MYOCARDIAL APOPTOSIS IN ACUTE DIABETES: ROLE OF LIPOTOXICITY AND OXIDATIVE STRESS

By

SANJOY GHOSH
B.Pharm, Bangalore University, 1999

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

MASTER OF SCIENCE

IN

THE FACULTY OF GRADUATE STUDIES

Faculty of Pharmaceutical Sciences
Division of Pharmacology and Toxicology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
JANUARY, 2003
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Department of Pharmaceutical Sciences

The University of British Columbia
Vancouver, Canada

Date 17th April, 2003
ABSTRACT

The general objective of this thesis was to examine the role of lipotoxicity and oxidative stress under control or high fat fed conditions in acute STZ diabetes. In the lipotoxicity study, we examined the impact of dietary manipulation of palmitic acid on rat cardiomyocyte apoptosis under hyperglycemic conditions in vivo. Feeding palm oil (PO) or sunflower oil (isocaloric control; SO) for 4 weeks induced hyperinsulinemia, with a moderate rise in serum glucose. PO magnified the palmitic acid whereas SO enhanced the linoleic and docosahexaenoic acid content within lipoproteins and heart. Following streptozotocin induced diabetes, total cardiac free fatty acid (FFA) and palmitic acid remained unchanged in PO rats despite a dramatic increase in serum FFA, and may reflect the activation of compensatory mechanisms that limit excessive accumulation of FFA in the heart. Indeed, lipoprotein lipase activity at the coronary lumen declined in high fat fed diabetic rats. Additional imposition of diabetes in PO animals resulted in the highest level of myocardial apoptosis and lipid peroxidation. Although SO decreased cardiac glutathione maximally following diabetes, lipid peroxidation remained unaltered. Our data suggest that although PO increases cardiac apoptosis, SO with its glutathione lowering and potential enhancement of fatty acid metabolites could also exert detrimental effects on the diabetic heart.

In the GSH study, we examined the regulation of GSH and its role in apoptosis and oxidative stress in the acutely diabetic rat heart. Acute diabetes induced changes in mitochondrial membrane potential and increased production of free radicals. Additionally, the role of cytosolic or mitochondrial GSH and its effects on oxidative stress and apoptosis in the acutely diabetic heart was investigated. Although myocardial GSH levels were not altered in diabetes, increasing GSH levels by ~2 fold
by exogenous supplementation attenuated the oxidative damage and apoptosis. Thus although total myocardial levels of GSH were unaltered, mitochondrial GSH, that controls both lipid peroxidation and apoptosis may have decreased following diabetes and was reversed by exogenous GSH. To test this hypothesis, we depleted either cytosolic GSH with L-buthionine-[S,R]-sulfoximine (BSO, 4 mmol/kg; 3 days) or and mitochondrial GSH with diethyl maleate (DEM, 4 mmol/kg; 3 days) following diabetes. Only DEM+BSO treatment augmented the oxidative stress and apoptosis. In conclusion, the oxidative stress and the resultant apoptosis demonstrated in the diabetic heart may probably occur as a consequence of depletion of mitochondrial GSH that can be reversed with exogenous supplementation of GSH.
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<td>ACC</td>
<td>acetyl CoA carboxylase</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end product</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
</tr>
<tr>
<td>AIN</td>
<td>American Institute for Nutrition</td>
</tr>
<tr>
<td>AR</td>
<td>aldose reductase</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>BSO</td>
<td>buthionine sulfoximine</td>
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<tr>
<td>CM-H$_2$DCFDA</td>
<td>5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester</td>
</tr>
<tr>
<td>DEM</td>
<td>diethyl maleate</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DR</td>
<td>death receptor</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis-2-nitrobenzoic acid</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas associated death domain</td>
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<tr>
<td>FADH$_2$</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FAS</td>
<td>fatty acid synthetase</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
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<td>GLUT 4</td>
<td>glucose transporter 4</td>
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<td>GPAT</td>
<td>glycerol phosphate acyl transferase</td>
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<td>GPX</td>
<td>glutathione peroxidase</td>
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<tr>
<td>GSH</td>
<td>glutathione</td>
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<tr>
<td>GSSG</td>
<td>glutathione disulfide</td>
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<tr>
<td>HF</td>
<td>high fat</td>
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<tr>
<td>HSPG</td>
<td>heparan sulfate proteoglycan</td>
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<tr>
<td>IAA</td>
<td>insulin auto antibody</td>
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<tr>
<td>IAP</td>
<td>inhibitor of apoptotic protein</td>
</tr>
<tr>
<td>ICA</td>
<td>islet cell auto antibody</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>IDDM</td>
<td>insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>JC-1</td>
<td>5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide</td>
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<tr>
<td>LADA</td>
<td>latent autoimmune diabetes in adult</td>
</tr>
<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
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<tr>
<td>mCICCP</td>
<td>m-chlorophenylhydrazone</td>
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<tr>
<td>MDA</td>
<td>malondialdehyde</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>NAC</td>
<td>n-acetyl cysteine</td>
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<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
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<tr>
<td>NF-kB</td>
<td>nuclear factor kappa b</td>
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<tr>
<td>NIDDM</td>
<td>non insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>pNA</td>
<td>para-nitroaniline</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SREBP-1C</td>
<td>sterol regulatory element binding protein 1c</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>TBA</td>
<td>thiobarbituric acid</td>
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<tr>
<td>TBARS</td>
<td>thiobarbituric acid reactive substances</td>
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<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>TNF-R</td>
<td>tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR-associated death domain</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT mediated dUTP nick end labeling</td>
</tr>
<tr>
<td>UCP</td>
<td>uncoupling protein</td>
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<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
</tr>
<tr>
<td>ZDF</td>
<td>Zucker diabetic fatty</td>
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<tr>
<td>γ-GCS</td>
<td>gamma glutamyl cysteine synthetase</td>
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ACKNOWLEDGEMENTS

I would like to thank Dr. Brian Rodrigues for all the guidance, support, and knowledge he has given me over the last couple of years.

I would also like to thank Ashraf and Jospy for their technical help and showing me the rules of the lab.

I am also grateful to the members of my supervisory committee for the valuable suggestions and encouragement they provided me.

I also would like to express thanks to Thomas for being a superb colleague and Roger for his help in the fatty acid studies.

Thanks also to Howard for being a very helpful summer student.

I would like to express my gratefulness to the CIHR/Rx&D for the financial support they provided me during my graduate studies.

