based on the absence of changes in permeability of mitochondria isolated from rat liver. The present section differs from others in that I examined cardiomyocytes rather than liver cells (Garcia Ruiz et al. 1997) and used DePsipher™ compared to the indirect assessment of mitochondrial permeability based on mitochondrial size (Pastorino et al 1999). It is noteworthy that the studies that failed to show an effect of ceramide on mitochondria were conducted in isolated mitochondria (Pastorino et al 1999) supporting the contention that cellular signal transduction pathways play a role in mediating the action of ceramide on mitochondria.

ii) Results with CMX-Ros

Antimycin A and rotenone both induced a loss of mitochondrial “negative sink”, and indirectly a loss of respiration, evidenced by a loss of CMX-Ros fluorescence. This loss of respiration was uninhibitable by cyclosporin A as these mitochondrial poisons affect the mitochondria upstream of MTP formation (Fig. 9). These results agree with previous reports of
antimycin- and rotenone-induced loss of mitochondrial respiration (Isenberg and Klaunig 2000). Isenberg and Klaunig (2000) reported that rotenone reduced CMX-Ros fluorescence. Thus, CMX-Ros is a valid method of observing mitochondrial “negative sinks” and respiration.

In contrast, I demonstrated a significant increase in CMX-Ros fluorescence in response to ceramide suggesting an increase in mitochondrial respiration (Fig 10). This observation does not follow the theory that decreased $\Delta \Psi_m$ would eventually lead to decreased mitochondrial respiration resulting in a lack of ATP generation. Hence, this observation is novel in that mitochondrial respiration activity was stimulated specifically by ceramide in a mechanism that is not dependent on $\Delta \Psi_m$. There has been a previous report by Poot and Pierce (1999) of a similar increase in CMX-Ros fluorescence concomitant with camptothecin-induced apoptosis. A possible explanation for this increased mitochondrial respiration is the mitochondria’s attempt to re-establish its potential by increased mitochondrial enzyme activity and respiration. Green & Reed (1998) reported that a transient opening of MTPs with only a transient mitochondrial depolarization might allow a repeated, respiration-driven reestablishment of $\Delta \Psi_m$. However, to my knowledge, there have been no previous reports of increased “negative sink” or respiration during ceramide treatment that occurs simultaneously as apoptosis. This finding, if valid, is novel and will require more work to elucidate its mechanism.

iii) Cyclosporin A-sensitivity

In contrast to results performed with palmitate, ceramide-induced loss of $\Delta \Psi_m$ (Fig. 8) and apoptotic DNA (Fig. 12) was through a cyclosporin A-insensitive mechanism. The concentration of cyclosporin A used in these studies was sufficient to inhibit MTPs (Halestrap & Davidson 1990). As well, the same concentration was used previously and shown to inhibit cyt C release and cell death in these cardiomyocytes induced by palmitate (see previous Section C or
Kong and Rabkin 2000). Arora et al. (1997) also reported that cyclosporin inhibited ceramide-induced effects in rat liver mitochondria.

Cyclosporin A has been shown to be both effective and ineffective in reversing changes to $\Delta \Psi_m$. Using JC-1 to observe mitochondrial depolarizations, Minamikawa et al. (1999) observed that cyclosporin A impeded the permeability transition and swelling in cultured human osteosarcoma cells upon treatment with a protonophore. However complete inhibition was not observed. Hoyt et al. (2000) reported that in cultured neurons, tamoxifen induced a loss of $\Delta \Psi_m$, assessed by JC-1, was partially blocked by cyclosporin A. This observation was echoed by White and Reynolds (1996) who observed mitochondrial depolarization, measured with JC-1, in neurons stimulated with glutamate to be cyclosporin A-sensitive. In contrast, cyclosporin A was shown to be ineffective in reversing spontaneous, partial, and transient depolarizations in the same cultured neurons by others (Buckman et al. 2001).

It is possible that ceramide induces MTP formation that did not include the cyclosporin-A sensitive cyclophilin D. However, the identities of MTP components were not determined in this thesis. It is also possible that cyclosporin A may not affect ceramide-induced mitochondrial effects if ceramide induces it mechanism independent of MTP formation as in the case with antimycin and rotenone.

Taking these results together, ceramide may induce a mitochondrial effect independent of increases in mitochondrial permeability or ceramide may involve formation of MTPs which are not cyclosporin A-sensitive. MTPs are comprised of a mixture of four various components: cyclophilin; the cyclosporin A-sensitive component, a member of the Bcl-2 family, the adenine nucleotide translocator, and the voltage-dependent ion channel (Beutner et al. 1996). Hence, mitochondria can have an increase of permeability via MTPs that is cyclosporin A-insensitive if the MTPs involved do not have a cyclophilin component. As well, ceramide-induced loss of
mitochondrial membrane potential may be due to its effect on the other MTP components, perhaps Bcl-2, as ceramide induces Bcl-2 dephosphorylation involving a mitochondrial phosphatase of the PP2A type (Ruvolo et al. 1999).

Comparison between palmitate- and ceramide-induced effects

My findings indicate distinct differences in the mitochondrial effects between palmitate and ceramide, as summarized in Figure 11 and Table 1. Palmitate and ceramide induce a loss of cell viability, DNA content, and ΔΨm as well as cyt C release (see in next section). However, the similarities between the two death mechanisms end there.

Palmitate-induced cell death is inhibitable by cyclosporin A whereas ceramide is not. This could be due to either i) ceramide-induced formation of MTPs do not have a cyclosporin-A sensitive component; or ii) ceramide-induced mitochondrial effects are upstream of MTP formation. Thus, resealing of MTPs may not have an effect on ceramide-induced mitochondrial effects since MTP formation is the end result and not the cause of ceramide-induced death.

Palmitate also induced a loss of mitochondrial respiration, evidenced by a loss of CMX-Ros fluorescence (Section C). In contrast, ceramide induced an increase in CMX-Ros fluorescence or respiration. I speculate that this “burst of respiration” before depolarization is key to ceramide’s mitochondrial effects. Also in consideration, ceramide-induced CMX-Ros effects differ from those induced by antimycin A and rotenone. This result suggests that ceramide-induced effects on mitochondrial respiration do not involve Complex I and II of the respiratory chain. Conversely, palmitate, which produced results similar to antimycin A and rotenone, may act through Complexes I and II. Nevertheless, further work must be done to elucidate the reason behind these putative effect on respiration.
Table F1 - Summary of mitochondrial responses

<table>
<thead>
<tr>
<th></th>
<th>Nuclear hypoploidy</th>
<th>DePsipher™ Green</th>
<th>DePsipher™ Red</th>
<th>CMX-Ros</th>
<th>Cyt C release</th>
<th>Cyclo. A - inhibitable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitate 100 μM</td>
<td>2 fold greater than control</td>
<td>Incr. vs. control</td>
<td>Unchanged</td>
<td>Decr. vs. control</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Ceramide 100 μM</td>
<td>4 fold greater than control</td>
<td>Incr. vs. control</td>
<td>Decreased vs. control</td>
<td>Incr. vs. control</td>
<td>Yes*</td>
<td>No</td>
</tr>
<tr>
<td>Antimycin A 100 μM</td>
<td>8 fold greater than control</td>
<td>Incr. vs. control</td>
<td>Decreased vs. control</td>
<td>Decr. vs. control</td>
<td>N/A</td>
<td>No</td>
</tr>
<tr>
<td>Rotenone 100 μM</td>
<td>6 fold greater than control</td>
<td>Incr. vs. control</td>
<td>Decreased vs. control</td>
<td>Decr. vs. control</td>
<td>N/A</td>
<td>No</td>
</tr>
</tbody>
</table>

Cyclo. A = cyclosporin A
vs. = compared to control
* = see next section
N/A = not available

CONCLUSION

In conclusion, it is unlikely that palmitate induces its death mechanism via ceramide. In contrast, ceramide induces several mitochondrial effects (increased mitochondrial respiration and insensitivity to cyclosporin A) distinct from palmitate which suggests that ceramide may induce an apoptotic program different from palmitate, at least from the perspective of mitochondria.
SECTION G - CERAMIDE INDUCES APOPTOSIS IN A MAPK-DEPENDENT MECHANISM WHICH DIRECTLY AFFECTS THE MITOCHONDRIA (Kong JY and Rabkin SW – SUBMITTED TO *FASEB J*)

SUMMARY

Ceramide induced a mitochondrial death mechanism that was distinct from palmitate. To explore the underlying cellular mechanisms of ceramide-induced cell death, cardiomyocytes from embryonic chick heart were treated with the cell permeable ceramide analogue C2-ceramide. Ceramide induced a significant amount of apoptosis as demonstrated by changes in DNA content, assessed by flow cytometric analysis of PI stained cells, indicative of apoptosis. Ceramide had significant effects on mitochondria, specifically, a loss of mitochondrial potential as indicated by the fluorescent probe DePsipher™.

MAP kinases were implicated in these effects of ceramide as SAPK phosphorylation increased as ERK phosphorylation decreased during ceramide treatment. In addition, p38 phosphorylation was observed in peri-mitochondrial fractions of cells after ceramide treatment. Inhibition of p38 with SB 202190 but not the MEK inhibitor PD 98059 significantly inhibited ceramide-induced apoptosis and loss of mitochondrial potential. These data suggest that mitochondria are involved in ceramide-induced apoptosis in part by a p38 sensitive pathway. Furthermore, a role for p38 through the mitochondria is suggested by the abilities of the p38 inhibitor SB 202190.

INTRODUCTION

The aim of this section was to dissect the mechanism by which ceramide induced the mitochondrial effects, distinct from palmitate, which resulted in apoptosis. As ceramide is
generated or added exogenously at the level of the membrane, it is logical to assume that a signaling mechanism must be in place to transmit ceramide’s signal at the membrane to the mitochondria. This signaling mechanism is key to ceramide-induced mitochondrial effects, loss of $\Delta \Psi_m$ and increase in intramitochondrial “negative sink” (i.e., respiration), seen in the previous Section F.

While there has been some support for a role for ceramide signaling to or within the mitochondria (Susin et al. 1997), other data do not support or refute a role for mitochondria in ceramide-induced apoptosis (Tepper et al. 1999). Ceramide was observed to induce apoptosis and induce MTP formation resulting in the release of AIF and cytochrome C in intact HeLa cells (Susin et al. 1997). Ceramide did not induce nuclear DNA fragmentation in the absence of mitochondria suggesting that ceramide requires mitochondria for apoptotic induction (Susin et al. 1997). However, Tepper et al. (1999) reported that ceramide induced apoptosis in two separate mechanisms, one mitochondrial dependent the other independent. Arora et al. (1997) reported that ceramide-induced cell death in isolated hepatocytes was associated with adenosine triphosphate (ATP) depletion and mitochondrial depolarization suggesting that ceramide caused mitochondrial dysfunction (Arora et al. 1997). Ceramide has been reported to act directly on mitochondrial complex III activity causing a rapid decline of mitochondrial oxidation in the electron transport chain (Gudz et al. 1997). Due to this inhibition of electron transport, reactive oxygen species are generated which damage the cell (Garcia-Ruiz et al. 1997). Furthermore, two separate groups reported that ceramide did not alter mitochondria permeability of isolated mitochondria from rat liver (Garcia-Ruiz et al. 1997; Pastorino et al. 1999). One potential explanation for these discordant findings is the examination of isolated mitochondria vs. intact cells. Intact cells may generate other pro-/anti-apoptotic factors that alter mitochondria upon ceramide treatment or generation. In contrast, apoptotic effects in isolated mitochondria will
only be observed if ceramide acts directly on the mitochondria itself. Further, the techniques used to measure mitochondrial size in isolated mitochondria due to swelling (Botla et al. 1995) only allows for observation of mitochondrial permeability, the end point of MTP formation, and not mitochondrial function or potential ($\Delta \Psi_m$). Thus this section sought to determine if ceramide affects mitochondria in intact cells through directly or indirectly a signaling pathway. As well, this section will also observe ceramide-induced changes to mitochondrial permeability, $\Delta \Psi_m$.

The mechanism by which ceramide induces apoptosis is not restricted to an action on mitochondria. Ceramide targets factors that transfer the apoptotic signal to the nucleus including, but not limited to kinases, phosphatases, protein kinase zeta ($\text{PKC}_\zeta$), phospholipases, Raf, mitogen activated protein kinases (MAPK), SAPK/JNK, caspases, and transcription factors (Mathias et al. 1998; Kolesnick & Kronke 1998). Ceramide may preferentially target one but not another within the same cell or activate different targets in different cell type. The MAPKs are intriguing signal transduction pathways which may be involved in the actions of ceramide. Ceramide has been shown to activate or inhibit MAPKs, depending on the cell type (Xia et al. 1995). Ceramide induced apoptosis in endothelial and U937 cells with a concomitant marked activation of stress-activated MAPK/c-Jun activated MAPK (SAPK/JNK), but not the extracellular-regulated MAPK (ERK) cascade (Verheij et al. 1996; Cuvillier et al. 1996). In general, ERK seems to be anti-apoptotic (Xia et al. 1995) whereas SAPK/JNK appears to function in a pro-apoptotic role (Basu & Kolesnick 1998). Another MAPK, p38 MAPK, has been implicated in ceramide-induced apoptosis but the results are conflicting. Tavarini et al. (2000) reported that ceramide induced apoptosis that was inhibitable by the p38 MAPK inhibitor SB 202190 in neuroblastoma cells. Similarly, Hida et al. (1999) reported that ceramide induced apoptosis in oligodendrocytes and preferentially activated p38$\alpha$ and that ceramide-induced apoptosis was attenuated with the p38 inhibitor SB 203580 and by expression of a p38$\alpha$. 

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dominant negative mutant. In contrast, Jain et al (1998) reported that ceramide had no effect on p38 MAPK and SAPK/JNK in apoptosis induction in adipocytes. The objective of this section was to determine whether ERK, SAPK, and p38 MAPK are involved in ceramide-induced apoptosis in cardiomyocytes. Specifically, I sought to investigate the ERK, SAPK, and p38 MAPK pathways becoming phosphorylated. In conjunction with MAPK inhibitors, MAPK activation and cell death will be linked.

The objective of this section was to examine in cardiomyocytes the hypothesis that ceramide alters mitochondrial membrane potential and induces apoptosis through a major MAPK pathways involving p38 with the mitochondrial effects of ceramide. The cardiomyocyte element of the heart is rich in mitochondria as metabolism within mitochondria generates the ATP necessary for actively functioning muscle cells (Chien 1999). Thus, this highlights the need to understand the mitochondrial mechanism(s) by which ceramide might induce cardiac cell death.

RESULTS

In the previous section, ceramide 300 μM for 6h was observed to induce a stronger death and mitochondrial response than ceramide 100 μM for 24 h (see Figs 3, 7, and 9 of Section F). As I expect kinase inhibitors would reduce ceramide-induced cell death, I believed that it was more advantageous to elicit a stronger death response at 300 μM ceramide for 6h to highlight any potential kinase-inhibited decreases to ceramide-induced death. In addition, I speculated that apoptotic signaling would occur before apoptotic development, I chose a shorter time point, 6 h, than the 24 h used in Section F.

Apoptosis was examined by quantitating the number of cells with low DNA content because apoptosis is characterized by DNA fragmentation into fragments of 180 bp which is measured by the number of cells with reduced PI fluorescence (Frey 1997).
experiment shows the cell population distribution according to the degree of PI staining (FL3) (Fig. 1 upper panels). Of the 10,000 cells analyzed in control, only a very small proportion showed PI (staining) fluorescence less than $10^2$. In contrast, the proportion of the population with this degree of low staining (hypoploidy) was increased in ceramide-treated cells. By analysis of the histogram of PI staining, the number of cells exhibiting low DNA content associated with apoptosis was quantitated, Ceramide, 300µM, for 6h, significantly (p<0.01) increased the number of cells exhibiting apoptosis (Fig. 1 lower panel) to $3835\pm399$ (N=11) from $856\pm91$ (N=11) in control (diluent treated) cells. This represents a 5.1±0.2 fold increase in apoptosis induced by ceramide.

Evaluation of the contribution of MAPK to ceramide-induced apoptosis was first studied by the use of the relatively selective p38 inhibitor SB 202190 (Singh et al 1999) and the relatively selective MEK inhibitor PD 98059 (Kultz et al 1998). SB 202190 was chosen over the other common p38 inhibitor SB 203580 because SB 203580 has a higher IC$_{50}$ (600 nM) than SB 202190 (IC$_{50}$ 350 nM) (Iwasaki et al 1999; Singh et al 1999). SB 202190, treatment beginning 60 min before ceramide, produced a modest but significant (p<0.01) 16.7±3.8% (N=10) reduction in ceramide-induced apoptosis compared to control (Fig.2). In contrast, the selective MEK inhibitor PD 98059, 1000 nM, did not significantly affect ceramide-induced apoptosis.

To further assess the MAPK signaling mechanisms involved in ceramide-induced cardiomyocyte apoptosis, ceramide-induced effects on MAPK phosphorylation was investigated. SAPK/JNK and ERK phosphorylation were first investigated. Immunoblots for phosphorylated SAPK/JNK and phosphorylated ERK, as well as native SAPK/JNK and ERK, were performed on whole cell lysates from cells treated with ceramide (Figure 3). There was a time-dependent
Figure G1 - Ceramide increases the population of cells with low DNA content
Cardiomyocytes were treated with C2-ceramide, 300μM or its diluent (control) for 6h and the number of cells with nuclei with low DNA content, was assessed by low PI fluorescence (FL3). The upper panels are representative histograms of 10 000 ceramide- (right) or diluent- (control) (left) treated cells. The apoptotic (Apo) population with low DNA content (PI fluorescence less than 10^2 is indicated by the arrow. The lower panels show the number of cells exhibiting apoptosis per 10 000 cells analyzed in each experiment. The data are presented as the mean ±S.E.M. (N= 10). Data analysis shows the difference of ceramide compared to control (***p<0.01).
Control

Ceramide

Number of cells with low DNA content per 10,000 cells

Control

Ceramide
Figure G2 - p38 inhibition, but not MEK inhibition, blunts ceramide-induced apoptosis. Cardiomyocytes treated with ceramide (300μM for 6h) (N=5), with either the MEK inhibitor PD 98059 (PD) 1μM (N=5) or the p38 inhibitor SB 202190 (SB) 500nM (N=5), were examined by flow cytometry using PI fluorescence as a measure of DNA content in permeabilized cardiomyocytes. The percent reduction in ceramide-induced apoptosis is shown compared the effects of each inhibitor with ceramide compared to ceramide alone (0%). The inset shows the number of cells with low DNA content per 10,000 cells. Data analysis shows the comparison with ceramide (** p<0.01) or ceramide (ΔΔp<0.01).
Figure G3 – *Ceramide induces a change in SAPK/JNK and ERK activation*. Cardiomyocytes, grown for 72h and serum starved for the last 16 h, were treated with ceramide 300 μM for 0, 15, 30, or 60 min. Cells were lysed and whole cell lysates were run on a 10% PAGE gel and immunoblotted for either phospho-ERK or native ERK, or phospho-SAPK/JNK or native SAPK/JNK. SAPK/JNK has a molecular weight of 54 and 46 kDa.
increase, up to 12%, in SAPK phosphorylation with a concomitant decrease in ERK phosphorylation that was evident 15 to 30 min after ceramide treatment and which returned to baseline thereafter. There was a small SAPK response in control cells that was attributable to the reported constitutive SAPK activity (Jain et al 1998).

I next sought to determine whether ceramide affected cardiomyocyte mitochondria.

Cardiomyocytes were stained with DePsipher™. DePsipher™ is a lipophilic mitochondria-specific dye which readily enters the intramitochondrial space, where it forms red aggregates in the presence of mitochondrial potential or remain as green monomers in the absence of a potential (Dedov et al. 2001). Red aggregates were readily visualized in control cells (Fig.4A). In contrast, a uniform green staining reflecting the loss of mitochondrial potential was observed in ceramide treated cardiomyocytes. Flow cytometric analysis of DePsipher™-stained cells showed that ceramide shifted the distribution of green monomers compared to control (diluent treated) cells (Fig 4B). The representative contour plot displays the population of 10,000 cardiomyocytes dependent on their mitochondrial state. As the mitochondria of a significant portion of the population of cardiomyocytes lose their potential, the population moves to the bottom and to the right representing an increase in green monomers and decrease in red aggregates. Ceramide, 300 μM for 6h, produced a significant (p<0.01) loss of mitochondrial potential, as indicated by the 1.8±0.4 fold (N=5) increase in the amount of green fluorescence, and the significant (p<0.01) decrease in the amount of red aggregate fluorescence, as indicated by a 0.5±0.2 (N=5) fold decrease in red fluorescence compared to control (Fig. 4C).

To assess whether p38 MAPK is directly interacting with the mitochondria to induce mitochondrial effects, both mitochondrial and cytosolic fractions of cardiomyocytes were prepared. Cardiomyocytes were treated with ceramide 300μM and both mitochondrial and cytosolic preparations were blotted for phosphorylated p38 MAPK and native p38 MAPK.
Figure G4 - Ceramide induces a loss of mitochondrial potential.
Cardiomyocytes were treated with ceramide 300μM or its diluent for 6 h and stained with DePsipher™.
Panel A - Cardiomyocytes, grown on coverslips, were examined on a fluorescent inverted microscope.
Panel B - Representative contour dotplot of cardiomyocytes analyzed by flow cytometry for red dye aggregates (FL3), denoting an intact mitochondrial potential, or green dye monomers (FL1), denoting a loss of mitochondrial potential. A downward shift in FL3 and a shift to the right in FL1 suggest a loss of mitochondrial potential. Representative contour plot of 6 separate experiments.
Panel C - The number of cells which exhibit green or red DePsipher™, relative to control. Ceramide induced a significant (**) p<0.01 compared to control) increase in the number of cells with mitochondria that have lost their mitochondrial potential (green) and a decrease in the number of cells with intact mitochondrial potential (red) (N=6).
These preparations were also immunoblotted for cytochrome C to validate mitochondrial purity and actin to validate equivalent protein loading (Figure 5C). p38 was evident in mitochondrial as well as cytosolic preparations with an expected greater amount of p38 in the cytosol. Ceramide treatment induced phosphorylated p38 in the mitochondrial fraction, the phosphorylated p38 having been identified by the positive control cell lysate (C6 glioma cells treated with anisomycin; provided by Cell Signaling Technologies). Densitometric analysis of mitochondrial samples show a modest and significant (p<0.01) 1.2±0.03 fold (N=4) increase in phosphorylated p38 in the mitochondrial fraction of ceramide-treated cells compared to control. To determine whether p38 was acting in mitochondria, cardiomyocytes were pretreated, for 6h, with either SB 202190 (500nM) or PD 98059 (1μM) before ceramide. There was a significant (p<0.01) reduction of phosphorylated p38 back to control levels by SB 202190 (Fig 5 B). In contrast, there was very little change in cytosolic phosphorylated p38 MAPK after ceramide treatment with the exception of the SB 202190 pretreated sample which had the expected reduced phosphorylation. Mitochondrial preparations were determined pure because cyt C immunoblots showed a strong cyt C presence in mitochondrial fractions with no cyt C presence in control cytosol (Panel C). Ceramide treated samples showed an increase in cytosolic cyt C consistent with a loss of cytochrome C from mitochondria. To validate equivalent protein loading, actin (α, β, and γ isoforms) was also examined in these samples (Panel C). No change in actin between lanes suggest that equal amounts of protein were loaded on the gel.

To determine whether the ceramide-induced p38 mechanism is upstream of ceramide-induced mitochondrial effects, cardiomyocytes were pretreated with PD 98059 or SB 202190 before ceramide and then examined by DePsipher™ flow cytometry (Figure 6). The increase in green monomers, indicative of a loss in mitochondrial membrane potential produced by
**Figure G5 - Ceramide induces a phosphorylation of p38 in the perimitochondrial fraction.**

Chick cardiomyocytes, grown for 72h and serum starved for the last 16h, were treated with ceramide (Cer) 300 µM for 6 h, with or without SB 202190 (SB) 500 nM or PD 98059 (PD) 1 µM pretreatment for 1 h. Cells were lysed and mitochondrial and cytosolic fractions were run on a 12% PAGE gel.

**Panel A** - Samples were immunoblotted for either p38 or phospho-p38. Phosphorylated p38 was identified with the positive control (C6 glioma cells treated with anisomycin; provided by Cell Signaling Technologies). Representative blot of 4 separate experiments.

**Panel B** - Densitometric analysis of phosphorylated p38 of 4 separate experiments are summarized. Sample integrated densities (Number of pixels/area) are expressed relative to control integrated density. ** p<0.01 compared to control; ΔΔ p<0.01 compared to ceramide.

**Panel C** - To determine the purity of the mitochondrial isolation, samples were immunoblotted the mitochondrial marker cytochrome C and actin to establish that all changes in protein expression were not due to unequal loading of proteins.
<table>
<thead>
<tr>
<th></th>
<th>SB+ PD+</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>C</td>
<td>Cer</td>
<td>Cer</td>
<td>Cer</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
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</table>

**Mitochondria**

17kDa

**Cytosol**

47kDa
Figure G6 - SB 202190, but not PD 98059, inhibited loss of mitochondrial potential induced by ceramide - Cardiomyocytes were treated with diluent (control) (N=4), ceramide, 300 μM for 6h (N=4) without, or with either the p38 inhibitor SB 202190 (SB) (N=4) or MEK inhibitor PD98059 (PD)(N=4). Cardiomyocytes were examined by flow cytometry using DePsipher™ and the number of cells exhibiting green/red were expressed relative to control. The data with ceramide alone are repeated from Fig 4C for ease of comparison. * p<0.01 compared to control; Δ p<0.05 compared to ceramide.
ceramide 300 μM for 6 h, was significantly reduced (p<0.05) from the 1.8±0.4 fold with ceramide to 1.4±0.1 (N=4) by treatment with the p38 inhibitor SB 202190.

DISCUSSION

This section presents several novel findings with p38 MAPK. First, the p38 MAPK inhibitor SB 202190, but not the MEK/ERK inhibitor PD 98059, reduced ceramide-induced loss of mitochondrial pore potential and cell death. Second, phosphorylated p38 MAPK was shown to be phosphorylated in mitochondria in the same time frame as the observed ceramide-induced mitochondrial effects. Ceramide induced phosphorylation of SAPK and reduced phosphorylation of ERK further implicates a role for MAPK in the mitochondrial pathway of apoptosis.

Ceramide was demonstrated to clearly alter mitochondria potential based on the use of a mitochondrial-specific dye. This dye or very similar structural analogues such as JC-1, have been shown to accurately reflect mitochondria membrane potential (Jiang S et al. 1999; Bowser et al. 1998; Zamzani et al. 1995). Importantly, comparative studies have shown that they are the preferred agent for assessment of in cardiac cells (Mathur et al. 2000).

It is possible that the previous studies which failed to show an effect of ceramide on mitochondria (Quillet-Mary et al. 1997; Garcia-Ruiz et al. 1997; Pastorino et al 1999) may have been due to differences in the methods of observing mitochondrial effects. In Quillet-Mary et al.’s study (1997), they reported that ceramide had no influence on mitochondrial membrane potential using the dye DiOC₆. However, an in depth comparative study performed by Salviolo et al. (1997) showed that DiOC₆ is not as reliable fluorescent probe to assess ΔΨₘ as JC-1. In García-Ruiz et al.’s study (1997), ceramide did not reveal any significant change in mitochondrial membrane permeability, assessed by spectrophotometric measurement of
mitochondrial swelling. There are two major issues regarding this method: use of isolated mitochondria and inference of mitochondrial permeability due to swelling. First, observation of mitochondrial size by spectrophotometry has been established (Botla et al. 1995) allows for a great degree of error as contamination by other cellular organelles will change absorbance readings. Rather, flow cytometric measurement of mitochondrial size is a more accurate measure without the possibility of contamination (De Gannes et al. 1998). Second, the use of isolated mitochondria does not permit the study of intracellular interaction with mitochondria resulting in mitochondrial effects. As in the study with Pastorino et al. (1999), ceramide did not induce any mitochondrial effects unless the pro-apoptotic member of the Bcl-2 family, Bax, was present. Hence, ceramide would not induce a pro-apoptotic mitochondrial effect in isolated mitochondria because of the absence of intracellular Bax. The present section is novel in that mitochondrial changes were observed with the most prescribed dye, the JC-1 analogue DePsipher, and in intact cells to permit intracellular component interaction with the mitochondria.

**P38 MAPK localization in the mitochondria**

This section showed a presence of p38 MAPK localizing to the mitochondria and activated, phosphorylated p38 MAPK upon ceramide treatment and that this localized activation was inhibitable by the p38 MAPK inhibitor SB 202190 but not by the MEK/ERK inhibitor PD 98059. I recognize that the isolation of mitochondria relied on ultracentrifugation which may not represent only the mitochondria constituents of the cells. However, the protocol that I used consistently yields a highly enriched mitochondrial fraction with little contamination by lysosomes or mitochondria as ascertained by electron microscopy or assessment of mitochondrial enzyme activity (Botla et al 1995). Further, these data demonstrated that it was rich in the mitochondrial marker cytochrome C which was not detectable in control sample (ie-intact mitochondrial prep) cytosolic preparation. This section also demonstrated that upon ceramide
treatment, there was a loss of mitochondrial cytochrome C with a concurrent significant increase in cytosolic cytochrome C, a phenomenon which has been previously reported by several groups (for review, see Richter and Ghafourifar 1999) Phosphorylated p38 in the mitochondria fraction upon ceramide treatment was coincident with the same concentration and time that elicited loss of ΔΨm and loss of DNA content, suggesting that p38 is active and necessary to participate in ceramide's mitochondrial effects. A ceramide-induced release of cytochrome C was observed at the same ceramide treatment conditions suggesting that ceramide also induces an increase in mitochondrial permeability and loss of cytochrome C from the mitochondria. Cytochrome C release activates the caspase cascade leading to apoptosis (Green & Reed 1998). These results suggest a physical link between p38 MAPK activity and mitochondrial effects.

This novel finding of phosphorylated p38 in the perimitochondrial fraction can be supported by the finding that p38 may affect mitochondria during apoptosis (Galan et al 2000; Zhuang et al 2000; Chen et al 2000). Although phosphorylated p38 has been reported to affect mitochondria during apoptosis (Galan et al. 2000; Zhuang et al. 2000; Chen et al. 2000), this is the first study, to my knowledge, that p38 has been located in or with mitochondria. Zhao et al (2001) reported p38 phosphorylation during the opening of mitochondrial ATP-sensitive K+ channels in mouse hearts. I speculate that p38 may directly interact with mitochondrial channels resulting in an increase in mitochondrial permeability and hence, a loss of ΔΨm. Similarly p38 may directly interact with a Bcl-2 family member as the Bcl-2 family is important in mitochondrial responses during apoptosis. The latter speculation is supported by Kharbanda et al (2000) who reported a translocation of SAPK to mitochondria for functional interactions with Bcl-xL in the apoptotic response to genotoxic stress.
Mitochondrial kinases

There have been previous studies of other kinases localizing to mitochondria. Ruvolo et al. (1998) reported mitochondrial localization of PKCα in association with Bcl-2 phosphorylation during resistance to drug-induced apoptosis in human pre-B REH cells. Similarly, Majumder et al. (2000) reported translocation of PKCδ from the cytoplasm to the mitochondria during cytochrome C release and apoptosis induced by the phorbol ester TPA. Harada et al. (1999) reported that PKA anchors to the mitochondria thus permitting kinase/substrate interaction which results in the phosphorylation and inactivation of BAD in response to a survival factor.