Last but not the least, thanks to my wife Mimi, without whose selfless support, this work could not have been completed.
1. INTRODUCTION

1.1. DIABETES – A Brief History

Diabetes, a disease of "frequent urination" and its 'remedy' existed in ancient Egypt around 3,500 years ago. In 100 AD, the Greek physician Aretaeus coined the term 'diabetes' (meaning siphon) for this disease. J. Von Mering and O. Minowski first proposed the role of the pancreas in development of diabetes in 1889. The first real progress in understanding the disease came in 1921 when Dr. Frederick G. Banting and Charles H. Best isolated crude insulin from animal pancreas and injected it into a morbidly diabetic 11 year old patient who survived. Subsequently, Dr. Banting shared the Nobel Prize in 1923, with John JR Macleod for the discovery of insulin, the 'magic cure' for diabetes. However, as diabetic patients started to live longer, new complications of the eyes, hearts, blood vessels, kidneys, limbs and nerves developed. More recently, the Diabetes Control and Complications Trial (DCCT) (1993) and the UK Prospective Diabetes Study (UKPDS) (Turner, 1998; 1998) demonstrated that other than high blood pressure and high blood glucose are the main culprits behind the complications and aggressive glycemic control with either insulin or oral glucose lowering agents could bring down their incidence in diabetic patients.

1.2. DIABETES MELLITUS – An Overview

As per the definition of diabetes mellitus from an expert committee (1997), diabetes mellitus is defined as "a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both." Diabetes is classified based on clinical etiology into two major types: a) type 1 diabetes (previously termed
as insulin-dependent diabetes mellitus or IDDM) characterized by a total loss of insulin secretion and therefore an obligatory necessity for exogenous insulin; b) type 2 diabetes, caused by a combination of impaired insulin action and an inadequate compensatory insulin secretion.

In Type 1 patients, pancreatic β cell islets are destroyed by a poorly understood mechanism probably due to autoimmunity at a very early age. Other than unknown factors that cause the destruction of the islets (idiopathic Type 1 diabetes), several auto antibodies including islet cell auto antibodies (ICAs) (Irvine et al., 1980), insulin auto antibody (IAAs), glutamic acid decarboxylase auto antibodies (GAD 65), and tyrosine phosphatase auto antibodies (IA-2 and IA-2β) have been identified in the serum of Type 1 diabetic patients (immune-mediated Type 1 diabetes) (Batstra et al., 2001). Type 1 diabetes comprises around 5-15% of all diabetic cases. Type 2 diabetics comprise over 85-95% of all diabetic cases. They are usually asymptomatic and are often diagnosed by fasting hyperglycemia and by oral glucose tolerance tests. Type 2 diabetes (previously known as non-insulin-dependent diabetes mellitus or NIDDM) is often characterized by insulin resistance and relative insulin deficiency. The exact cause of such a disease is not well known but genetics, obesity and/or abdominal body fat are strongly associated with type 2 diabetes. The risk of developing type 2 diabetes increases with age (thus previously known as ‘adult onset diabetes’). The development of hyperglycemia is gradual over years after the demonstration of insulin resistance. As glycemic control is important in these patients, diet and exercise is often advocated initially, together with oral anti-diabetic drugs. In more advanced stages, some individuals do require insulin (thus the terms IDDM / NIDDM became redundant).
Unfortunately the epidemiology of Type 2 diabetes is fast changing in the Western world including North America. Recently, apart from an increase in the adult population, there has been a huge upsurge in the cases of obesity, insulin resistance and type 2 diabetes in children and adolescents (Rocchini, 2002; Invitti et al., 2002). Interestingly, in the adult population, an increasingly large number of people are being identified with autoimmune diabetes (latent autoimmune diabetes in adults, or LADA) and are being classified as the new 'Type 1.5' diabetes (5-10% of all diabetic cases) (Schernthaner et al., 2001). Although these patients have a low body mass index, they demonstrate high triglyceride levels and insulin resistance comparable to classical Type 2 diabetic patients. The low titer of GAD, IA2, ICA antibodies is thought to be responsible for this less aggressive form of the disease which is often misinterpreted as 'Type 2' cases (Schernthaner et al., 2001).

Diabetes is fast taking the form of a pandemic. An estimated 30 million people had diabetes in 1985. In the next 15 years, the figure jumped to 150 million worldwide. An alarming 300 million diabetic cases have been projected for the year 2025. Needless to say, diabetes is also a growing concern in North America, especially, Canada. The total burden of diabetes and its chronic complications in Canada in terms of cost was between US$4.76 and $5.23 billion in 1998 (Dawson et al., 2002). In newly diagnosed diabetic patients, the direct costs for management of just the disease (without any complications) was US $573 million (Dawson et al., 2002).
1.3. DIABETES MELLITUS – Experimental Models

Experimental models of diabetes can be divided into two main classes:

a) Chemically induced diabetes: Agents like streptozotocin (STZ) and alloxan induce diabetes in rodent models. STZ [2-deoxy-2- (3-methyl-3-nitrosourea) 1-D-glucopyranose] is a broad-spectrum antibiotic secreted by Streptomyces achromogenes, which by virtue of its selective β-cell toxicity and longer half-life is the preferred agent (Rodrigues B et al., 1999). Another advantage of STZ is that the severity of the diabetic condition can be varied depending on the dose used (in rats, the dose being 25–100 mg/kg STZ). The chemically induced diabetic models predominantly represent Type1 and/or poorly controlled Type 1 diabetes.

b) Genetic models of diabetes: Different models both for Type 1 (BioBreeding (BB) diabetic rats) and Type 2 (Zucker diabetic fatty (ZDF) rats, db/db mice) are available. These animals develop diabetes spontaneously upon aging (Leiter, 1989). In all of these models, secondary complications resembling clinical diabetes often follow the onset of the disease.

1.4. ‘HEART MUSCLE-SPECIFIC’ DISEASE- Evidence in Diabetes

After the discovery of insulin and a range of oral hypoglycemics, it is uncommon to have uncontrolled hyperglycemia for extensive periods. Although diabetic patients generally keep their blood sugar under control, chronic complications such as retinopathy, autonomic neuropathy, nephropathy, cerebrovascular diseases and cardiovascular diseases like angiopathy, atherosclerosis, and cardiomyopathy still prevail. Out of all these disorders, diabetic heart diseases are probably the most predominant (Marks and Raskin, 2000). In Canada, diabetic cardiovascular disease was the greatest contributor to the total economic burden of diabetes at $637 million
Clinically, diabetes is often linked with hyperlipidemia, hypertension and obesity. However, these factors do not entirely account for the increased morbidity and mortality in diabetes (Fuller et al., 1980). In 1972, autopsies on four diabetic patients with congestive heart failure and nephropathy demonstrated an absence of coronary atherosclerosis (Rubler et al., 1972). In another study with diabetic patients, scarring and interstitial collagen deposition was observed without arterial obstruction in hearts, suggesting the presence of a congestive/constrictive heart muscle specific disease (cardiomyopathy) (Cai and Kang, 2001).

Cardiomyopathy is defined as diseases of the myocardium associated with cardiac dysfunction (Richardson et al., 1996). Among the various types of cardiomyopathies, diabetic cardiomyopathy is classified as a ‘specific cardiomyopathy’ characterized by impaired diastolic function (Francis, 2001). Other than the Framingham study (Kannel et al., 1974), a more recent study including 1810 diabetic patients and 944 age-matched controls further demonstrated the impact of diabetes on heart failure in the absence of any coronary artery disease or hypertension (Devereux et al., 2000). Even three decades after it was first identified, the etiology of diabetic cardiomyopathy is not completely understood (Cai and Kang, 2001). The origin of this multifactorial disorder seems to originate from defects in organelles like the sarcoplasmic reticulum and mitochondria, and/or from metabolic derangements (Rodrigues et al., 1995).

The heart requires high levels of adenosine triphosphate (ATP) for its energy, which it derives from a combination of glucose, free fatty acids (FFA), ketone and pyruvate (van der Vusse et al., 1992). Mainly glucose transporter 4 (GLUT4) mediates glucose uptake in the heart (and other insulin sensitive tissues like the skeletal muscle and fat). In animal models of diabetes, the levels of GLUT4 mRNA and protein decrease by 50% and both basal and insulin stimulated glucose uptake is hindered (Garvey et al.,
Augmented levels of ketone bodies, which can further suppress glucose utilization and lead to an increased intracardiac glycogen stores, further compromises glucose utilization. Thus, during diabetes, in the absence of insulin or its function, the heart switches almost exclusively towards FFA as its major energy source (Chatham JC et al., 1996). But the heart has limited potential to synthesize FFA. Hence, this metabolic substrate is supplied to cardiac cells from three sites: a) fragmentation of triglyceride (TG) in adipose tissue with an ensuing increase in albumin-bound FFA in plasma, b) breakdown of intracellular cardiac TG stores, and c) lipolysis of TG in circulating lipoproteins with the assistance of lipoprotein lipase (LPL). To provide for this augmented utilization of FFA by the diabetic heart, a multitude of events occurs. For instance, adipose tissue lipolysis is enhanced, resulting in elevated circulating plasma FFA; in turn, hepatic very low-density lipoprotein (VLDL) secretion and circulating VLDL-TG concentrations increase (Rodrigues et al.; 1992). There is an increased activity of cardiac enzymes that catalyze the synthesis of TG, together with a rise in CoA levels; this promotes the accumulation of intracellular TG stores (Murthy et al., 1983) and subsequent hydrolysis of this TG store could also lead to high tissue FFA levels. Finally, significantly elevated lipoprotein lipase activity at the coronary endothelial surface in STZ-diabetic rats has been recognized (Rodrigues et al., 1997a; Sambandam et al., 1999). Accordingly, this abnormally high capillary LPL could provide excess FFA to the diabetic heart. Augmented myocardial palmitic acid, a saturated fatty acid, in conjunction with hyperglycemia has also been proposed to play a role in the etiology of this disease (Dyntar et al., 2001). Although debatable, because FFA oxidation leads to oxygen ‘wastage’, a high level of fatty acid oxidation may lead to ischemic alteration in the diabetic heart (Pogatsa, 2001; Feuvray and Lopaschuk, 1997). Diabetes also causes depressed mitochondrial function and a decrease in
creatine kinase activity, which may lead to decreased energy supply to the myopathic heart in diabetes (Spindler et al., 1999).

Early diastolic changes and a late systolic alteration characterize diabetic cardiomyopathy. Such changes can directly arise from alteration in the regulation of intracellular cations, which in turn regulate the contractile properties of the heart (Mahgoub and Abd-Eifattah, 1998). In spite of considerable research in the area, conclusive evidence is lacking regarding the exact status of Ca\(^{++}\) in the diabetic heart. A depressed Na\(^+\)/K\(^+\) ATPase increases intracellular Na\(^+\) that can decrease Ca\(^{++}\) and K\(^+\) levels in the heart (Golfman et al., 1998). In addition, the Na\(^+\)/Ca\(^{++}\) exchanger mechanism is impaired together with a decreased myofibrillar Ca\(^{++}\) ATPase activity (Chatham JC et al., 1996). Decreased Ca\(^{++}\) binding to the sarcolemma and decreased Ca\(^{++}\) intake in the sarcoplasmic reticulum are characteristics of rodent models of diabetes (Cai and Kang, 2001). On the other hand, Ca\(^{++}\) overload may be a defect in the diabetic heart that could be significantly reversed by the administration of verapamil, a Ca\(^{++}\) channel blocker (Afzal et al., 1988). Diabetic hearts also demonstrate reduced sensitivity to norepinephrine and \(\beta\)-adrenoceptor mediated stimulation (Tanaka et al., 1992). This may result from a decreased number of receptors or a defect in signaling (Dincer et al., 2001). Lately, oxidative stress has also been implicated in the etiology of diabetic cardiomyopathy (Dhalla et al., 1998) along with other diabetic complications (Brownlee, 2001b). Oxidative stress arises due to an imbalance between the production of reactive oxygen species (ROS) and its neutralization by antioxidants. Oxidative damage by ROS and reactive nitrogen species (RNS) have been found in the hearts of diabetic rats (Kajstura et al., 2001). Lately, sustained apoptosis, or physiological cell death' in the myocardium has also
been correlated with diabetic cardiomyopathy, both in human patients and rodent models of diabetes (Fiordaliso et al., 2000; Frustaci et al., 2000).

1.5. APOPTOSIS IN HEART—An Overview

Apoptosis or 'programmed cell death' is a process of cell death that occurs subsequent to the activation of a genetically programmed, energy-dependent mechanism. In apoptosis, mitochondrial functional alterations, cytoskeletal alterations and membrane budding is observed initially. These lead to nuclear condensation and fragmentation (karyorrhexis) and formation of small apoptotic bodies that are engulfed and subsequently eliminated by phagocytic cells (macrophages) (Haunstetter and Izumo, 1998). Under natural circumstances, programmed cell death functions to maintain normal cell mass, and is a balance against mitotic proliferation of cells. The other form of cell death known as necrosis or 'accidental cell death' is not energy dependent and is typically activated by toxins, ischemia and other physical insults. In the cardiovascular system, apoptosis operates normally to control development of the rat heart (Kajstura et al., 1995). As adult cardiomyocytes are terminally differentiated cells, they have limited capacity for regeneration. Therefore, augmented levels of myocyte death may well lead to destruction of the contractile myofibrils, which can disrupt the syncitium of the heart during the pathogenesis of various diseases like cardiomyopathy, myocardial infarction, and myocarditis (Bennett, 2002; Haunstetter and Izumo, 1998). Various pro-apoptotic stimuli that contribute towards apoptosis include β-adrenergic stimulation, elevated glucose, oxygen free radicals, activation of the local renin-angiotensin system, hypoxia, ischemia-reperfusion, cytokines like TNF-α, calcium overload (Sabbah, 2000), and free fatty acids (FFA) (Sparagna et al., 2000; Zhou et al., 2000; Haunstetter and Izumo, 1998). Lately, it has been proposed that
cardiac apoptosis may not proceed towards DNA degradation and death (apoptosis interruptus) but may lead to the disintegration of myofibrils such that the contractile properties are lost ('zombie' myocytes) (Communal et al., 2002; Narula et al., 2001). Thus, although the incidence of apoptosis at any one time is low, prevention of cardiomyocyte apoptosis either by restraining pro-apoptotic pathways or stimulating the cell survival apparatus have become important therapeutic options, for the coming years (Haunstetter and Izumo, 2000b; Haunstetter and Izumo, 2000a). An example of an anti-apoptotic remedy in the heart is caspase inhibition (Fauvel et al., 2001; Yaoita et al., 1998).