Hexokinases have also been reported to interact with the MTP component voltage-dependent anion channel (Gottlob et al. 2001)

There have also been reports of intramitochondrial kinases involved in apoptosis. Kohler et al. (1999) reported of adenylate kinases localized in the mitochondrial intermembrane space and mitochondrial matrix in which their release from them mitochondria during apoptosis may be involved in the apoptotic process. Damuni and Reed (1988) reported of a soluble mitochondrial kinase with type II casein kinase properties. Thus, it is possible that p38 may localize to the mitochondria to induce an intramitochondrial signaling mechanism to induce apoptosis.

The role of p38 MAPK pathway in apoptosis

Although p38 has been implicated in apoptosis in some cell types (Jain et al. 1998; Hida et al. 1999; Tavarini et al. 2000), the role of p38 in ceramide-induced apoptosis in heart remains to be defined. The present section sought to identify the role of p38 in ceramide-induced apoptosis in cardiomyocytes with the use of the p38 inhibitor SB 202190 at concentrations (500 nM) in excess of its IC50 for p38 MAPK (350 nM) (Singh et al. 1999). This inhibition significantly decreased ceramide-induced low DNA content and loss of ΔΨm. This thesis also showed that
MEK/ERK inhibition with PD 98059 had no effect on ceramide-induced apoptosis. This is to be expected considering ceramide reduced ERK phosphorylation. These data suggest that ceramide induced effects involve the p38 MAPK activation and that inhibition of an already deactivated ERK pathway has no effect on ceramide-induced cell death. My findings can be supported by previous reports (Zhuang et al 2000; Galan et al 2000; Iwama et al 2001). Zhuang et al (2000) reported, in human leukemia cells, inhibition of p38 phosphorylation also inhibited DNA fragmentation (assessed by DNA laddering), decrease in $\Delta \Psi_m$ (assessed by JC-1), and caspase-3 activation (assessed by western blot of cleaved caspase-3) induced by reactive oxygen species. In addition, Zhuang et al (2000) hypothesized that p38 signals to activate caspase-3 resulting in apoptosis. This study's results are also consistent with the observation of Galan et al (2000) who reported a role for p38 in cadmium-induced apoptosis in promonocytic U937 cells. Galan et al (2000) reported that there was a rapid phosphorylation of p38 concomitant with apoptotic DNA laddering and that p38 inhibition with SB 203580 decreased i) apoptotic DNA degradation, assessed by DNA content observed by flow cytometry, ii) phosphatidyl serine exposure, assessed by annexin V staining observed by flow cytometry and iii) loss of $\Delta \Psi_m$, assessed by flow cytometric analysis of JC-1 dye loading. Although apoptosis was attenuated by p38 inhibition with SB 203580, apoptosis was not affected by PD 98059, suggesting that the MEK/ERK pathway was not involved (Galan et al. 2000). The present section also agrees with results of Iwama et al. (2001) who reported in arsenic-treated U937 cells of a concomitant p38 activation and a deactivation in ERK during loss of $\Delta \Psi_m$, cytochrome C release, and apoptosis.

P38 MAPK has been previously studied in the heart. Myocardial ischemia and reperfusion were shown to activate p38 MAPK in vivo (Wang et al. 1998b; Bogoyevitch et al. 1996; Yin et al. 1997). Ischemia alone caused a moderate increase in p38 MAPK while reperfusion after ischemia further increased p38 MAPK (Ma et al. 1999). Administration of the
p38 MAPK inhibitor, SB 203580, before the ischemic insult resulted in dose-dependent inhibition of p38 MAPK, decreased apoptosis, and diminished consequences of ischemia/reperfusion such as recovery of coronary flow, cardiac contractility and left ventricular pressure (Ma et al. 1999). Considering the increase in ceramide levels and cytochrome C release from mitochondria in myocardial ischemia, infarction, and cardiomyopathy (for review, see Gottlieb & Engler; Narula et al. 1999), may provide new evidence to link the process of ischemic injury and ceramide formation to p38 activation then to the mitochondria death pathway involving cytochrome c release.

Wang et al (1998b) reported a role for p38 MAPK in cardiac apoptosis. Wang et al (1998b) reported that in mouse hearts after chronic transverse aortic constriction, p38 MAPK activity was significantly increased. Dissecting the p38 response into p38α and p38β pathways with adenoviral vector expression, p38β was found to be responsible for the hypertrophic response whereas p38α was responsible for apoptosis. Hence, further studies need to be performed to determine which p38 isoform is responsible for the observed p38 mitochondrial and apoptotic effects. This thesis has improved on these studies by observing in vitro, a direct link between p38 activation and mitochondria, an observation that is difficult in lengthy (i.e. time greater than 24 h) in vivo samples. As well, this thesis used in vitro cells (>20%) whose rates of apoptosis are greater than that observed biologically (<0.2%) (Olivetti et al. 1997). Hence, reductions in apoptosis are more easily observed when there is significant apoptosis.

Role of SAPK/JNK and MEK/ERK pathways in apoptosis

My findings that ceramide increases SAPK/JNK phosphorylation in cardiomyocytes is consistent with the contention that SAPK/JNK may play a major role in apoptosis in heart (for review, see Feuerstein & Young 2000). The SAPK/JNK cascade is a stress response system activated by stresses similar to those signaling ceramide generation (for review, see Paul et al. 2000).
SAPK/JNK phosphorylation in response to ceramide is robust and ceramide’s lethality is associated with a strong stimulation of SAPK/JNK in some cell lines (Jarvis et al. 1997). Data suggest that mitochondria are influenced by pro-apoptotic signal transduction through the SAPK/JNK pathway (Tournier et al. 2000; Takada et al. 2001, Jendrossek et al. 2001). Similar to this section, co-activation of both SAPK/JNK and p38 MAPK induced apoptosis in the rat heart (Bogeyevitch et al. 1996).

My findings are consistent with a current hypothesis that apoptosis is produced by an activation of a pro-apoptotic kinase (SAPK) program with a deactivation of the anti-apoptotic (ERK) program (Xia et al. 1995). The ERK deactivation observed in this section agrees with previous reports of active MEK/ERK pathway conferring protection against apoptosis (Erhardt et al. 1999). In this section, the decrease in ERK phosphorylation in response to ceramide is consistent with the lack of an effect of PD 98059 on ceramide-induced apoptosis, as a further inactivation of ERK with PD 98059 would not be anticipated to affect ceramide-induced apoptosis. Similar to these results, Cartee et al. (2001) reported a sustained activation of MEK/ERK that was insensitive to the MEK inhibitor U0126 during mitochondrial dysfunction and apoptosis in U937 cells exposed to cyclin-dependent kinase inhibitor and PMA in U937 cells. Taken together, I speculate that a deactivation of the ERK pathway permits a pro-apoptotic response.

CONCLUSION

In summary, part of the pathway through which ceramide induces apoptotic cell death involves p38 MAPK. This section demonstrated that ceramide-induced apoptosis and loss of $\Delta \Psi_m$ occurs in part through p38 MAPK as the p38 MAPK inhibitor, SB 202190, reduced ceramide-induced loss of DNA content and loss of $\Delta \Psi_m$. As well, because phosphorylated p38
was observed in the mitochondrial-enriched fraction, I speculate that p38 MAPK in the mitochondria or in close proximity to the mitochondria plays an important role in ceramide’s mitochondrial effects. The changes in p38 MAPK are part of the effect of ceramide on MAPK that includes simultaneously induction of the pro-death SAPK/JNK and p38 MAPK pathways and deactivation of the anti-apoptotic, pro-growth ERK pathway. Taken together the presence of MAPK in mitochondria, the similar extent of the reduction in p38MAPK phosphorylation and reduction in apoptosis plus the ability of SB 202190 to reduce ceramide-induced changes in $\Delta \Psi_m$, suggest a role for MAPK in the mitochondrial pathway wherein ceramide induces apoptosis.
SUMMARY

As palmitate oxidation yields acetyl CoA which is the substrate for cholesterol synthesis, the objective of this chapter was to determine the effect of interruption of cholesterol synthesis on cardiomyocyte cell death. In embryonic chick cardiomyocytes, palmitate was co-treated with the HMG-CoA reductase inhibitor lovastatin. Lovastatin inhibits cholesterol biosynthesis, a pathway which uses the fatty acid oxidation byproduct acetyl CoA. It was hypothesized that a combination of lovastatin and palmitate would increase fatty acid oxidation but also cause an accumulation of oxidation byproducts. The combination of lovastatin and palmitate produced a significant amount of apoptosis that was greater than lovastatin or palmitate-induced apoptosis alone. These results suggest that palmitate-induced apoptosis may also occur due to an accumulation of fatty acid oxidation byproduct acetyl CoA.

As lovastatin was observed to induce apoptosis on its own, lovastatin’s other effects were investigated. Lovastatin-treated cardiomyocytes displayed distinct changes in cellular morphology that coincided with a loss of F-actin. As lovastatin induced apoptosis, I examined the possible role of another apoptotic signaling pathway, the caspases. In particular, caspase 2 and -3 were investigated as representatives of initiator and executioner caspases, respectively. Cardiomyocytes were pretreated with caspase 2 and caspase 3 inhibitors prior to lovastatin and the resulting apoptosis and F-actin effects were observed. Lovastatin induced apoptosis and loss of F-actin was significantly blocked by caspase 2 and 3 inhibitors, suggesting that lovastatin-induced changes in cellular structure and apoptosis were mediated via a caspase pathway.
Due to the ability of lovastatin to inhibit HMG-CoA reductase, downstream events such as prenylation and cholesterol synthesis were inhibited. The addition of mevalonic acid during lovastatin treatment allows events downstream of HMG-CoA reductase to continue, even though HMG-CoA reductase continues to be inhibited by lovastatin. Mevalonic acid pretreatment blocked lovastatin-induced apoptosis and loss of F-actin suggesting that the downstream events of HMG-CoA reductase, such as prenylation, are involved in cell survival.

**INTRODUCTION**

Increased palmitate metabolism results in the production of acetyl CoA (for review, see Lopaschuk 1994a&b), a precursor of cholesterol synthesis. A competing hypothesis for palmitate-induced cell death include that palmitate may induce cell death by its inability to be completely metabolized or there is an accumulation of oxidation byproducts. It is hypothesized that these partially metabolized fatty acids, such as 3-ketoacyl-CoA, or oxidation byproducts, such as acetyl CoA, are toxic to cardiomyocytes. (Liedtke et al. 1978; Hendrickson et al. 1997).

Cholesterol synthesis has been widely investigated for its role in cardiovascular disease. HMG-CoA reductase is an important enzyme in the synthesis of cholesterol as it converts 3-hydroxy-3-methylglutaryl CoA, a trimer of acetyl CoA molecules generated from fatty acid oxidation, to mevalonic acid which is then metabolized leading ultimately to the production of cholesterol and other products used in prenylation. Statins are a class of inhibitors of HMG-CoA reductase. Statins have been studied in the past to inhibit HMG-CoA reductase in heart (Shmeeda et al. 1994). Statins are currently in clinical use for the inhibition of cholesterol synthesis. However, statins have been shown to induce apoptosis in various cell types including vascular smooth muscle cells (Guijarro et al. 1998), HL60 cells (Perez-Salsa & Mollinedo 1994), macrophages (Rogers et al 1996), prostate cells (Marcelli et al 1998), osteoclasts (Luckman et
al 1998), and mouse proximal tubular cells (Iimura et al. 1997). The loss of cardiomyocytes is considered to be the underlying basis for the complications of myocardial infarction and the development of heart failure. Lovastatin, as with other statins such as simvastatin and atorvastatin, has been implicated in apoptosis (Guijarro et al. 1998, Perez-Salsa et al. 1994, Rogers et al. 1996, Padayatty et al. 1997, Marcelli et al. 1998, Luckman et al. 1998).

As mentioned in the Introduction, mevalonic acid is a product of HMG-CoA reductase which can be further converted to metabolites within the cholesterol synthesis pathway. As lovastatin’s clinical effect is to inhibit HMG-CoA reductase, lovastatin treatment is associated with a deficiency of mevalonic acid (Repko and Maltese 1989). Although lovastatin has been observed to induce apoptosis (Guijarro et al. 1998, Perez-Salsa et al. 1994, Rogers et al. 1996, Padayatty et al. 1997, Marcelli et al. 1998, Luckman et al. 1998), it is unclear whether this is due to the inhibition of HMG-CoA reductase causing an accumulation of upstream products or to the absence of a HMG-CoA downstream products. The present section investigated this question.

Caspases have been implicated in both the induction and prevention of apoptosis (for review, see Green and Reed 1998). Caspases have also been implicated in the morphological changes associated with apoptosis (Kothakota et al. 1997). Caspase-2 and caspase-3 will be investigated in this chapter. Caspase-3 was investigated as it is an established member of the pathway leading to apoptosis (for review, see Green and Reed 1998). Caspase-3, in particular, has been observed to rapidly cleave gelsolin, an actin-modulating protein (Kothkota et al. 1997). In contrast, caspase-2 was investigated due to its incorporation into the membrane. It was postulated that as lovastatin affects the prenylation and hence membrane incorporation of important signaling molecules such as Rho (Koch et al. 1997), caspase-2 may be involved because of its proximity to the membrane.
In some cells, statin-induced apoptosis and structural alterations are due to effects on filamentous, polymerized F-actin in HL-60 and rat renal proximal tubular cells (Levee et al. 1996; Fenton et al. 1992; Koch et al. 1997). F-actin is the backbone of microfilaments and influences overall cellular morphology so that changes in actin polymerization and depolymerization are vital to the cell. Broad spectrum stresses, such as oxidant stress and ATP depletion, cause depolymerization of F-actin that is associated with changes in cellular morphology similar to that observed with lovastatin (Hinshaw et al. 1991 & 1993).

The objective of this section is to determine whether the accumulation of acetyl CoA by the concomitant inhibition of HMG-CoA reductase and increased fatty acid oxidation with palmitate is responsible for palmitate-induced cell death. In the second objective, I will determine whether lovastatin's effects are due to its inhibition of HMG-CoA reductase alone or whether the interruption of cholesterol biosynthesis is involved using mevalonate to re-initiate cholesterol biosynthesis during lovastatin treatment. I will also determine the role of capsase-2 and -3, if any, in the signaling involved in lovastatin-induced apoptosis and changes in morphology.

**RESULTS**

To first establish that inhibition of cholesterol synthesis will induce apoptosis in these cardiomyocytes, a dose curve of lovastatin-induced cell death was determined previously in this laboratory (Figure 1). Concentrations of 50μM and greater induced a significant increase in cell death, as assessed by the MTT assay. Lovastatin 100μM for 24h was chosen for further experiments as it induced significant (p<0.01) 19.8±5.1% (N=18) cell death without eradicating all viable, adherent cardiomyocytes.

To assess whether lovastatin 100 μM will induce apoptotic cell death, a dose curve was established. Cardiomyocytes were treated with lovastatin 10, 50, and 100 μM for 24h and their
Figure H1 - Lovastatin induced a loss of cell viability. Cardiomyocytes were treated with lovastatin at 1 (N=9), 10 (N=12), 50 (N=12), 100 (N=18), 200 (N=5) and 300 µM (N=8) for 24h and the cell viability assessed by MTT assay. A loss of MTT absorbance at 570nm signifies a loss of cell viability (inset). The loss of A570 were reported as percentages of loss of cell viability, or alternatively, percentage increases in cell death. ** p<0.01 compared to control. This figure was produced previously in this laboratory.
nuclei examined for the loss of DNA content, indicative of apoptosis (Fig. 2). Using the amount of PI staining within the nucleus as an indicator of DNA content, it was determined that the amount of PI fluorescence was a measure of nuclear DNA content. By analysis of histogram of PI (FL3) staining (inset), the number of cells with low DNA content was quantitated. (Fig. 2).