1.6. APOPTOSIS IN HEART- Effect Of Diabetes

Characteristics of diabetic cardiomyopathy include, myocardial hypertrophy and interstitial fibrosis at a later stage (Grossman et al., 1992). It has been proposed that this hypertrophy may be an insufficient compensatory response to increased cell death in the diabetic heart, which may lead to dilated cardiomyopathy with contractile impairment. In Type 2 diabetic patients, an 85-fold increase in apoptosis and 4-fold increase in necrosis was observed in the hearts (Frustaci et al., 2000). Although morphologically apoptosis and necrosis are distinct entities, they can be initiated by the same stimuli in the heart (Shiraishi et al., 2001). Recently various factors including protein kinase C activation (Shizukuda et al., 2002), oxidative stress, upregulation of renin-angiotensin system (Fiordaliso et al., 2000), and proapoptotic free fatty acids (Dyntar et al., 2001) have been implicated in causing myocardial apoptosis in diabetes. Other than the myocytes, hyperglycemia has also been proposed to cause apoptosis in other cell types such as fibroblasts, endothelial cells (Baumgartner-Parzer et al., 1995), neural cells (Barber et al., 1998), germ cells (Cai et al., 2000), neurons
(Srinivasan et al., 2000) and blastocysts (Keim et al., 2001). Hyperglycemia induced myocardial apoptosis is predominantly regulated by the mitochondrial pathway and is often dependent upon the release of cytochrome c and the caspase-3 pathway. Additionally, prevention of the p53-signaling pathway can prevent cardiomyocyte apoptosis. It should be noted that apoptosis, which is evident acutely in diabetes, progressively decreases with time (Cai et al., 2002; Fiordaliso et al., 2000).

1.7. APOPTOSIS IN HEART: Basic Mechanisms

Although initiated by a number of stimuli, apoptotic pathways in the heart can be classified into two major types based on the initial steps. The first one involves apoptosis via 'death' receptors. These are membrane bound receptors of the tumor necrosis receptor family (TNF-R) such as Fas (CD95), TNF-R1 or death receptors (DR) 3-6, that bind their trimerized ligands, and cause receptor aggregation upon apoptotic stimuli. They subsequently engage adapter proteins like FADD and TRADD via protein-protein interactions (Ashkenazi and Dixit, 1998; Chinnaiyan et al., 1995). These adapters engage the inactive forms of cysteine containing proteases (Pro-caspases) 8 and 2 to the complex (Cohen, 1997). Caspase 8 becomes proteolytically cleaved and 'activated' within this complex (thus known as the death inducing signaling complex or DISC). Once caspase 8 is activated, it initiates a caspase cascade leading to the 'activation' of other downstream effector caspases (caspases 3, 6 and 7). These caspases then disintegrate intracellular proteins and causes DNA fragmentation and cell death.

The second pathway also known as the mitochondrial pathway can be initiated by a number of ways. The mitochondria may lose its membrane potential, which signals the translocation of pro-apoptotic Bcl-2 family members like Bax, Bak or Bid to the
mitochondria. These in turn can release cytochrome c from the intramembrane space into the cytosol. As a protective mechanism, antiapoptotic Bcl-2 family members like Bcl-2, Bcl-X<sub>L</sub> interact with the proapoptotic members to prevent this release of cytochrome c (Gross et al., 1999). The cytochrome c once released gets associated with an adapter protein AIF-1 (apoptosis inducing factor-1) and procaspase 9 to form an apoptosome. Subsequently, caspase-9 gets activated and initiates the caspase cascade (Bennett, 2002; Haunstetter and Izumo, 1998). Upon apoptotic stimuli, the mitochondria can also release Smac/DIABLO which promotes apoptosis by neutralizing IAP (inhibitors of apoptosis proteins), responsible for cellular defense against caspases (Shi, 2001). Interestingly, caspase 8 can activate Bid, thus connecting the two pathways (Creagh and Martin, 2001) (See Figure 9, pg.72). As caspase activation and subsequent proteolysis in apoptosis requires energy, adequate ATP levels are required, in the absence of which the cells may be directed towards necrosis, by the same initiating steps (Nicotera et al., 1999).
2. LIPOTOXICITY AND DIABETES