Lovastatin significantly (p<0.01) increased the number of nuclei with low DNA content: from control 1 030±65 to 4 794±384 (N=18) per 10 000 cells with 100 μM lovastatin.

Inferential data on the accumulation of acetyl CoA due to increased palmitate oxidation and HMG-CoA reductase inhibition, was examined as cardiomyocytes were co-treated with palmitate 100μM and lovastatin 100μM for 24h. Apoptosis was observed by flow cytometric analysis of both annexin V binding (Panel A) and nuclear DNA content (Panel B) (Figure 3). As both palmitate and lovastatin induced significant (p<0.01) increases in annexin V binding (1.3±0.1 N=8 and 1.6±0.1 N=4 fold greater than control, respectively) and low DNA content (2.0±0.1 N=14 and 4.5±1.1 N=4 fold greater than control, respectively), the combination of lovastatin plus palmitate induced a significant degree of apoptosis which was significantly (p<0.05) greater than palmitate (1.7±0.2 N=4 for annexin V and 6.8±1.9 N=4 for DNA content). This suggests that the combination of HMG-CoA reductase inhibition and increased palmitate oxidation with palmitate, produced an accumulation of acetyl CoA, can induce apoptosis in cardiomyocytes greater than palmitate alone.

Lovastatin not only causes an accumulation of acetyl CoA by inhibition of HMG-CoA reductase, but it also inhibits cholesterol biosynthesis. To determine whether it is lovastatin itself or the inhibition of cholesterol biosynthesis that is responsible for cell death, mevalonic acid was co-treated with lovastatin. The addition of mevalonic acid, a downstream product of the point of lovastatin inhibition, should help determine whether lovastatin-induced effects are reversible if cholesterol synthesis was allowed to continue but still retain an inactive HMG-CoA insert Fig H2.
Figure H2 - **Lovastatin induced a loss of DNA content** - Cardiomyocytes treated with lovastatin for 24 h (N=3-18) were examined by flow cytometry using PI fluorescence as a measure of nuclear size in permeabilized cardiomyocytes. Lovastatin induced a significant (**p<0.01**) increase the number of cells exhibiting low DNA content. Inset - Representative histogram from one of 18 separate experiments.
Figure H3 - *Combination of lovastatin and palmitate induce a significant enhancement of death* - Cardiomyocytes were co-treated with 100 μM palmitate (N=14), 100 μM lovastatin (N=4), or the combination of the two (N=4) for 24h. Cells were then stained with either annexin V (Panel A) or permeabilized and then stained with PI to assess DNA content (Panel B) and analyzed by flow cytometry. Data are presented as the fluorescence observed with either annexin V or PI relative to control fluorescence. **p<0.01 compared to control; Δ p<0.05 compared to palmitate.
reductase. Mevalonic acid significantly (p<0.01) but only partially reduced lovastatin-induced loss of DNA content by 18.3±5.5% (N=8) (Fig 4). This suggests that lovastatin-induced apoptosis is in part, but not totally, due to inhibition of cholesterol biosynthesis.

To identify which form of death is induced by lovastatin, cells were dually stained with FDA and PI (Figure 5). Early apoptosis is detected in cells which still express membrane integrity (low PI) with a loss of intracellular enzyme activity (low FDA) whereas oncosis is identified by cells which do not express membrane integrity (high PI) with a loss of intracellular enzyme activity (low FDA). Lovastatin induced an increase in apoptosis, as evidenced by the increased population of low FDA and low PI (Figure 5 - Panel A). Lovastatin induced a significant (p<0.01) increase in apoptotic cells, from control 251±27 (N=6) to lovastatin 2874±993 (N=6) (lower left, Panel B). Mevalonic acid pretreatment significantly (p<0.01) inhibited lovastatin-induced apoptosis to 2185±903, or by 33.5±7.2% (N=6) (upper left, Panel B). Mevalonic acid, however, did not affect lovastatin-induced oncosis (right side, Panel B). These results suggest that lovastatin may also produce apoptosis via another pathway, independent of inhibition of cholesterol biosynthesis.

As caspases are involved in apoptotic signaling (for review, see Green & Reed 1998), the role of caspases in lovastatin-induced apoptosis was investigated, in particular caspase-2 and -3. To identify the role of caspase-2 and -3 in lovastatin treatment, the caspase-2 and -3 inhibitors, z-VDVAD-fmk and Ac-DEVD-CHO respectively, were used. Both inhibitors were added 1 hour before lovastatin treatment and the nuclear DNA content was determined (Figure 6). zVDVAD, 100 μM, significantly (p<0.05) reduced lovastatin-induced loss of DNA by 19.1±8.3% (N=6) whereas AcDEVD, 100μ M, significantly (p<0.01) reduced it by 25.1±6.6% (N=3) (Fig. 6b).

To determine whether caspase-2 and -3 were involved in lovastatin-induced apoptosis and oncosis, cardiomyocytes were treated with lovastatin 100 μM with or without zVDVAD 100
Figure H4- Lovastatin induced a loss of DNA content that is partially blocked by mevalonic acid - Cardiomyocytes treated with lovastatin, 100 μM for 24 h (N=8), with or without a 1 h pretreatment with mevalonic acid 100 μM, were examined by flow cytometry using PI fluorescence as a measure of nuclear size in permeabilized cardiomyocytes. Considering lovastatin induced loss of DNA content is 5 fold greater than that of control (inset), lovastatin is expressed as maximal death or 0% survival. Mevalonic acid pretreatment significantly (ΔΔp<0.01) reduced the amount of apoptotic nuclei induced by lovastatin.
Figure H5 - *Lovastatin induces apoptosis that is blocked by mevalonic acid* - Cardiomyocytes treated with lovastatin, 100 μM for 24h (N=6), with (N=6) or without (6) a 1 h pretreatment with mevalonic acid 100 μM, were examined by flow cytometry using PI fluorescence as a measure of membrane integrity and fluorescein diacetate as a measure of intracellular esterase activity. Early apoptosis is determined by cells which still express membrane integrity (low PI) with a loss of intracellular enzyme activity (low FDA).

Panel A - Representative dotplots from one of 8 separate experiments.
Panel B - Left side is dedicated to apoptotic cells (low FDA and low PI) whereas right side is dedicated to oncotic cells (low FDA and high PI). Lower panels are the number of cells exhibiting apoptotic or oncotic characteristics in a population of 10 000 cells. Considering lovastatin-induced apoptosis is almost 30 fold greater than that of control (2.5 fold for oncosis), lovastatin is expressed as maximal death or 0% survival. Upper panels are the % reduction of lovastatin-induced apoptosis/oncosis due to mevalonate. Mevalonic acid significantly (ΔΔp<0.01) reduced the amount of apoptosis induced by lovastatin.
A

Control

Lovastatin

Mevalonic Acid

Mev + Lov

Apoptosis

Oncosis
**APOPTOSIS**

![Graph showing % reduction in lovastatin-induced apoptosis](image)

**ONCOSIS**

![Graph showing % of lovastatin-induced oncosis](image)

![Graph showing number of cells exhibiting apoptosis per 10,000 cells](image)

![Graph showing number of cells exhibiting oncosis per 10,000 cells](image)
Z-VDVAD + Lovastatin  
Ac-DEVD + Lovastatin

Figure H6 - Lovastatin induced a loss of DNA content that is partially blocked by caspase inhibitors - Panel A - Representative histograms of cardiomyocytes treated with lovastatin, 100 μM for 24 h (N=8), with or without a 1h pretreatment with either caspase-2 (zVDVAD) or caspase-3 inhibitor (Ac-DEVD) (100μM for both) (N=4), using PI fluorescence as a measure of nuclear size in permeabilized cardiomyocytes. Panel B - Lovastatin is expressed as maximal death or as 0% survival. Both caspase 2 and caspase 3 inhibitor significantly (Δp<0.05, ΔΔp<0.01) reduced the amount of apoptotic nuclei induced by lovastatin.
\% reduction in lovastatin-induced apoptosis (nuclei with low DNA content)

<table>
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<th>Lovastatin</th>
<th>Z-VDVAD+Lov</th>
<th>Ac-DEVD+Lov</th>
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<td>% Reduction</td>
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<td>P-Value</td>
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**Figure B**
μM or AcDEVD 100μM pretreatment and dually stained with FDA and PI (Figure 7).

Lovastatin induced a significant (p<0.01) increase in the number of cells exhibiting apoptosis, from control 249±31 (N=7) to lovastatin 3150±793 (N=7), which was significantly (p<0.01) reduced by zVDAVD and AcDEVD to 1806±487 (N=6) and 2804±999 (N=3), respectively (Panel B), or by 42.7±7.1 and 11.0±6.7% (Panel C). Although lovastatin induced a significant increase in oncosis, from control 1 557±457 to 3 520±183 (N=7) per 10 000 cells with lovastatin, caspase-2 and -3 inhibition with zVDVAD or AcDEVD did not affect the development of oncosis.

In addition to inducing cell death, lovastatin has been reported to induce changes in cell morphology (Koch et al. 1997). Effects of changes to cell morphology by F-actin were visualized by staining with NBD Phallacidin (Figure 8). Lovastatin (Panel C) induced a dramatic loss of intact F-actin compared to another apoptosis inducer palmitate (Panel B). As palmitate has been shown to induce apoptosis in these cardiomyocytes (Kong and Rabkin 2000b), it was used as a positive control for the effects of F-actin on cell death via apoptosis. As palmitate did not show a disruption in F-actin, the loss of F-actin induced by lovastatin may be attributed to lovastatin’s mechanism of action. In addition, it can be concluded the inhibitors zVDVAD (Panel D) and AcDEVD (Panel E) recovered some of the loss of F-actin due to lovastatin.

To quantitate lovastatin-induced effects on cell morphology, cardiomyocytes were stained with the F-actin specific dye NBD-phallacidin and its fluorescence measured on flow cytometry. Lovastatin 100μM induced a significant (p<0.01) increase in number of cells exhibiting low F-actin fluorescence (FL1<10^2): from control 998±76 per 10 000 cells to 4 172±459 per 10 000 cells (N=14) with lovastatin (Figure 9). Caspase-2 and -3 inhibition with zVDVAD and AcDEVD reduced lovastatin-induced loss of F-actin by 21.4±7% (N=8) (p<0.01) and 20.9±8.8% (N=4 (p<0.05), compared to that of lovastatin (Figure 10).
Figure H7 - Lovastatin induced apoptosis that is partially blocked by caspase inhibitors - Cardiomyocytes treated with lovastatin, 100 μM for 24h (N=8), with or without a 1h pretreatment with either caspase-2 (zVDVAD) or caspase-3 inhibitor (AcDEVD) (100 μM for both), were examined by flow cytometry using PI fluorescence as a measure of membrane integrity and fluorescein diacetate as a measure of intracellular esterase activity.

Panel A - Representative dotplot from one of 8 separate experiments.
Panel B - The number of cells exhibiting either apoptosis (low FDA, low PI) or oncosis (low FDA, high PI) in a population of 10 000 cells.
Panel C - Apoptosis or oncosis induced by lovastatin is expressed as maximal death or 0% survival. Both zVDVAD and AcDEVD significantly (ΔΔp<0.01) reduced the amount of apoptosis, or increased the amount of survival, induced by lovastatin. In contrast, neither zVDAVD nor AcDEVD affected the amount of oncosis induced by lovastatin.
Figure H8 - *Lovastatin induced a loss of morphology and F-actin* - Cardiomyocytes, grown on coverslips, were treated with diluent (A), palmitate 100 μM (B), lovastatin 100 μM (C), zVDVAD 100 μM pretreatment + 100μM lovastatin (D), or AcDEVD 100 μM pretreatment + 100 μM lovastatin (E) for 24h. Cells were fixed and stained with NBD-Phallacidin and visualized in a fluorescent microscope.
Figure H9 - Lovastatin induced a loss of F-actin - Panel A - Cardiomyocytes treated with lovastatin, 50 (N=3) or 100 μM for 24h (N=14) and were examined by flow cytometry using NBD-Phallacidin fluorescence as a measure of intact F-actin. Inset - Representative histogram of 14 separate experiments.
**Z-VDVAD + Lovastatin**

**Ac-DEVD + Lovastatin**

Figure H10 - *Lovastatin induced a loss of F-actin structure that is partially blocked by caspase inhibitors* - Cardiomyocytes treated with lovastatin, 100 μM for 24 h (N=14), with or without a 1 h pretreatment with either zVDVAD (N=8) or AcDEVD (N=4) (100μM for both), were examined by flow cytometry using NBD-Phallacidin fluorescence as a measure of intact F-actin.

Panel A - Representative histograms of one of 4 separate experiments.

Panel B - Considering lovastatin is expressed as maximal death or 0% survival, both zVDVAD and AcDEVD significantly (Δp<0.05, ΔΔp<0.01) reduced the loss of F-actin induced by lovastatin.
% reduction in lovastatin-induced loss of F-actin

Lovastatin  Z-VDVAD+Lov  Ac-DEVD+Lov

B

ΔΔ  Δ
To determine whether lovastatin-induced effects on F-actin are due to the inhibition of cholesterol biosynthesis, the effects of mevalonic acid on lovastatin-induced loss of F-actin was observed by and quantitated with NBD-phallacidin (Figure 11). Mevalonic acid significantly (p<0.01) but partially reduced lovastatin-induced loss of F-actin by 29.8±8.6% (N=7). These results suggest that lovastatin induced changes to cell morphology via F-actin by preventing a downstream cholesterol-related mechanism to occur. As mevalonic acid inhibition was partial (29.8%), these results suggest lovastatin is inducing apoptosis in another mechanism independent of cholesterol biosynthesis. Perhaps, lovastatin-induced apoptosis and changes to morphology may be due to a caspase-2 and -3 dependent mechanism.

DISCUSSION

These experiments determined that HMG-CoA reductase inhibition with lovastatin co-treated with palmitate, induced significant (p<0.01) apoptosis that was significantly (p<0.05) greater than palmitate alone. To evaluate whether it is the accumulation of upstream substrates (e.g. acetyl CoA) produced by HMG-CoA reductase inhibition or whether the inhibition of cholesterol synthesis is responsible for lovastatin-induced apoptosis, mevalonic acid was co-treated with lovastatin to permit cholesterol biosynthesis to re-activate with concomitant HMG-CoA reductase inhibition with lovastatin. Mevalonic acid was found to significantly (p<0.01) inhibit approximately 20-30% of lovastatin-induced apoptosis and F-actin effects. This suggests that lovastatin induces a separate apoptotic mechanism, independent of the mevalonate pathway. Further investigation determined that lovastatin induced cell death, in part, via caspase-2 and caspase-3. Caspase-2 and -3 inhibition with zVDVAD and AcDEVD, respectively, significantly (p<0.05) for caspase-2, but partially inhibited lovastatin-induced apoptosis and loss of F-actin by approximately 20-30%. Taken together, these results raise the
Figure H11 - **Lovastatin induced a loss of F-actin structure that is partially blocked by mevalonic acid** - Cardiomyocytes treated with lovastatin, 100 μM for 24 h (N=8), with or without a 1h pretreatment with mevalonic acid (N=8), were examined by flow cytometry using NBD-Phallacidin fluorescence as a measure of intact F-actin. Inset - The number of cells exhibiting low NBD-Phallacidin in a population of 10,000 cells. Considering lovastatin induced loss of F-actin is 4 fold greater than that of control (inset), lovastatin is expressed as maximal death or as 0% survival. Mevalonic acid significantly (ΔΔp<0.01) reduced the loss of F-actin induced by lovastatin.
possibility that lovastatin induced apoptosis partially by inhibition of cholesterol biosynthesis (20-30%), by a caspase-2 sensitive mechanism (20-30%), and by a caspase-3 sensitive mechanism (20-30%). In other words, lovastatin induces apoptosis and changes in cell morphology in more than one death pathway. An alternate postulate is that these pathways are linked in series so that blockade of any one of them produced a 30% reduction.

This is the first work, to my knowledge, to observe lovastatin-induced apoptosis in cardiomyocytes. However, lovastatin, as well as other HMG-CoA reductase inhibitors, have been observed to induce apoptosis in other cell models including mouse proximal tubular cells (Iimura et al. 1997), HL-60 (Perez-Sala et al. 1994), vascular smooth muscle cells (Guijarro et al. 1998), macrophages (Rogers et al. 1996), prostate cells (Marcelli et al. 1998), and osteoclasts (Luckman et al. 1998). Lovastatin has also been reported to induce oncosis in HL-60 cells (Hunakova et al. 1997). Lovastatin was found to inhibit HMG-CoA reductase activity at EC_{50} 80 μM (Rao et al. 1999) and the concentration used in the present section, 100 μM, has also been used in previous reports (Guzman et al. 1993; Guijarro et al. 1998). Marcelli et al. (1998) and Perez-Sala et al. (1995) used 10-50 μM of lovastatin for 24h and also showed significant apoptosis. Thus, I speculate that the apoptosis observed in this section with 100 μM lovastatin induces significant HMG-CoA reductase inhibition. The proposed mechanisms for lovastatin’s effects are numerous, including lovastatin-induced interruption of 1) prenylation (Repko and Maltese et al. 1989), 2) protein synthesis (Coxon et al. 1998; Vitale et al. 1999), and 3) translocation of G-proteins such as Ras and Rho (Koch et al. 1997; Kahiwagi et al. 1998).

Another proposed mechanism for lovastatin’s effect is discussed by Rao et al. (1999). Rao reported that lovastatin, particularly its pro-drug form, arrests cells by inhibiting the proteasome which leads to cell cycle arrest at G1. As this is a documented observation in MDA-MB-0157 breast cancer cell line, my study contradicts Rao’s results as my results did not observe
any growth arrest upon lovastatin treatment. Figure 2 displays a representative cell cycle DNA content histogram with distinct apoptotic, G0/G1 (quiescent), S (DNA synthesis), and G2/M (mitosis) peaks. If lovastatin were inducing G1 arrest in cardiomyocytes, then I would expect to see an increase in G0/G1 peak with a lack of a S or G2/M peak. Instead, I observed, upon lovastatin treatment, that there was a marked S and M peak with an actual decrease in G0/G1 which is presumably attributed to the increase in apoptotic cells. Thus, I do not believe that lovastatin induces apoptosis by cell cycle arrest in cardiomyocytes.

In this thesis, pretreatment of cells with mevalonic acid, a product of HMG-CoA reductase, partially blocked lovastatin-induced effects, suggesting that the presence of mevalonic acid to continue cholesterol biosynthesis pathway is beneficial. The concentrations of mevalonate used in these experiments were proven effective to reduce the effects of lovastatin in other cell models (Wang & Macauley 1999; Sindermann et al. 2000; Rubins et al. 1998; Padayatty et al. 1997). Although the mevalonate effects presented in this section are significant but modest, this concentration of mevalonate reduced lovastatin’s effects by 20-30%. These results imply two concepts: i) part of lovastatin-induced effects are due to the interruption of cholesterol biosynthesis (approx. 20-30 %) which is attenuated with mevalonate, and ii) lovastatin-induced apoptosis is mediated by a different mechanism independent of cholesterol biosynthesis.

Inhibition of cholesterol biosynthesis results in several biologically significant effects. There are cholesterol requirements, particularly for membrane synthesis, as in cellular hypertrophy or cell division, and most cells meet this requirement primarily through endogenous cholesterol biosynthesis (Dietschy 1993). Importantly, this pathway leads to the synthesis of numerous nonsterol compounds that have roles in electron transport, glycoprotein biosynthesis and cell growth. Cell growth effects are mediated through isoprenylation, or attachment of cholesterol biosynthesis byproducts. Isoprenylation of several key cellular proteins, such as small GTPs,
lead to cell growth (Goldstein and Brown 1990; Russell et al. 1992). Other cholesterol byproducts such as dolichol is required for glycoprotein synthesis and ubiquinones are important in mitochondrial electron transport (Maltese et al. 1990). Hence, interruption of cholesterol biosynthesis can lead to several deleterious effects which can cause a cell to eventually die.

As mevalonate only reduced lovastatin-induced effects by 20-30%, this implies that lovastatin may induce apoptosis in another pathway independent of its effects on cholesterol biosynthesis. One such pathway may be caspases. As previously mentioned in the Introduction chapter, caspases have been established to be involved in mediating apoptosis. Caspases -2 and -3 were investigated, using their inhibitors zVDVAD and AcDEVD respectively, because caspase-2 is an example of a membrane-based initiator caspase and caspase-3 is a known apoptotic executioner caspase (for review, see Green and Reed 1998). zVDVAD is an established inhibitor of caspase-2 and has been used in ascertaining the role of caspase-2 in apoptosis in various studies (Gamen et al. 2000, Kasof et al. 2000). The results with these caspase inhibitors agree with previous reports of caspases in cardiomyocyte death and in heart disease (Holly et al. 1999; Harsdorf et al. 1999; Koglin et al. 1999; Narula et al. 1999; Black et al. 1998). As well, caspase inhibitors have been shown to prevent cardiomyocyte death in response to ischemia (Gottlieb et al. 1996; Holly et al. 1999; Yaoita et al. 1998). There is growing evidence of caspase-3 involvement in lovastatin-induced cell death (Coxon et al 1998; Wang et al. 2000; Vitale et al. 1999). Coxon et al. (1998) reported an increase in caspase-3-like activity was associated with statin-induced apoptosis. Wang et al (2000) also reported activation of caspase-3 during lovastatin treatment of HL-60 cells which led to the activation of DNAse II responsible for DNA fragmentation. Similarly, Vitale et al. (1999) report that caspase-3 activates a protease during cell death in thyroid cells. However, to my knowledge, there has been no work with caspase-2 and lovastatin-induced cell death. Hence, the data from this thesis will add to the
understanding of the mechanism of lovastatin-induced cell death. Nonetheless, more work will have to be done to elucidate the role for other caspases. There was a preliminary report of a role for caspase-7 in lovastatin-induced apoptosis (Marcelli et al. 1998).

In addition to inducing apoptosis, lovastatin has also been reported to affect the cytoskeleton in HL60 cells (Levee et al. 1996), renal proximal tubule cells (van De Water et al. 1996), renal carcinoma cells (Fenton et al. 1992) and NIH323 cells (Koch et al. 1997). The results in this chapter agree with previous reports by Koch et al. (1997) that lovastatin affects F-actin with no effect on the actin molecule. In addition, I observed that this mechanism involves caspase-2 and -3. There were previous reports of lovastatin-induced cell death and changes to actin were inhibitable by AcDEVD (Wang et al. 2000; Meijerman et al. 1999; Huot et al. 1998). Iimura et al (1997) reported that actin filament disruption preceded DNA fragmentation, suggesting a possible causal relationship between actin filament disruption and DNA fragmentation during apoptosis. Actin disruption may activate DNAses, via caspases, responsible for apoptotic nuclear degradation. Hence, it is possible that as caspase-3 executes apoptosis by cleavage of various substrates including actin, caspase-3 may simultaneously cause apoptosis and changes in cell morphology as actin is not longer present to maintain cellular morphology.

Taking the results with mevalonic acid and caspase inhibition together, I observed that lovastatin-induced effects can be partially inhibited by mevalonic acid, the caspase-2 inhibitor zVDVAD and the caspase-3 inhibitor AcDEVD. These results raise the possibility that lovastatin-induced effects involve at more than one pathway to apoptosis and changes in morphology.
The results from this chapter may have clinical implications in that lovastatin therapy in high concentration may be causing cardiomyocyte cell death concurrently with lowering cholesterol levels. As discussed in the Introduction, apoptosis will have grave consequences as cardiomyocyte apoptotic death will reduce the number of viable cells for cardiac function.

In summary, the work in this thesis is the first to observe lovastatin-induced apoptosis and changes in cellular cytoskeleton in cardiomyocytes, perhaps by HMG-CoA reductase inhibition. Lovastatin-induced effects are partially mediated by a caspase-2 and caspase-3 dependent mechanisms.
SECTION I - ASSOCIATION OF RHO B AND CASPASE-2: CHANGES WITH LOVASTATIN-INDUCED APOPTOSIS IN CARDIOMYOCYTES (Kong JY and Rabkin SW. Submitted to Biochimica Biophysica Acta)

SUMMARY

Lovastatin was shown to induce apoptosis in a previous chapter and to produce significant changes in cell morphology and F-actin content in a caspase-2 and -3 dependent manner in embryonic chick cardiomyocytes. Because cytoskeletal actin is regulated in part by RhoB and because caspases are involved in apoptosis, I sought to determine whether there was an association between RhoB and caspase-2 in neonatal mouse cardiomyocytes. Because all immunoreactive experiments performed in this chapter are specific to mouse rather than chick, neonatal mouse cardiomyocytes were used in this chapter. In neonatal mouse cardiomyocytes, lovastatin induced a significant (p<0.01) concentration dependent reduction in cardiomyocyte viability with an increase in apoptosis. Caspase-2 was shown to be involved in lovastatin-induced apoptosis and alteration in cytoskeletal F-actin as zVDVAD abrogated lovastatin’s effects. Lovastatin-induced changes to morphology were shown to be restricted to changes in F-actin rather than the actin molecule.

Having established a role for caspase-2 in lovastatin-induced apoptosis in mouse cardiomyocytes, the association between RhoB and caspase-2 was investigated. RhoB and caspase-2 were shown to associate as demonstrated by an immunoprecipitation with caspase-2 followed by immunoblot with RhoB and by an immunoprecipitation with RhoB followed by immunoblot with caspase-2. RhoB/caspase-2 association was observed mainly in the nuclear fraction, and to a lesser extent in the cytosolic fraction of the cell. Although Rho B presence was constant during lovastatin treatment, there was a time dependent caspase-2 association with
RhoB, with the peak occurrence at 2h of lovastatin treatment, in the nuclear fraction. This association was shown to be independent of caspase-2 activity as zVDVAD had no effect on RhoB/caspase-2 association. RhoB/caspase-2 association was determined to be specific as several controls (immunoprecipitates with the irrelevant antibody Troponin I-C, in the absence of cell lysate, and without the primary antibody) did not yield any associated complex. These data suggest that the novel Rho B/caspase-2 complex may modulate the role of Rho B or caspase-2 or both in the maintenance of F-actin and apoptosis.

INTRODUCTION

As discussed in the previous chapter, lovastatin produced significant changes to cell structure and F-actin concomitant with apoptosis. Thus, it was logical to determine whether the two phenomena were regulated by a common mechanism.

As mentioned in the Introduction, actin in its filamentous, polymerized form (F-actin) is the backbone of microfilaments so that changes in actin polymerization and depolymerization influence overall cellular morphology and have a critical impact on certain vital cellular functions. Cytoskeletal actin is regulated in part by members of the small GTP binding protein family Rho (Jahner & Hunter 1991) which associate with the cytoplasmic side of the cellular membranes where they perform a regulatory role (Ingber et al. 1994; Lo & Chen 1994). All members of Rho undergo prenylation as the first step that leads to their membrane association (Casey & Seabra 1996). Rho can activate multiple signaling pathways (Gomez et al 1998) and enhance apoptotic cell death (Jiminez et al 1995).

Caspases play a central role in apoptosis as they comprise a group of highly regulated molecules that mediate cell death as activation of these proteases damages or destroys crucial cell structures (Thornberry et al. 1997; Wolf & Green 1999). Caspases exist in a pro-precursor form
that is enzymatically converted to their mature, active enzyme form which can then activate other caspases or directly induce cell death (Enari et al. 1998). Pro-caspases differ in length and sequence of their N-terminal prodomain. Some procaspases such as pro-caspase-2 use their long N-terminal pro-domain to anchor within the cell membrane. Oligomerization of the long N-terminal pro-caspases can activate these membrane-bound caspases via autoproteolysis (Yang et al. 1998). Caspase-2 can be activated by oligomerization-induced autoactivation (Butt et al. 1998; Muzio et al. 1998). Caspase-2 is also of interest because it has two subtypes Ich-1L which induces apoptosis and Ich-1S which suppresses apoptosis (Duan & Dixit 1997). To induce one of the characteristic cellular structural changes of apoptosis, caspases need to transmit their signal to the nucleus, either directly or by initiating a caspase cascade involving a downstream executioner. Caspase-2 has a nuclear localization suggesting it acts directly or in close proximity to effectors of the nuclear fragmentation of apoptosis (Zhivotovsky et al. 1999; Colussi 1998).

Concomitant induction of apoptosis and depolymerization of F-actin is produced by HMG-CoA reductase inhibitors, such as lovastatin (Levee et al. 1996; Fenton et al. 1992; Koch et al. 1997) and is not shared by all inducers of apoptosis. HMG-CoA reductase is an important enzyme in the synthesis of cholesterol as it converts 3-hydroxy-3-methylglutaryl CoA, a trimer of acetyl CoA molecules, to mevalonic acid which is further metabolized to cholesterol. Inhibitors of HMG-CoA reductase, in clinical use for the management of hypercholesterolemia, induce apoptosis in various cell types including vascular smooth muscle cells (Guijarro et al. 1998), HL60 cells (Perez-Salsa & Mollinedo 1994), macrophages (Rogers et al. 1996), prostate cells (Padayatty et al. 1997), osteoclasts (Luckman et al. 1998) and mouse renal proximal tubular cells (Iimura et al. 1997). Indeed apoptosis-induced by HMG-CoA reductase inhibitors has been suggested to be responsible in part for their beneficial effects in atherosclerosis (Bellosta et al. 1998).
Considering that cytoskeletal actin is regulated in part by Rho and caspases are involved in apoptosis (Thornberry et al. 1997; Wolf & Green 1999), I sought to determine whether there was any relationship between Rho B and caspase-2. Because of lovastatin’s reported induction of apoptosis and alteration of F-actin in other cell types (Guijarro et al. 1998; Perez-Salsa & Mollinedo 1994; Rogers et al. 1996; Padayatty et al. 1997; Luckman et al. 1998; Iimura et al. 1997), I next sought to determine whether lovastatin-induced apoptosis with alteration in cardiomyocyte actin. This study reports the novel finding of an association between caspase-2 and Rho B that is altered by lovastatin.

RESULTS

Dose range for lovastatin in neonatal mouse cardiomyocytes

The present chapter used neonatal mouse cardiomyocytes as all the subsequent immunoprecipitation reactions use antibodies which recognize the mouse, but not chick, sequences for RhoB and caspase-2. A dose curve for lovastatin was previously established by this laboratory using the MTT cell viability assay (Figure 1). This laboratory has previously established that lovastatin induced cell death in neonatal mouse cardiomyocytes but at concentrations greater than seen with embryonic chick. Lovastatin 200 to 300 μM were determined to be a comparable concentration to the 100 μM lovastatin used in chick because in mouse, 200 to 300 μM induced a 12.8±3.8% (N=7) to 37.5±3.8% (N=4) loss of cell viability whereas 100 μM lovastatin in chick induced 19.8±5.1% (N=18) (see previous chapter, Figure H1).