2.1. DIABETIC HEART: Role of Lipoapoptosis

The adult mammalian heart is dependent heavily on fatty acid supply and its oxidation for normal metabolic function. But the heart like other non-adipose tissues has an inadequate ability to handle excesses FFA, over and above its capacity for utilization (Unger and Orci, 2000). For this reason, an enlargement in intracardiac FFA concentration can overwhelm the process of fatty acid breakdown. Pharmacological inhibition of β-oxidation in rat hearts by 2-tetradecylglycidic acid (Litwin et al., 1990) or oxfenicine (Bachmann and Weber, 1988) leads to lipid accumulation, uncoupling of oxidative phosphorylation, and altered ATPase activities. In this situation FFA accumulate and can, either by themselves, or via their channeling towards the production of second messengers like ceramide, provoke lipotoxicity and cell death (Sparagna et al., 2000). In most studies, FFA induced apoptosis was mostly, but not always (Listenberger et al., 2001) dependent on the de novo synthesis of ceramide, a naturally occurring sphingolipid (Paumen et al., 1997). As palmitoyl CoA is the precursor for ceramide biosynthesis, FFA induced cardiomyocyte apoptosis is predominantly mediated by saturated fatty acids like palmitic acid (Listenberger and Schaffer, 2002) Likewise, incubation of isolated control myocytes with saturated FFA (palmitate-C16:0; stearate-C18:0) led to apoptosis whereas treatment with monounsaturated FFA (oleate-C18:1) was ineffective (de Vries et al., 1997). Under in vitro conditions, co-incubation of adult cardiomyocytes with glucose and palmitic acid induced extensive apoptosis via ceramide formation and cytochrome c release (Dyntar et al., 2001). Interestingly, in a recent study using mildly diabetic animals (injected with
35 mg/kg STZ), the only dramatic finding was that a single molecular species of TG, tripalmitin (mobilizable intracellular storage depot of apoptotic palmitic acid) increases over 5 fold in the diabetic heart (Han et al., 2000). As the adult mammalian heart is composed predominantly of mitochondria, mitochondrial dysfunction can alter the energy homeostasis of the heart. It has long been known that both human and animal models of diabetes suffer from mitochondrial damage during diabetes (Ueno and Shiotani, 1999; Turko et al., 2001). Like FFA, ceramides also interact with cardiac mitochondria to induce the release of free radicals that extensively damage cellular proteins and membranes (Di Paola et al., 2000). It can also lead to the formation of a non-selective pore on the inner mitochondrial membrane, known as mitochondrial pore transition (MPT) that permits the passage of intra-mitochondrial molecules into the cytosol. This not only leads to a loss of membrane potential ($\Delta \psi_m$) (Hirsch et al., 1998a) and mitochondrial respiration, but also to the discharge of a host of pro-apoptotic factors into the cytosol like cytochrome c and apoptosis inducing factor (AIF-1). Cytochrome c has been implicated in the activation of caspases, the effectors of apoptosis. Hence, loss of $\Delta \psi_m$ and release of cytochrome c are central events during FFA-induced apoptosis in myocytes (Sparagna and Hickson-Bick, 1999) (See Figure 10, pg.76). Hearts from Zucker diabetic fatty rats have been observed to be filled with lipids which predispose these organs to increased ceramide levels and apoptosis (Shimabukuro et al., 1998). A 3.7 fold increase in myocyte TG has been reported in STZ diabetic rats (Paulson and Crass, III, 1982) together with a 13-fold increase in apoptosis (Fiordaliso et al., 2000). Finally, human diabetic patients have also been reported to have an increase in myocardial concentrations of triglyceride (Rubler et al., 1972) together with a 85-fold increase in myocyte apoptosis (Frustaci et al., 2000). To
compound this problem of elevated cardiac FFA, decreased antioxidant levels have been reported during diabetes, which could increase oxidative stress on the myocyte (Doi et al., 2001b; Davi et al., 1999). As oxidative stress can also arise due to FFA or ceramide-induced ROS release from the ETC (Quillet-Mary et al., 1997), palmitate mediated apoptosis is often perceived to occur via oxidative stress, although this is controversial (Hickson-Bick et al., 2002).

Other than increased supply of fatty acids, two other pathways also play contributory roles in the development of lipotoxicity. Increased steatosis of the heart along with the skeletal muscle, pancreas and liver of leptin resistant obese fa/ fa ZDF rat (Lee et al., 2001; Unger et al., 1999) has been observed which can be reversed with transgenic overexpression of functional leptin receptors. This is thought to be largely due to increased lipogenesis (Zhou et al., 1998a). In pancreatic islets, under a lack of leptin action, de novo synthesis of fatty acids from glucose also takes place. Leptin resistant pancreatic islets express a range of lipogenic enzymes like sterol regulatory element binding protein 1c (SREBP-1c), peroxisome proliferator-activated receptor (PPAR)-γ, acetyl CoA carboxylase (ACC), fatty acid synthetase (FAS) and glycerol phosphate acyl transferase (GPAT). In leptin deficiency or resistance, which often accompanies diet induced or genetic obesity, de novo lipogenesis takes place in non-adipocytes and can also take place in the heart. Additionally, when non-adipocytes are exposed to a high level of FFA, a compensatory rise in β-oxidation ensues. FFA can act as endogenous ligands for nuclear transcription factors like PPARs and induce excess fatty acid oxidation. Leptin seems to play an important role in this regard (Unger, 2002). Under excess FFA supply, if leptin action is maintained, it leads to upregulation of uncoupling protein (UCP)-2, which disposes off the extra fatty acids through the
generation of heat. However, under lipotoxic conditions, as leptin action is compromised, even 1 mM FFA cannot elicit this compensatory upregulation of fatty acid oxidation (Zhou et al., 1998b), and the excess FFA is channeled towards increased lipogenesis via FAS and ACC action. Recently inhibitors of FAS and ACC have been put forward as a therapeutic approach in treating lipotoxicity (Bergeron et al., 2001; Loftus et al., 2000).
3. OXIDATIVE STRESS and DIABETES

3.1. OXIDATIVE STRESS- Basic Mechanisms

Glycolysis is the first step of intracellular glucose oxidation and generates reduced nicotinamide adenine dinucleotide (NADH) and pyruvate. NADH, other than reducing pyruvate to lactate can donate reducing equivalents to the mitochondrial electron transport chain (ETC). Pyruvate can also be utilized by the tricarboxylic acid (TCA) cycle, to yield carbon dioxide, water, an additional four molecules of NADH and one molecule of reduced flavin adenine dinucleotide (FADH$_2$). NADH and FADH$_2$ are utilized for the generation of ATP by the ETC. The ETC is comprised of four inner membrane-associated complexes (termed as complex I to IV), cytochrome c and ubiquinone (also known as coenzyme Q). NADH, generated from glycolysis and TCA cycle donates electrons to NADH:ubiquinone oxidoreductase (complex I), that transfers the electron to ubiquinone. Ubiquinone can also accept electrons from FADH$_2$ containing dehydrogenases like glycerol-3-phosphate dehydrogenase and succinate: ubiquinone oxidoreductase (complex II). Electrons from reduced ubiquinone are then diverted to ubiquinol: cytochrome c oxidoreductase (complex III) and get transferred to cytochrome c, cytochrome c oxidase (complex IV). The final acceptor of the electron is oxygen, which gets converted to water. The proton gradient generated by such a flux of electron across complex II, IV and I activates ATP synthase (complex V) (Brownlee, 2001b). As in hyperglycemia, intracellular glucose concentrations increase, increased levels of electron donors from the TCA cycle in the form of NADH and FADH$_2$ causes an increased efflux of protons outside the mitochondria and leads to increased mitochondrial membrane potential. As a consequence, mitochondrial ETC gets blocked at complex III and the half-life of free
radical intermediate of ubiquinone is extended. The latter converts mitochondrial molecular oxygen to the free radical, superoxide (O$_2^-$).

It has been recently shown that overexpression of manganese-superoxide dismutase, (Mn-SOD) which dismutates superoxide to hydrogen peroxide (H$_2$O$_2$) in the mitochondria, prevented the three main alterations in diabetes like polyol pathway induction, protein kinase c activation and increased formation of advanced glycation end products (AGE) in endothelial cells (Nishikawa et al., 2000a; Du et al., 2001). Further, in neurons, overexpression of Mn-SOD also blocked apoptosis in response to hyperglycemia. SOD mimetics also decrease collagen induced platelet activation and aggregation demonstrated during hyperglycemia (Yamagishi et al., 2001b). As superoxide radicals can also leak out into the cytoplasm, the cytoplasmic SOD or Cu$^{++}$/Zn$^{++}$-SOD also demonstrates beneficial effects in preventing diabetes induced glomerular injury (Craven et al., 2001). Superoxide per se is not a good oxidizing agent. But excess superoxide radicals may generate hydrogen peroxide, which in turn may generate hydroxyl radicals (OH$^-$) via Fenton chemistry with the help of Fe$^{++}$ (Betteridge, 2000c) (See Figure B).

\[
2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2
\]

\[
Fe^{++} + H_2O_2 \rightarrow Fe^{+++} + OH^- + OH^*
\]

Hydroxyl radical is a very potent oxidant and usually acts at the site of generation, due to its extremely short half-life. Two distinct antioxidant systems have been developed:

a) Catalase present in the peroxisomes of the heart can neutralize hydrogen peroxide to water and oxygen.

\[
Catalase
2H_2O_2 \rightarrow H_2O + O_2
\]
b) Glutathione peroxidase (GPx) can reduce hydrogen peroxide to water with the help of reduced glutathione (GSH), that itself gets oxidized to glutathione disulfide (GSSG). (Griffith, 1999)

\[ \text{2GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{GPx}} \text{GSSG} + \text{2H}_2\text{O} \]

Out of the various free radical induced biochemical processes, the mechanism of lipid peroxidation is the most extensively studied pathway. ROS, like the hydroxyl radical, extracts a hydrogen atom (H*) from a methylene group (---CH₂---) of fatty acids. This leaves an unpaired electron on the carbon of the fatty acid, which by molecular rearrangement is converted to conjugated diene. Subsequently, the diene reacts with oxygen to form a peroxyl radical. This peroxyl radical can extract another hydrogen atom from a different fatty acid to initiate a chain reaction. A chain breaking antioxidant such as vitamin E that scavenges the intermediate lipid peroxyl radicals can terminate such a chain reaction. Unrestrained lipid peroxidation causes changes in lipid membrane fluidity and affects cell membrane permeability, mitochondrial respiratory function and various other biochemical functions (Betteridge, 2000b).

3.2. APOPTOSIS – Initiation And Modulation By Oxidative Stress

Historically it was perceived that treatment of cells with oxidants like hydrogen peroxide causes necrosis (Chandra et al., 2000). But other studies demonstrated that low doses of oxidants could also initiate apoptosis (Hampton and Orrenius, 1997). Intracellular ROS production is often an integral part of the apoptotic machinery initiated by ionizing radiation and chemotherapy. Additionally intracellular depletion of antioxidants like glutathione (GSH) has often been linked to apoptosis in various cell systems (Hall, 1999b). Apoptosis initiated by insults other than oxidative injury can
also be blocked by exogenous treatment with antioxidants like catalase, SOD (Galang et al., 2000) and N-acetyl cysteine (generates intracellular GSH) (Buttke and Sandstrom, 1994). Even the predominantly anti-apoptotic proteins like BCl2 and p35 are known to function as antioxidants in cells undergoing apoptosis (Hockenbery et al., 1993; Sah et al., 1999). Although the role of ROS in inducing apoptosis is well established, an integrated mechanism for the initiation of apoptosis has not yet been established. Most reports suggest the direct role of hydrogen peroxide (H2O2) on the mitochondria. H2O2 causes a collapse of the mitochondrial Δψm and opens a pore on the mitochondrial membrane called the mitochondrial permeability transition (MPT) and causes the release of cytochrome c (Stridh et al., 1998; Hirsch et al., 1998b). An alternative role for ROS induced Fas mediated cell death has also been proposed (Maher et al., 2002), although this claim is debatable (Hug et al., 1994). Whatever may be the initiating mechanism, H2O2 mediated caspase-3 activation has been extensively reported (Matsura et al., 1999). Finally, activation of transcription factors like NFκB and AP-1 and translocation of proapoptotic signals by p53 have been documented (Uberti et al., 1999; Bowie and O'Neill, 2000). These genes may induce the transcription of pro-apoptotic proteins or may suppress the survival promoting factors in the cell. Besides initiation, ROS can also modulate the apoptotic fate of the cell. Caspases can be inactivated by oxidants via oxidation of the thiol groups (in cysteine residues) (Samali et al., 1999) or S-nitrosylation (Melino et al., 1997) in their active site. Excess generation of ROS can also lead to the decrease in mitochondrial ATP (Kajikawa et al., 2002) generation, which under a critical limit promotes necrosis and not apoptosis (Das, 2001).
3.3. GLUTATHIONE – Synthesis, Metabolism And Action

Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinyl glycine, GSH) present in almost all mammalian cells. Its primary role is in the protection of cells and tissues against oxidative and nitrosative stress and against reactive electrophiles (Meister and Anderson, 1983). It has a low molecular weight and is synthesized in vivo from the three amino acids- cysteine, glycine and glutamate. De novo synthesis and the degradation of GSH represent the γ-glutamyl cycle, which is critical in maintaining the cellular redox potential. GSH is synthesized from its constituent amino acids by the action of two ATP-consuming enzymes- γ-glutamylcysteine synthetase (γ-GCS) (Reaction a) and GSH synthetase (Reaction b). The γ-GCS mediated step is rate limiting for the synthesis of GSH (Griffith, 1999).

Reaction a: L-glutamate + L-cysteine + ATP ⇒ L-γ-glutamyl-L-cysteine + ADP + Pi

Reaction b: L-γ-glutamyl-L-cysteine + glycine + ATP ⇒ glutathione + ADP + Pi

Reduced GSH is oxidized to GSH disulfide (GSSG) either non-enzymatically by auto-oxidation in the presence of oxygen or through the activity of the selenium containing enzyme glutathione peroxidase (GPx), which catalyzes the reduction of H₂O₂. This is an important mechanism in the neutralization of ROS (Exner et al., 2000). Normally, GSSG can be reduced back to GSH in an enzymatic recycling reaction by GSH reductase and NADPH. The level of GSH in the cell usually varies, depending on the role of the particular function of the cell, but is usually in the range of 1-8 mM. Adequate NADPH levels and GSH reductase activity maintains a GSH/GSSG ratio of > 100. Thus, under normal levels of ROS generation, there is practically no net loss of GSH. However, if stress levels increase along with an increase in the production of
ROS, and/or a fall in NADPH levels, then GSSG may accumulate. Subsequently, the redox status of the cell shifts activating certain transcription factors like NF-κB (Sen and Packer, 1996; Muller et al., 1997).

GSH (in the form of conjugates) and GSSG can be effluxed out of the cell by transporters and multidrug resistance proteins (MRPs) (Coppola and Ghibelli, 2000; Leslie et al., 2001). Thus, intracellular GSH levels are maintained by three other factors: a) level of γ-GCS b) GSH reductase activity, and c) rates of GSH and GSSG efflux from the cell (See Figure 11, pg.82).

### 3.4. GLUTATHIONE – Role in Mitochondria and Apoptosis

Mitochondrial H₂O₂ can have four fates: a) get converted to hydroxyl radical, b) get neutralized by catalase, c) get neutralized by GPx with the help of GSH and d) leak out of the mitochondrial matrix to accumulate in the cytoplasm (Nohl and Jordan, 1980).