Lovastatin-induced apoptosis in cardiomyocytes

To investigate whether lovastatin will induce apoptosis in neonatal mouse cardiomyocytes as with embryonic chick cardiomyocytes, mouse cardiomyocytes were treated
Figure 11 - Lovastatin induced a loss of cell viability in neonatal mouse cardiomyocytes - Cardiomyocytes were treated with lovastatin at 0 (N=9), 100 (N=3), 200 (N=7), 300 (N=4), 500 (N=7), 700 (N=9) and 1000 μM (N=2) for 24h and the cell viability assessed by MTT assay. A loss of MTT absorbance at 570nm (A_{570}) signifies a loss of cell viability. The loss of A_{570} were reported as percentages of loss of cell viability, or alternatively, percentage increases in cell death. ** p<0.01 compared to control. This figure was produced previously in this laboratory.
with lovastatin 200 μM for 24h and their nuclei examined for the loss of DNA content, indicative of apoptosis (Figure 2). Using the amount of PI staining within the nucleus as an indicator of DNA content, the amount of apoptotic nuclei was determined by analyzing the histogram of PI (FL3) staining (Panel A). Lovastatin increased in the number of apoptotic nuclei. Even at low concentrations of lovastatin, 200 μM for 24h, there was a significant (p<0.01) 1.9±0.4 (N=4) fold increase in apoptotic nuclei compared to control (Panel B).

Lovastatin induced apoptosis involves caspase-2 more than caspase-3 - As shown in the previous chapter with embryonic chick cardiomyocytes, lovastatin-induced apoptosis involves caspase-2. To determine whether mouse cardiomyocytes involve the same mechanisms, cardiomyocytes were grown on coverslips, treated with lovastatin 200 μM for 24 h, and then stained with antibodies specific for either active caspase-2 or active caspase-3 (Figure 3). Compared to control, there was a marked increase in active caspase-2 fluorescence whereas there was only a small amount of active-caspase-3 fluorescence. These results also suggest that caspase-2 is active during lovastatin treatment and appear to localize around the nucleus.

To further investigate the role of caspase-2 in lovastatin-induced cell death, mouse cardiomyocytes were treated with the caspase-2 inhibitor zVDVAD, 100 μM beginning 1 h prior to lovastatin 200 μM for 24 h (Figure 4). zVDVAD pretreatment, significantly (p<0.01) blunted lovastatin-induced apoptotic nuclei to 79.0±7.0% (N=4) of that induced by lovastatin (Figure 4). zVDVAD reduced lovastatin-induced apoptosis as the relative number of cells with low DNA content compared to control decreased from 1.9±0.4 with lovastatin alone to 1.4±0.3 (N=4) in lovastatin treated cells pretreated with zVDVAD (Figure 4 inset).

Lovastatin alters cytoskeletal actin- To examine whether the lovastatin-induced effects on cytoskeletal actin seen in embryonic chick cardiomyocytes are similar in mouse, neonatal mouse cardiomyocytes were treated with lovastatin 200 μM for 24 h and then stained with NBD-
Figure 12 - *The effect of lovastatin on apoptotic cell death.*
Panel A - Cardiomyocytes treated with lovastatin 200 μM for 24 h (N=4) were examined by flow cytometry using PI fluorescence as a measure of nuclear DNA content in permeabilized cardiomyocytes. The amount of apoptotic cardiomyocytes (Apo) is indicated by the population with low fluorescence (FL3). The amount of apoptosis in the control (diluent treated) cells is in part likely due to the trypsinization of the cardiomyocytes. Representative histogram of 4 separate experiments.
Panel B - Relative amount of apoptosis (i.e.- proportion of the population with low DNA content) compared to control. **p<0.01 compared to control.
A

Control

Lovastatin

B

Relative number of cells with low DNA content compared to control

**
Figure 13 - *Lovastatin induces an activation of caspase-2* - Cardiomyocytes were treated with lovastatin 200 μM for 24 h and stained with antibodies specific to either active caspase-2 (linked to FITC) or active caspase-3 (linked to PE).
Figure 14 - **Caspase 2 inhibition blocksLovastatin-induced apoptotic nuclei** - Cardiomyocytes were treated with lovastatin 200μM for 24h, with or without the caspase-2 inhibitor zVDVAD 100μM pretreatment, and the cells were analyzed for DNA content by FACS analysis. Represented as lovastatin-induced nuclei with low DNA content (apoptotic nuclei) as 100%, zVDVAD significantly blocked lovastatin-induced effects to 79.1±7% (N=4) that of lovastatin. ΔΔ *p<0.01* compared to lovastatin. **Inset** - zVDVAD bluntedLovastatin-induced relative amount of cells with low DNA content. *p<0.01* compared to control.
phallacidin. These stained cells were then examined by fluorescent microscope (Figure 5 - Panel A) or by flow cytometry (Panels B & C). Control (diluent treated cells) showed fine actin filaments whereas cells treated with lovastatin displayed a dramatic loss of intact actin fibers evident on microscopy (Panel A). Flow cytometry was used to measure the amount of loss of F-actin and showed that the majority of actin in these cardiomyocytes was present in one distribution (~$10^3$ on FL1) with a smaller population with low fluorescence <$10^2$ (FL1) (Panel B). Considering low actin staining values of <$10^2$, the number of cells displaying low amounts of actin was 2885±316 per 10,000 (N=4) in control cells and was increased significantly (p<0.01) to 7557±511 per 10,000 cells (N=4) with lovastatin treatment (Panel C).

**Effect of zVDVAD on F-actin**

As caspase-2 was shown to be involved in lovastatin-induced apoptosis in embryonic chick cardiomyocytes, I next determined whether caspase-2 is involved in neonatal mouse cardiomyocytes. Cardiomyocytes were treated with the caspase 2 inhibitor, zVDVAD 100 μM beginning 1 h prior to lovastatin 200 μM for 24 h, stained with NBD-phallacidin, and examined by flow cytometry (Figure 6). zVDVAD significantly (p<0.01) blocked lovastatin-induced loss of F-actin to 63.0±11.5% (N=4) of lovastatin’s effect (Figure 6). zVDVAD significantly (p<0.05) reduced the number of cells with low F-actin staining from 7557±511 with lovastatin to 4389±1182 (N=4) with lovastatin plus zVDVAD pretreatment (Figure 6 inset).

**Lovastatin-induced effects on F-actin are not due to actin molecule degradation**

To confirm that lovastatin acted on F-actin filaments, rather than degrading cellular actin, cells treated with 200 μM lovastatin for 24 h, with or without zVDVAD or AcDEVD, and the lysates were immunoblotted for actin (Figure 7). In all samples, there were no signs of actin degradation suggesting that the effect of lovastatin is presumably on F-actin filaments.
Figure 15 - **Lovastatin induces loss of F-actin structure** - Cardiomyocytes were treated with lovastatin 200 μM for 24 h (N=4), stained with NBD-phallacidin, and then examined either by microscopy or flow cytometry.

Panel A - Cardiomyocytes, grown on coverslips were fixed and stained with NBD phallacidin. Representative photomicrographs are shown for cardiomyocytes treated with diluent (Control) or lovastatin 200 μM for 24 h (Lovastatin).

Panel B - Representative histograms from one of 4 separate experiments of NBD phallacidin staining and examined by flow cytometry. M1 marks the position of the median value for NBD phallacidin fluorescence in the control (diluent) treated cells.

Panel C - Number of cells exhibiting low NBD-phallacidin fluorescence (FL1<10²). **p<0.01 compared to control.
Figure 16 - *Caspase-2 inhibition reduces lovastatin-induced changes in F-actin*. Cardiomyocytes were treated with lovastatin 200 μM for 24 h, with or without caspase-2 inhibitor zVDVAD 100 μM pretreatment (N=4 for all). Cells were fixed and stained with 0.16 μM NBD phallacidin and measured by flow cytometry. Represented as lovastatin-induced changes in F-actin as 100%, zVDVAD significantly reduced lovastatin-induced effects. ΔΔp<0.01 compared to lovastatin. Inset - Number of cells exhibiting low NBD-phallacidin fluorescence and hence, low F-actin. Δ p<0.05 compared to lovastatin.
Figure 17 - **Lovastatin does not alter cellular actin** - Cardiomyocytes were treated with lovastatin 200 μM for 24 h, with or without zVDVAD or AcDEVD 100 μM pretreatment. Cells were immunoblotted with anti-actin.
**RhoB is associated with caspase-2**

Because of the relationship of RhoB to actin (Jahner & Hunter 1991; Ingber et al., 1994; Lo & Chen 1994) in the maintenance of cytoskeleton (Koch et al. 1997), and the role of caspase-2 in lovastatin-induced effects, cardiomyocytes were examined for the presence of RhoB/caspase-2 association. I hypothesized that any association between RhoB and caspase-2 would occur before changes in F-actin and apoptosis. Thus, I examined the presence of a RhoB-caspase-2 association in a short, 2 h, time frame. Whole cell lysates were prepared from control and cells treated with lovastatin, 200 μM for 2 h. Lysates were immunoblotted for caspase-2 and a 46 kDa caspase-2 band was visualized (Figure 8 - Panel A). To verify an association between RhoB and caspase-2, whole cell lysates were also immunoprecipitated with RhoB followed by immunoblotting with caspase-2 (Fig. 8 Panel B). Again, a caspase-2 band was visualized. To validate that the caspase-2 antibody was specific and was not detecting all immunoprecipitated proteins, the cell lysates were immunoprecipitated with the irrelevant (to the RhoB/caspase-2 hypothesis) antibody Troponin I-C, followed by caspase-2 immunoblotting (Fig. 8 Panel C). No caspase-2 was visualized in Panel C. A control blank “B”, containing the immunoprecipitation reagents sans cell lysate, was also performed.

To validate the caspase-2/Rho B association, reciprocal immunoprecipitations were performed. Whole cell lysates were prepared from control and cells treated with lovastatin, 200 μM for 2 h. Lysates were immunoblotted for RhoB and a 21 kDa band was visualized (Figure 9 - Panel A). To verify an association between RhoB and caspase-2, whole cell lysates were also immunoprecipitated with caspase-2 followed by immunoblotting with RhoB (Fig. 9 Panel B). Again, a RhoB band was visualized. A control blank “B”, containing the immunoprecipitation reagents sans cell lysate, was also performed.
Figure 18 - *Caspase2-Rho B association complex* - Cardiomyocytes treated with diluent (C) or lovastatin (L), 300 µM for 2h. Cell lysates were either immunoblotted for caspase-2 (Panel A), immunoprecipitated with RhoB followed by immunoblotting with caspase 2 (Panel B), or immunoprecipitated with the irrelevant antibody Troponin I-C followed by immunoblotting with caspase-2 (Panel C). Representative blots of 3-14 separate experiments.
Figure 19 - Caspase-2-Rho B association complex - Cardiomyocytes treated with diluent (C) or lovastatin (L), 300 μM for 2h. Cell lysates were either immunoblotted for Rho B (Panel A) or immunoprecipitated with caspase-2 followed by immunoblotting with RhoB (Panel B). Immunoprecipitation reagents were run in lane “B”.
Due to the relative greater abundance of polyclonal RhoB antibody, further experiments involved immunoprecipitation with Rho B and immunoblotting with caspase-2. I next sought to examine the potential time dependency of the Rho B/caspase-2 association. Cells were treated with lovastatin 300 μM for 1, 2, and 3 h and the cells were lysed, immunoprecipitated with RhoB and immunoblotted for caspase-2 (Figure 10). A caspase-2 band was visualized with a time-dependent increase of expression, 2 h being maximal expression. A control (S) to determine the “stickiness” of Protein-sepharose beads was also performed. “S” refers to a control of cell lysate reacted with protein A-sepharose in the absence of the immunoprecipitation antibody RhoB. There was no association evident caspase-2 band in the “S” control.

To elucidate the cellular localization of the RhoB/caspase-2 association, cells were treated with lovastatin 300 μM for 1, 2, or 3 h. Cells were lysed and the nuclear and cytosolic fractions were separated by means of differential centrifugation before immunoprecipitation with RhoB and immunoblotting with caspase-2 (Figure 11 - Panel A). To validate whether the nuclear isolation was pure, a concomitant immunoblotting with an antibody raised against the nuclear marker Histone-3 was performed (Panel B). Both nuclear and cytosolic fractions showed the caspase-2 band after RhoB immunoprecipitation at the expected 46 kDa molecular weight (Panel A). Controls for the presence of immunoprecipitation reagents and non-specific binding to Protein A-sepharose beads were performed and presented in lane “B”. RhoB and caspase-2 association showed a greater caspase-2 signal in nucleus by 2-3 h of lovastatin treatment whereas there was no change in RhoB presence in the nuclear fractions. Densitometric analysis showed that lovastatin increased the caspase-2 association, with a significant (p<0.05) peak increase in the nucleus at 2h, of 1.2±0.1 fold greater than at time 0 (N=3) (Panel C). There was very little RhoB presence in the cytosolic fractions with the greatest presence occurring at
Figure 110 - Lovastatin induces a time dependent increase of a Rho B-Caspase 2 complex - Cardiomyocytes were treated with lovastatin 300μM for 1, 2, 3, or 4 h. Cells were lysed and the immunoprecipitated with αRho B and Protein A-Sepharose and then immunoblotted for caspase-2. “S” refers to blank control reacted with Protein A Sepharose without the Rho B antibody.
Figure II1 - **Lovastatin induces a time dependent increase of a Rho B-Caspase 2 complex** - Cardiomyocytes were treated with lovastatin 300µM for 1, 2, or 3 h. Cells were lysed and the nuclear and cytosolic fractions were separated by differential centrifugation.

Panel A - Samples were then immunoprecipitated with αRho B and Protein A-Sepharose and then immunoblotted for caspase-2. B refers to blank control which contains immunoprecipitation reagents with the exception of cell lysate. Molecular weight markers (kDa) are shown on the left. Representative blot of 3 separate experiments.

Panel B - To ensure nuclear separation from cytosol was pure, samples were immunoblotted with the nuclear marker Histone-3.

Panel C - Densitometric analysis of caspase-2 of the caspase-2/RhoB complex in nuclear samples. Densitometry was determined by the number of pixels divided by the area of the caspase-2 band (N=3 for all). * p<0.05 compared to control (Time 0 h).
1 h of lovastatin treatment. These results suggest that there is a lovastatin-induced time-dependent association of caspase-2 and RhoB in nuclear fractions.

*RhoB-caspase-2 associated complex is not dependent on caspase-2 activity*

To explore the possibility that caspase-2 activation might affect the association of RhoB/caspase-2, cardiomyocytes were treated with lovastatin, 300 μM, for 1 or 4 h, with or without the caspase 2 inhibitor, zVDVAD 100 μM pretreatment for 1 h prior. Cardiomyocytes were fractionated into cytosolic and nuclear fractions and immunoprecipitated with RhoB followed by immunobloting with caspase-2. The resulting immunoblots were subjected to densitometric analysis for both the RhoB and caspase-2 components (Fig. 12). zVDVAD had no effect on expression of either RhoB or caspase-2. This result suggests that caspase-2 activity is not integral to caspase-2 association with RhoB.

Considering all experiments, the association of RhoB and caspase-2 was found in each of 14 independent experiments.

**DISCUSSION**

These experiments have several novel findings including the major new finding of an association of RhoB with caspase-2. These experiments also reported an alteration of this association in the cytosolic and nuclear fractions with lovastatin treatment, the loss of cardiac F-actin content with lovastatin, and the induction of significant apoptotic cell death in neonatal mouse cardiomyocytes that is reduced by the caspase-2 inhibitor z-VDVAD-fmk.

*Experimental considerations*

This thesis presents the first demonstration of the association between RhoB and caspase-2. The validity of the association was demonstrated by its consistent presence in all independent experiments (N=14). The same RhoB/caspase-2 association was observed during
Figure 112 - The Rho B/caspase-2 association is not influenced by treatment with the caspase-2 inhibitor z-VDVAD Cardiomyocytes were treated with lovastatin 300 μM for 1 h (N=3) or 4 h (N=2), with or without caspase-2 inhibitor, z-VDVAD, 100μM pretreatment. Cells were lysed and the nuclear and cytosolic fractions were separated. Samples were then immunoprecipitated with αRho B and Protein A-Sepharose and then immunoblotted for caspase-2. Densitometric analysis of the caspase-2 and RhoB components evaluated the number of pixels/area in each protein band (integrated density).
immunoprecipitation with caspase-2 and immunobloting with Rho B as well as immunoprecipitation with Rho B and immunoblotting with caspase-2 (Fig. 8b and Fig. 9b). Two different lots of Rho B antibody were used to address potential confounding issues and the results were similar. Appropriate controls for the immunoprecipitation reaction were performed. Specifically, a “blank” which contained immunoprecipitation reagents in the absence of cell sample were run simultaneously with every experiment. Thus, the molecular weights of all immunoprecipitation components were noted and excluded when observing the RhoB/caspase-2 complex. As well a “sample blank” was run which involved the cell sample and Protein-A sepharose in the absence of the primary antibody to determine that the protein immunoprecipitated was not directly binding non-specifically to the Protein A Sepharose (Fig 10). In addition, there were no commonalities of sequences between caspase-2 and Rho B antigen excluding the possibility of a nonspecific cross reactivity, as confirmed by the technical services departments of Pharmingen and Santa Cruz, the suppliers of caspase-2 and RhoB antibodies, respectively. As well, the possibility of caspase-2 immunoblotting non-specifically detecting anything that was immunoprecipitated was excluded as immunoprecipitation with the irrelevant antibody Troponin I-C did not yield any caspase-2 associated complex (Fig 8C). Furthermore, the change in the Rho B/caspase-2 association with lovastatin argues against a nonspecific cross reactivity that should remain constant regardless of the intervention. Interesting the RhoB/caspase-2 association was observed in both cytosolic and nuclear fractions of cardiomyocytes but predominated in the nuclear fraction (Fig. 11).

**Speculative purposes of RhoB/caspase-2 association**

The constitutive association of Rho B/caspase-2, observed in untreated cells, suggests a functional interaction. An attractive possibility is that Rho B interacts with caspase-2 for the purpose of intracellular transport. Rho B’s perinuclear localization led to the suggestion that Rho
B is involved in regulating transport and or assembly of components necessary for cytoskeleton remodeling (Gomez et al. 1998; Zalcman et al. 1995). Another Rho isoform, RhoA, plays a role in actin filament bundling into stress fibers through its action on actin cytoskeleton organization in neonatal rat cardiomyocytes (Wei et al. 2001). Caspase-2 is localized to the nucleus and its pro-domain, integral to nuclear localization, may function in nuclear transport (Colussi et al. 1998). Another potential explanation for the RhoB/caspase-2 association is that caspase-2 may interact with RhoGDI to cleave GDI hence forming a RhoB/caspase-2 complex. RhoGDI, in the cytosol translocates to the nucleus during apoptotic DNA digestion (Krieser & Eastman 1999). These and other possible consequences of the Rho B/caspase-2 association require further investigation. However, support for my observations and its potential significance is highlighted by the finding that in cardiomyocytes angiotensin II induces translocation of Rho A from the cytosolic fraction to the particulate (including nuclear) fraction (Aoki et al. 1998).

**Time frame of RhoB/caspase-2 association**

I suggest a link between Rho and caspase-2 in apoptosis because of the role of caspases in apoptosis and the increase in the RhoB and caspase-2 association after treatment with lovastatin, that induces apoptosis. My observation of a link between Rho and caspase-2 is supported by the connection between Rho and caspase-3 activity (Subauste et al. 2000). Rho maybe involved in caspases assemble into filamentous cytoplasmic structures during apoptosis (Martin et al. 1998). Indeed, the RhoB/caspase-2 association may play a role in initiating an early nuclear response to lovastatin, as the association was more evident after 1 h of lovastatin treatment. The association of RhoB/caspase-2 may dissociate after 2 h or there may be a cessation of new RhoB/caspase-2 association. Dissociation of the complex may be due to Rho inactivation as RhoB is unstable with a 2 h half-life (Lebowitz et al. 1995). Rho B can be functionally depleted from cells within 2 to 3 h of drug treatment (Lebowitz et al. 1995). RhoB and caspase-2 are not dependent on
activated caspase-2 for their association or dissociation, as a caspase-2 inhibitor did not alter the
detection of the RhoB/caspase-2 complex. This is also consistent with its presence in untreated
cells in which presumably there is no capase-2 activation.

RhoB and caspase-2 are not dependent on activated caspase-2 for their association or
dissociation, as the caspase-2 inhibitor zVDVAD did not alter the detection of the RhoB/caspase-
2 complex. These data are also consistent with the presence of the association in untreated cells
in which presumably there is no caspase-2 activation. However, the role of active caspase-2 in
lovastatin-induced apoptosis and loss of F-actin was established. It is possible that lovastatin-
induced caspase-2 association with RhoB by 2 h occurs for a transport role - i.e. to move
caspase-2 to the nucleus to induce the apoptotic and F-actin effects. More in depth studies need
to be performed to determine whether this hypothesis is the case.

Implication of Rho B on lovastatin-induced cytoskeletal effects

Although it was not the intention of this thesis to address the issue of protein prenylation
in response to lovastatin, HMG-CoA reductase is a control site for the isoprenoid biosynthetic
pathway and is important in the post-translational modifications of proteins via prenylation (for
review, see Hill et al. 2000). As RhoB can be prenylated, the cellular localization and activity of
Rho is dependent on this modification, (Hill et al. 2000). Lovastatin-induced toxicity in muscle
cells correlates with a depletion of prenylated proteins, which likely include members of the Ras
superfamily (Flint et al. 1997). Lovastatin inhibition of Rho prenylation alters its cellular
localization and perhaps its function as well (Guijarro et al. 1998; Koch et al. 1997).

The loss of cytoskeletal actin is not a universal feature of apoptosis but rather is restricted
to apoptosis induced by only certain agents. My experiments demonstrated that lovastatin
produced a loss of NBD-phallacidin fluorescence, a measure of intact F-actin content. Early
speculation of lovastatin’s cellular mechanism of action proposed an interaction with RhoB to
induce disassembly of cytoskeleton (Ridley & Hall 1992). Also, chemical activation of RhoB produced a slight reversion of lovastatin-induced rounding of cells (Koch et al. 1997). The mechanism how Rho achieves cytoskeletal control is varied. A RhoB-activated kinase inactivates the myosin-binding subunit of myosin-phosphatase leaving active myosin complex bound to actin (Kimura et al. 1996) and another Rho B-activated kinase directly phosphorylates the myosin light chain thereby affecting actomyosin interaction (Amano et al. 1996). As well, Rho regulates phosphatidyl-inositol 4-phosphate 5-kinase and the phospholipids which control numerous actin-binding proteins (Chong et al. 1994). As lovastatin inhibits Rho prenylation, hence Rho functionality and membrane incorporation, it is possible that lovastatin-induced loss of F-actin is due to the inactivity of Rho.

Implication of caspase-2 on lovastatin-induced effects

This thesis demonstrated that the caspase-2 inhibitor zVDVAD reduced lovastatin-induced cell death implicating a role for this caspase in the caspase cascade leading to apoptosis. The specificity of zVDVAD for caspase-2 inhibitor is relatively high and its $K_i$ for inhibition of caspase-2 is approximately 1,000 fold less than the tetrapeptide caspase-3 inhibitor DEVD (Talanian et al. 1997). While there is relatively little data on the role of caspase-2 in the heart, its activation has already been linked to the presence of heart failure and cardiac apoptosis (Jiang et al. 1999).

Actin disruption and apoptotic effects - a link?

The RhoB/caspase-2 complex observed in this section suggest a link between apoptotic induced loss of cell morphology via disruption of actin and apoptotic DNA cleavage. Previously, Iimura et al (1997) reported that actin filament disruption precedes DNA fragmentation, suggesting a causal relationship between actin filament disruption and DNA fragmentation. Actin disruption may activate DNAses, via caspases, leading to apoptotic nuclear
degradation. This thesis reported that the caspase-2 inhibitor zVDVAD significantly (p<0.01) reduced lovastatin induced apoptosis and the loss of F-actin provides data linking the two processes because inhibition of apoptosis in the absence of an effect on actin would have suggested that actin degradation was an unrelated phenomenon. The two processes, apoptosis and loss of F-actin, however, appear to be intertwined, suggesting the need for other kinds of investigations to determine whether caspase-2 plays a role in the inactivation of RhoB or whether it affects cardiomyocyte F-actin disassembly in an independent manner.

In summary, the finding of a previously unrecognized association of RhoB and caspase-2 in the cytosolic and nuclear fractions of cardiomyocytes suggests important ramifications for processes regulated by RhoB and caspase-2 including apoptosis. This suggestion is supported by several observations including changes in the association with lovastatin treatment that induces cardiomyocyte apoptosis. The caspase-2 inhibitor, zVDVAD blunted lovastatin-induced apoptosis and changes in F-actin. Caspase-2 and RhoB are associated mainly in the nuclear fractions in a time-dependent manner with lovastatin treatment, suggesting a lovastatin-specific nuclear response. Thus, the roles of caspase-2 and RhoB in apoptosis may be benefited by the closeness of their association.
CHAPTER IV

DISCUSSION

A) THESIS RESULTS

1. Observation of apoptosis and oncosis in cardiomyocytes (Section A)

This thesis demonstrated that cell death by both apoptosis and oncosis was observable in embryonic chick and neonatal mice cardiomyocytes. These cardiomyocytes readily underwent apoptosis and displayed the classic morphologic characteristics of apoptosis including nuclear fragmentation, cell shrinkage and membrane bleb formation (Kong and Rabkin 1999). Apoptosis was consistently demonstrated utilizing six different techniques specially designed to identify apoptosis: namely i) flow cytometric analysis of membrane permeability to FDA and PI, ii) flow cytometric analysis of nuclear DNA content, iii) Annexin V binding, iv) DNA gel electrophoresis, v) TUNEL staining, and vi) cell microscopy. Early apoptotic cells exhibit no active esterases but good membrane integrity (low FDA and low PI) whereas oncotic cells exhibit no active esterases and a loss of membrane activity (low FDA and hi PI) thus making FDA+PI dual staining a quantitative and selective measure of the mode of cell death. PI staining of permeabilized nuclei allows PI to bind to the DNA and hence, quantitate the amount of DNA present and lost during apoptosis but allows for exclusion of oncotic DNA loss (Darzynkiewicz et al. 1992). Annexin V binding allows the detection of early apoptosis as phosphatidylserine exposure occurs during early apoptosis (van Heerde et al 2000). End-stage DNA fragmentation was observable with agarose gel electrophoresis and TUNEL which detect the apoptotic patterned DNA fragments. Finally, microscopic observation permits the visualization of the cell structural hallmarks of apoptosis. While each of these techniques have limitations in sensitivity and specificity for the detection of apoptosis (discussed in depth in Section A), the findings on
apoptosis were consistent with each technique. However, flow cytometry and microscopy were shown to be the most efficient and quantitative methods available for apoptotic detection in cardiomyocytes. Experiments to test various hypothesis used these methods of detection.

2. Palmitate induces apoptosis and oncosis concomitant with an increase in nuclear size that is not attributable to cell division. (Section B)

Palmitate, a C16:0 saturated fatty acid, induces significant cell death, both apoptotic and oncotic, at 100 μM or greater. This exogenous addition of palmitate was chosen to mimic the elevated fatty acid levels in patients after myocardial infarct (Oliver 1968, Opie et al. 1977).

As well, palmitate induced an increase in nuclear size with structural abnormalities that have some similarities to cardiomyocyte nuclei in patients with cardiomyopathies (Scholz et al. 1994). However, I determined that the increase in nuclear size was not due to an increase in DNA content due to cell cycle. These results suggest that palmitate may play a role in cardiomyocyte death after myocardial infarction and cardiomyopathy.

3. Palmitate-induced apoptosis operates, in part, by a mitochondrial mechanism (Section C)

Palmitate induced a loss of CMX-Ros fluorescence as well as apoptosis. CMX-Ros fluorescence is dependent on the intramitochondrial “negative sink” generated during mitochondrial respiration by production of electrons in mitochondrial electron transport. Hence, indirectly, palmitate is inducing a loss of mitochondrial respiration. As well, palmitate induced a loss of cyt C from the mitochondria into the cytosol implicating an increase in mitochondrial permeability, likely through MTPs. As cyclosporin A inhibits MTP formation and reduced palmitate-induced loss of cyt C, these results suggest that palmitate induced MTP formation during apoptosis.

4. Palmitate induces apoptosis via CPT-1 if sufficient carnitine is available (Section D)
Palmitate and carnitine are both necessary for palmitate’s transport into the mitochondria, via CPT-1, for fatty acid oxidation. My results demonstrate that palmitate plus carnitine (30 mM) induced significant apoptosis that was attenuated with the CPT-1 inhibitor oxfenicine. Oxfenicine did not reduce palmitate-induced apoptosis but did reduce carnitine-induced apoptosis. Another method of CPT-1 inhibition, stimulation of carbohydrate metabolism, also proved ineffective in reducing palmitate-induced apoptosis. These results suggest that carnitine availability is integral to CPT-1 mediated apoptosis: when excess carnitine is available, palmitate induces apoptosis via a CPT-1 dependent mechanism, possibly by putting CPT-1 into “overdrive”. However, when there is no additional carnitine, palmitate induces apoptosis in a CPT-1 independent mechanism. The McMillin group (Sparagna et al. 2000 and Hickson Bick et al. 2000) hypothesized that palmitate induces CPT-1 to become insensitive to palmitate resulting in decreased palmitate oxidation within the mitochondria. The results from this section agree with this hypothesis: palmitate can not be metabolized due to the insensitivity of CPT-1 and its accumulation induces apoptosis. However, with the addition of carnitine, CPT-1 mediated palmitate oxidation is enhanced. I speculate that carnitine plus palmitate-induced apoptosis is greater than that of palmitate and carnitine alone because the combination induces at least 2 death mechanisms: CPT-1 sensitive and CPT-1 insensitive mechanism.

5. Palmitate-induced apoptosis is reduced by the peroxisome proliferators fenofibrate and WY 14643 (Section E)

Fenofibrate induces peroxisome proliferation that would allow for more peroxisomal metabolism of palmitate. As well, peroxisomal metabolism does not require CPT-1. Palmitate-induced apoptosis was reduced by fenofibrate, suggesting that palmitate metabolism inside the mitochondria is part of palmitate’s apoptotic mechanism. However, fenofibrate is also a PPAR ligand. If fenofibrate’s beneficial effects are due to a PPAR mechanism, then another, more
potent, PPAR ligand (WY 14643) should have an even greater beneficial effect than fenofibrate. This thesis demonstrated that WY 14643, at equivalent concentration as fenofibrate, did reduce palmitate-induced apoptosis, but in a lesser extent than fenofibrate. These results suggest that fenofibrate reduces palmitate-induced apoptosis by at least 2 different mechanisms: i) induction of peroxisomal fatty acid oxidation; and ii) a PPAR-independent mechanism. Peroxisomal metabolism of palmitate would help metabolize palmitate and hence reduce apoptosis, irrespective of possible palmitate-induced CPT-1 insensitivity.

6. Palmitate-induced apoptosis is not by ceramide generation. Ceramide induces apoptosis by its own, separate mechanism. - Section F

Palmitate was shown to induce apoptosis irrespective of ceramide synthesis via the fumonisin B1-inhibitable enzyme ceramide synthase. However, to examine ceramide’s potential role in palmitate-induced apoptosis at times greater than 48 h, exogenous C2-ceramide was used and its death effects were compared to those of palmitate. Like palmitate, ceramide induced significant cell death, loss of ΔΨm, and cyt C release. However, distinct from palmitate, ceramide-induced loss of ΔΨm was insensitive to cyclosporin A suggesting that ceramide induced an increase of mitochondrial permeability that does not have a cyclosporin A-sensitive component. As well, ceramide induced an increase in CMX-Ros fluorescence in contrast to palmitate-induced loss of fluorescence. This result suggests that ceramide induced an increase in the mitochondrial “negative sink” (indirectly, respiration) whereas palmitate induced a decrease in mitochondrial respiration. Taken together, these results suggest that ceramide and palmitate act through different mechanisms in apoptotic induction with dramatic differences in mitochondrial effects.