Thus, for detoxification of the mitochondrial H₂O₂, there are only two candidates- catalase and GPx (Antunes et al., 2002). It has been shown before that catalase is not as effective as GPx in prevention of the lipid peroxidation of the heart (Simmons and Jamall, 1989; Molina and Garcia, 1997). As mitochondria lack γ-GCS mRNA, it depends totally on the transport of the cytosolic GSH into the mitochondria which accounts for 10-15% of the total cellular GSH pool (Griffith and Meister, 1985b). Glutathione being negatively charged at physiological pH is transported actively into the mitochondria, also having a negative pH. Ordinarily, the relative concentration of cytosolic and mitochondrial glutathione does not differ, but under oxidative stress, even though the cytosolic GSH is depleted, mitochondrial GSH is preserved, confirming the active nature of GSH transport against the concentration gradient (Hall, 1999a). It has been observed that antimycin A, a drug that increases ROS is more
cytotoxic towards cells with GSH depleted in the mitochondria compared to cytosolic GSH depletion alone (Garcia-Ruiz et al., 1995b). Further, treatment with buthionine sulfoximine (BSO), an inhibitor of $\gamma$-GCS, decreases 90% of the intracellular GSH but is non-toxic in most cells. This may be partly due to the fact that BSO is often unable to deplete mitochondrial glutathione, as under cytosolic GSH depletion, the mitochondria actively accumulates the remaining GSH within the matrix as a protective mechanism. However, diethyl maleate (DEM), which can permeate into the mitochondria and bind irreversibly to all preformed GSH is more cytotoxic (Watson et al., 1996). Interestingly, treatment with DEM can in turn trigger $\gamma$-GCS activity (Cai et al., 1997a). Thus GSH levels return to normal levels within 8-12 hours after DEM administration (Gerard-Monnier et al., 1992a). However co administration of BSO and GSH in vivo has been shown to achieve chronic glutathione depletion in the liver (Gallagher et al., 1992).

It has been also been hypothesized that GSH extrusion from the cell is irreversibly related to the apoptotic process (van den Dobbelsteent et al., 1996). Inhibition of GSH efflux has also been shown to rescue cells from apoptosis and cause them to undergo regeneration instead (Ghibelli et al., 1998). Additionally, glutathione depletion has been linked directly with cytochrome c release from the mitochondria. In the heart, during reperfusion after ischemia, the heart effluxes high levels of GSSG, which persists long after the generation of ROS has ceased (Tritto et al., 1998). Such a loss in the glutathione pool may be prevented by preadministration of N-acetyl cysteine (NAC), a source of cysteine, which increases the glutathione pool, and has been shown to be beneficial in preventing changes in the ischemic-reperfused hearts (Cuzzocrea et al., 2000; Sochman et al., 1990).
3.5. DIABETIC HEART- Role of Oxidative Stress

The predominance of the ROS induced oxidative processes in the pathogenesis of diabetic complications is well established (Nishikawa et al., 2000a). Blocking mitochondrial superoxide production has been shown to reverse hyperglycemic damage (Brownlee, 2001b; Brownlee, 2001b; Yamagishi et al., 2001a). Antioxidants like superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) are responsible for the neutralization of ROS or RNS induced damage (Betteridge, 2000a). Interestingly increases in SOD, GPx and catalase have been observed in the diabetic heart (Kakkar et al., 1996; Mak et al., 1996), this compensatory response against the augmentation of oxidative stress in diabetic rats may be insufficient in protecting the heart over a prolonged period. Supplementation with antioxidants reversed myocardial (Na\(^+\), K\(^+\))-ATPase and calcium ATPase depression in diabetic rat hearts (Kowluru et al., 2000). Reports have suggested that oxidative stress can lead to the development of perivascular fibrosis and alterations of the autonomic nerves and endothelial cells in the diabetic heart (Rosen et al., 1995). An impaired endothelium can increase ROS and reduce the effects of nitric oxide (NO) and increase the risk of coronary artery disease (Giugliano et al., 1995). Conversely, overproduction of NO coupled with excess ROS can lead to the formation of peroxynitrite, which can modify several proteins and amino acids by oxidation. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMP) regulate the degradation of the collagen network of the myocardium (Monnier et al., 1996). Blockade of ROS generation can prevent the upregulation of MMPs in the diabetic hearts (Uemura et al., 2001). Finally apoptosis in the diabetic myocardium has also been linked to increased oxidative stress (Kajstura et al., 2001).
RATIONALE OF THE PROPOSED EXPERIMENTS

In diabetes, since glucose transport and oxidation are insufficient, energy production in the heart is almost entirely via oxidation of free fatty acids (FFA). To achieve this, the heart increases its capacity to take up FFA. This may overwhelm the oxidative pathway such that FFA is directed towards assembly of triglyceride (TG) or production of ceramide, a pro-apoptotic factor. In our model of STZ-diabetes, which has already been established to augment lipoprotein lipase (LPL) at the coronary luminal surface, we attempted to provide an excess of the pro-apoptotic palmitic acid to the heart by feeding a palm oil rich diet. Palmitic acid by itself or through its conversion to ceramide can disrupt the ETC to cause the release of pro-apoptotic factors like cytochrome c (cyt. c) and reactive oxygen species leading to oxidative stress. Following diabetes, such lipotoxicity and attendant oxidative stress may predispose the heart towards increased apoptosis or 'physiological cell death' that may systematically kill cardiomyocytes, thus leading to contractile dysfunction and possible cardiomyopathy (See Fig. A). Lower levels of antioxidants like SOD and GSH could further exacerbate oxidative stress (amplificatory loop) in the STZ-diabetes model. Glutathione is the most important endogenous antioxidant that protects the cardiac mitochondria from such ROS induced damage. However, the cardiac glutathione level is decreased in long term diabetic rats and in humans which may cause an augmentation of oxidative stress and myocardial apoptosis under such conditions.

Overall Hypothesis: Acute diabetes may cause increased myocardial apoptosis due to lipotoxicity and oxidative stress.
Two separate studies were conducted to determine the mechanisms of cardiac apoptosis in acute diabetes. In the Lipotoxicity Study, the role of palmitic acid was investigated by feeding a tripalmitin rich (palm oil rich) diet. In the GSH Study, the role of GSH was studied in causing cardiac apoptosis in acute STZ-diabetes. Further, the therapeutic potential of GSH was studied in ameliorating cell death in the diabetic heart.

**SPECIFIC GOALS OF THE PRESENT INVESTIGATIONS**

In the Lipotoxicity Study, using high fat feeding prior to inducing diabetes, our objectives were to:

1. Determine the role of palmitic and other fatty acids in high fat diets on the diabetic heart.
2. Determine the mechanism behind cardiac apoptosis following high fat feeding and diabetes.