It is also noteworthy that this section examined methods of observing mitochondrial changes. This thesis used 2 separate methods to observe changes to mitochondria during apoptosis: i) CMXRos as an indirect measure of mitochondrial respiration and ii) DePsipher™
as a measure of $\Delta \Psi_m$.

6. Ceramide-induced apoptosis involves p38MAPK, SAPK/JNK, and ERK - Section G

Ceramide induced an increase in phosphorylation of the pro-apoptotic p38 MAPK and SAPK and a concomitant decrease in the phosphorylation of the anti-apoptotic ERK. P38 MAPK was further implicated in ceramide’s death mechanism as the p38 MAPK inhibitor SB 202190 significantly reduced ceramide-induced loss of DNA content and $\Delta \Psi_m$. In contrast, the MEK/ERK inhibitor PD 98059 had no effect on ceramide-induced apoptosis. In addition, phosphorylated p38 MAPK was observed in the mitochondrial enriched fraction upon ceramide treatment which was significantly reduced upon SB 202190 pretreatment. Immunoblotting of cyt c in mitochondrial and cytosolic fractions demonstrated ceramide-induced cytochrome C release and hence, an mitochondrial permeability. These results suggest that p38 MAPK plays a role in ceramide-induced apoptosis and mitochondrial effects and may directly interact with the mitochondria to execute its mechanism.

7. Palmitate-induced apoptosis does not occur through HMG-CoA reductase inhibition - a study with lovastatin - Section G

It was hypothesized that palmitate may induce apoptosis due to an increased amount of cholesterol biosynthesis because acetyl CoA, a byproduct of palmitate metabolism and the starting substrate for cholesterol, would be in greater amounts due to increased palmitate metabolism. To examine this, I combined the cholesterol biosynthesis inhibitor lovastatin with palmitate. Lovastatin plus palmitate induced significant apoptosis suggesting that palmitate’s death mechanism was not by cholesterol biosynthesis. However, as lovastatin induced significant apoptosis and a change in cell morphology distinct from palmitate, I examined lovastatin’s signaling mechanism.
Lovastatin not only inhibits HMG-CoA reductase, leading to a reduction in the production of all cholesterol byproducts, but it also inhibits processes such as prenylation. To examine the effect of HMG-CoA reductase inhibition but with re-activated cholesterol biosynthesis, mevalonic acid was co-treated with palmitate. Mevalonic acid significantly reduced lovastatin’s death effects but did not abolish them suggesting that lovastatin’s death effects are only partially due to HMG-CoA reductase inhibition. To investigate lovastatin’s other apoptotic mechanism, I examined caspase-2 and -3. Both the caspase-2 and -3 inhibitors zVDVAD and AcDEVD, respectively, were found to reduce lovastatin-induced apoptosis suggesting that these caspases are involved.

9. Lovastatin induced effects are mediated through caspase-2 and RhoB which associate upon lovastatin treatment - Section I

Lovastatin induced apoptosis and changes in cell morphology in a mainly caspase-2 dependent manner in mouse cardiomyocytes. As well, caspase-2 was observed to associate with RhoB upon lovastatin treatment, predominantly in the nuclear fraction. This association was independent of caspase-2 activity as zVDVAD, a caspase-2 inhibitor, had no effect on this association. These results suggest that RhoB/caspase-2 complex may be a mediator for both of lovastatin’s cytoskeletal and apoptotic effects.

B) CHAIN LENGTH VS. UNSATURATED BONDS? OLEIC AND CAPRIC ACID DO NOT INDUCE APOPTOSIS

The possibility of palmitate’s death effects were due to chain length were addressed by studies with oleic acid whereas the possibility of non-specific fatty acid effects (such as a detergent action) were addressed using capric acid.

The unsaturated fatty acid oleic acid did not induce apoptosis although oleic acid is also a
long chain fatty acid like palmitate. Unlike palmitate and other saturated fatty acids, unsaturated fatty acids require an extra enzymatic step to remove the unsaturated bond and convert the unsaturated fatty acid to a saturated fatty acid to permit oxidation (for review, see Lopaschuck et al. 1998). Several studies have cited unsaturated fatty acids to be anti-apoptotic. Oleic acid has been reported to be non-toxic and that it also inhibited apoptosis induced by palmitate in neonatal rat heart (DeVries et al. 1997) and in MDA-MB-231 breast cancer cells (Hardy et al. 2000). Palmitoleic acid, the unsaturated version of palmitic acid, also inhibited palmitate-induced apoptosis in pancreatic β-cells (Maedler et al. 2001). Mu et al. (2001) reported that the addition of arachadonic acid, a polyunsaturated fatty acid, induced a protective effect on palmitate-induced apoptosis in human granulosa cells.

The present thesis also observed that capric acid did not induce apoptosis. As capric acid (C10:0) is a shorter chain length fatty acid than palmitate and therefore does not require CPT-1 for mitochondrial transport for oxidation (for review, see van der Vusse 1992), capric acid’s lack of toxic effect suggests that fatty acid oxidation alone is not responsible for fatty acid-induced apoptosis. These results agree with Abdel-Aleem et al. (1998) who also reported the lack of death effect with octanoate, a C8:0 fatty acid.

Thus, the results with both oleic and capric acid suggest that palmitate’s death effects are specific and not due to a nonspecific action of fatty acids on the cell.

C) IMPLICATIONS OF THIS WORK IN CLINICAL CARDIOLOGY

These experiments sought to elucidate the mechanism by which fatty acids cause cell death in cardiomyocytes. This thesis determined that palmitate induces apoptosis in a specific manner dependent in part on the mitochondria. Therapeutic interventions to reduce palmitate-induced apoptosis would include attenuating its metabolism within mitochondria by CPT-1
inhibition with drugs such as oxfenicine, or by inducing peroxisomal fatty acid oxidation with a peroxisome proliferator with drugs such as fenofibrate. In addition the results from this thesis demonstrate that palmitate induces serious mitochondrial changes which are involved in apoptotic induction. Therapeutic intervention of these mitochondrial changes with cyclosporin A may blunt some of palmitate’s death effects clinically.

Although it was shown that palmitate did not owe its death effects to the de novo synthesis of ceramide, chronic exposure (ie - over a lifetime) of palmitate may increase synthesis of ceramide. Ceramide induced cell death in a mitochondrial mechanism that was not circumvented by cyclosporin A, as was the case with palmitate. This suggests that potential treatment with cyclosporin A would not be effective in circumstances of increased ceramide generation.

Ceramide’s apoptotic effects were determined to be attributable, in part, by signaling mechanisms involving p38, SAPK, and ERK. This thesis demonstrated that inhibition of p38 with SB 202190 attenuated some of ceramide’s death action. Therapeutic intervention of these kinase cascades, especially an inhibition of the p38 and SAPK pathways or a stimulation of the ERK pathway, is a possible avenue to controlling ceramide-induced apoptosis.

Lovastatin is currently in use to inhibit cholesterol synthesis. The results from this thesis demonstrate that lovastatin can induce apoptosis, an observation with potentially serious clinical ramifications. Lovastatin induces significant changes in cardiomyocyte morphology. This observation may have clinical implications as rounding of cardiomyocytes which reflect changes in cytoskeleton may contribute to reduced contractility associated with cardiac failure.

Lovastatin’s mechanism of death was determined to involved caspase-2 and caspase-3. Therapeutic inhibitions of these caspases (zVDVAD or AcDEVD) as well as reactivation of cholesterol biosynthesis with mevalonic acid may be promising in attenuation lovastatin-induced
cardiac apoptosis that may occur during lovastatin therapy.

In summary, this thesis provides evidence that fatty acids can induce cell death in cardiomyocytes. Though the identification of the underlying the mechanisms behind this kind of cell death, several therapeutic interventions were demonstrated to prevent cardiac apoptosis.

D. LIMITATIONS OF THIS STUDY

As with all *in vitro* studies, there are limitations to the interpretations of this thesis’ results. Although the role of fatty acid oxidation in embryonic chick is comparable to human fetal stage, this model may not be comparable to adult human. Although the present model demonstrates the effects of exaggerated fatty acid oxidation, the embryonic model differs in mitochondrial maturity compared to adult human mitochondria. Adult heart has more mitochondria with well developed cristae than in embryo, thus emphasizing the importance of fatty acid oxidation in adult. As a result, mitochondrial effects in the embryonic model may not be exactly translatable into adult human due to these mitochondrial differences.

Use of kinase inhibitors such as PD 98059 and AcDEVD, although significant in their effects, are not necessarily completely inhibiting their target kinase. Although all concentrations of inhibitors were either suggested by previous studies (see Materials and Methods, Section VII-C) or personally established with kinase assays (eg. PD 98059), I am assuming that the kinase inhibitors used are significantly inhibiting, but not necessarily abolishing, kinase activity. Extensive work with kinase assays would have to be performed to validate kinase inhibition in these cardiomyocytes.

Examination of apoptosis by flow cytometry has limitations. Although I am using two separate mechanisms to observe apoptosis (FDA+PI for early apoptosis and DNA content for late apoptosis), it is difficult to ensure that all apoptosis was measured. For example, the percentage
apoptosis obtained from FDA+PI staining is a measurement of early apoptosis. Cells that are undergoing DNA fragmentation in late apoptosis may not be measured in the apoptotic population in the FDA+PI method. Conversely, cells with low DNA content are measured but would not include the cells beginning apoptosis but not yet started fragmenting DNA. However, irrespective of which method used to measure apoptosis, be it caspase assays or cell viability assays, it will be difficult to measure total apoptosis as apoptosis occurs over a long time: at one time point, you would find cells beginning, in the midst of, and finishing apoptosis.

E) IDEAS FOR FUTURE RESEARCH

This thesis provides the background for future research. Although some MAPKs have been investigated in palmitate-induced apoptosis (Sparagna et al. 2001), there is still a need to link the signaling molecule to the physical loss of mitochondrial potential, mitochondrial respiration, and mitochondrial membrane integrity. Tracking radioactively labeled palmitate within mitochondria would prove useful in determining palmitate’s fate within the mitochondria and its role in changing mitochondrial bioenergetics.

Further investigation should be done with regards to why ceramide and palmitate share some mitochondrial effects yet differ in others. In particular, ceramide-induced increase in respiration with a contradictory loss of mitochondrial potential needs further investigation. I also speculate that p38 MAPK is directly interacting with mitochondria, but this needs to be confirmed.

The effects of lovastatin, although surprising with respect to palmitate-induced apoptotic pathways, should be further investigated. The novel association between RhoB and caspase-2 needs to be addressed and its function within the cell established. It is possible that the function of this complex may be to keep caspase-2 inactive, akin to the pro-domain of caspases. Thus, the nuclear localization of caspase-2 of the RhoB/caspase-2 complex during lovastatin treatment
may be a sign of caspase-2 activation. Inhibition of these complex components, individually as well as in conjunction, may provide valuable data with regards to the function of this complex.
CHAPTER V

SUMMARY AND CONCLUSIONS

1. Palmitate induced apoptosis in 13% of the dead cell population and the remainder was identified as oncosis.

2. Palmitate induced an increase in nuclear size which was not attributable to DNA synthesis or mitosis.

3. Palmitate induced a loss of mitochondrial respiration, assessed by loss of CMX-Ros fluorescence, and an increase in mitochondrial permeability, evidenced by loss of mitochondrial cyt c into the cytosol. These mitochondrial effects were attenuated with cyclosporin A.

4. Palmitate-induced apoptosis was enhanced with the addition of carnitine implicating a role for CPT-1. These effects were diminished by the CPT-1 inhibitor oxfenicine. However, in the absence of carnitine, palmitate induced apoptosis occurs in a CPT-1 insensitive mechanism.

5. Palmitate-induced effects were attenuated by the pretreatment with fenofibrate and WY 14643, both peroxisome proliferators which promotes fatty acid oxidation to occur within the peroxisomes. In addition, fenofibrate’s beneficial effects likely also involve PPARα-independent mechanisms.

6. Palmitate-induced apoptosis is not attributable to the de novo synthesis of the apoptotic inducer ceramide.

7. Ceramide induced apoptosis in a mechanism distinct from palmitate. Ceramide induced a cyclosporin A-insensitive mitochondrial effect and increased CMX-Ros fluorescence that
may represent an increase in mitochondrial respiration.

8. Ceramide induces an activation of the SAPK and p38 MAPK with a concurrent decrease in ERK activity. Ceramide’s mitochondrial effects involve p38 MAPK signaling as inhibition of p38 resulted in a decrease in ceramide-induced loss of $\Delta \Psi_m$ and apoptosis and phosphorylated p38 MAPK was observed to localize to the mitochondria upon ceramide treatment.

9. Palmitate-induced apoptosis is not due to increased cholesterol biosynthesis as the HMG CoA reductase inhibitor lovastatin did not reduce palmitate-induced cell death.

10. Lovastatin induces apoptosis and dramatic changes in cell morphology, in parts, by inhibition of cholesterol biosynthesis and caspase-2 and caspase-3 activation.

11. There is a novel association between the actin filament regulator RhoB and caspase-2. The association of RhoB and caspase-2 is independent of caspase-2 activity.

12. Methodological considerations

This thesis utilized several methods of observing cell death, apoptosis and oncosis, in a cardiomyocyte model and found in cardiomyocytes that:

a) Dual staining with FDA & PI - this method allows easy separation of apoptotic and oncotic populations. As well, it allows quantitation of the presence of apoptotic and oncotic cells in a population of cardiomyocytes

b) DNA fragmentation

i) TUNEL - this method allows quantitation of only fragmented DNA as the TdT enzyme only labels nicked/fragmented DNA strands that occur during apoptosis

ii) Agarose Gel - this method allows visualization of DNA fragments of 180 bp size and multiples known as the apoptotic DNA ladder.
iii) Nuclear hypoploidy by flow cytometry - this method allows quantitation of a population of cells exhibiting low DNA content associated with apoptotic DNA fragmentation.

c) Annexin V - Annexin V binds to phosphatidylserine that is exposed on the outside of the cell during early apoptosis

d) Microscopic analysis

i) Cell morphology with Acridine Orange - this method allows the visualization of cell morphology and intracellular pH conditions.

ii) Nuclear morphology with Giemsa - this method allows the visualization of nuclei and nucleolus and any changes associated with these structures.

iii) Mitochondrial changes with the J-aggregate dye 5′6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyaninine iodide, or DePsipher™ - this method allows the visualization of the state of $\Delta \Psi_m$ within a cell’s mitochondria.

e) Mitochondrial Changes

i) CMX-Ros - this method indirectly demonstrates intramitochondrial enzyme respiration based on its accumulation in the “negative sink”

ii) DePsipher™, or 5′6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyaninine iodide - this method allows quantitation of the loss of $\Delta \Psi_m$ by observing the amount or red aggregate (intact potential) vs. green monomer (loss of potential) fluorescence.

iii) Cyt C detection in cytosol - this method detects the aftermath of increased mitochondrial permeability as cyt c release into the cytosol can only occur if the mitochondria is permeable.

13. Cell models - embryonic chick and neonatal mouse

Embryonic chick cardiomyocytes were used in experiments to observe apoptosis and oncosis. However, it is acknowledged that this model is less readily extrapolated to human as
the chick model is an avian one. Hence, the mammalian neonatal mouse cardiomyocyte model was used in more detailed experiments in which the data can be extrapolated more easily to humans. Both cell models consistently produced apoptosis and the results with the two models, with the exception of some concentration effect responses, were similar.
Fig. 1
CONCLUSIONS
CHAPTER VI

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