In the GSH Study, using acutely diabetic rats, our objectives were to:

3. Investigate the status of GSH in the heart.
4. Determine the role of cytosolic and mitochondrial GSH on myocardial apoptosis and oxidative stress.
5. Demonstrate the effect of exogenous GSH in ameliorating oxidative damage.
4. RESEARCH DESIGN AND METHODS

Experimental Protocols

STUDY # 1: LIPOTOXICITY STUDY

The objective of the study was to determine the impact of dietary manipulation of palmitic acid in the lipoprotein fractions on cardiomyocyte apoptosis in the rat heart under normoglycemic and hyperglycemic conditions in vivo. Male Wistar rats (220-240 g) were obtained from the University of British Columbia Animal Care Unit and maintained under a 12-h light (0700-1900)/dark cycle. Animals were fed either standard laboratory chow (PMI Feeds, Richmond, VA) or a high fat (HF) diet (AIN\textsuperscript{1}-76A supplemented with either 20% w/w palm oil or 20% w/w sunflower oil) (Research Diets Inc., New Brunswick, NJ) for 4 weeks (for diet composition, please refer to Table 1). Water was provided \textit{ad libitum}. All animals in the study were cared for in accordance with the principles promulgated by the Canadian Council on Animal Care and The University of British Columbia.

STUDY # 2: GSH STUDY

The objective of this study was to establish a role for oxidative stress and GSH in the regulation of apoptosis in the acutely diabetic STZ-rat heart. Male Wistar rats (220-240 g) were obtained from the University of British Columbia Animal Care Unit and maintained under a 12-h light (0700-1900)/dark cycle during the course of their treatment and after the induction of diabetes. They were fed standard laboratory chow diet (PMI Feeds, Richmond, VA). Water was provided \textit{ad libitum}. Other than the untreated (UTX) groups, animals were injected with daily doses of GSH (400mg/kg; ip)

\textsuperscript{1} American Institute for Nutrition
for 18 days prior to the induction of diabetes and also for 3 days subsequent to diabetes induction (van Dam et al., 2001). GSH was dissolved in 0.65N NaOH as and neutralized to pH 7.4 with 0.2N HCl. In different groups, the animals were depleted of their myocardial glutathione subsequent to diabetes with either L-buthionine sulfoximine (BSO; 4mmol/kg, ip; dissolved in saline) (Leichtweis and Ji, 2001) or diethyl maleate (DEM; 4 mmol/kg, ip; dissolved in corn oil) (Connaughton et al., 1996) or both. Parallel control groups were treated with either saline or corn oil was kept and the myocardial GSH levels were similar in both groups (data not shown).

**Induction of Diabetes**

Selective β-cell death and the ensuing diabetic state can be produced after a single intravenous dose of STZ (Junod et al., 1969). A dose-dependent increase in severity of diabetes is produced by 25-100 mg/kg STZ (Rodrigues B et al., 1999). After an I.V., injection of 55 mg/kg STZ, stable hyperglycemia develops within 24-48 hrs and remains 2-3 times higher than normal in concert with a ≈50% reduction in plasma insulin levels. Although these animals are insulin deficient, they do not require insulin supplementation for survival and do not develop ketoacidosis. For both the studies, rats were randomly divided into nondiabetic and diabetic groups. Halothane-anesthetized rats were injected with STZ (55 mg/kg IV, Sigma Chemical Co.) or an equivalent volume (1 ml/kg) of saline. Glycosuria was determined 24 hours after STZ injection, and hyperglycemia was tested at 48 hours with a glucometer. All STZ-treated rats displayed hyperglycemia (>13 mmol/L).
Isolated Whole Heart Perfusion for LPL Assay

For the lipotoxicity study, rats were anesthetized with 65 mg/kg of sodium pentobarbital i.p. (MTC Pharmaceuticals, Cambridge, Ont.), the thoracic cavities were opened and hearts were removed. Rats were not injected with heparin prior to killing, as it displaces LPL bound to HSPGs on the capillary endothelium. Therefore, it was necessary to remove and cannulate the heart as quickly as possible to avoid clotting of blood in the coronary arteries. Immediately upon excision, the beating heart was immersed in ice-cold (4°C) calcium-free Joklik minimal essential medium (pH 7.4) supplemented with 2 g NaHCO₃, 1.2 mM MgSO₄, and 1 mM L-carnitine. After cannulation of the aorta, the hearts were perfused retrogradely by the non-recirculating Langendorff technique for 5 min (or until the perfusate was clear of blood). The perfusion fluid was continuously gassed with 95% O₂-5% CO₂ in a double-walled water-heated chamber that was kept at 37°C with a temperature-controlled circulating water bath. The rate of coronary flow (7-8 ml/min) was controlled by a Masterflex® pump (Cole-Parmer Instrument Co., IL USA). To measure the release of LPL activity into the medium, the perfusion solution was changed to Joklik containing 1% BSA (w/v, 0.15 mM, Fraction V, Boehringer Mannheim Germany), 1 mM CaCl₂ and heparin (5 U/ml, Hepalean, Organon Teknika, Toronto, Canada). This concentration of heparin was previously shown to maximally release cardiac LPL from its binding sites. The coronary effluent was collected for 5 minutes in timed fractions and frozen until assayed for LPL activity (Rodrigues et al., 1997a; Sambandam et al., 1999; Sambandam et al., 2000).
Lipoprotein Lipase Assay

LPL catalytic activity in coronary perfusate and incubation medium of cardiac myocytes was determined by measuring the \textit{in vitro} hydrolysis of a sonicated $[^{3}\text{H}]$ triolein substrate emulsion. The standard assay conditions included 0.6 mM glycerol, tri $[^{9,10-3}\text{H}]$ oleate (1 mCi/mmol), 25 mM PIPES (pH 7.5), 0.05% (w/v) albumin, 50 mM MgCl$_2$, 10% (v/v), heat-inactivated chicken serum (containing the LPL activator, apolipoprotein CII), and 100 µl of either myocyte medium or heart perfusate in a total volume of 400 µl. The release of $[^{3}\text{H}]$ oleate was measured after incubation for 30 min at 30°C. The released $[^{3}\text{H}]$ oleate in the reaction mixture was determined by adding 3 ml of fatty acid extraction solution (methanol: chloroform: heptane; 1.41:1.25:1.0 and 100 µl of oleic acid) and 100 µl of 0.1 M NaOH (Ramirez et al., 1985). After vortex mixing and centrifugation (TY JS-4.2 rotor, 2,500 x g for 30 minutes) using a Beckman J-6B centrifuge, the radioactive sodium $[^{3}\text{H}]$ oleate in a sample (0.5 ml) of the upper phase was determined by liquid scintillation counting. All LPL assays were performed in duplicate where the reaction rate was linear with respect to time and volume of medium assayed. Results were expressed as nanomoles of oleate released per hour per ml (coronary perfusate) or $10^6$ cells (myocyte medium or cells).

Separation of Lipoproteins

For the Lipotoxicity study, prior to removal of the heart, blood was withdrawn from the inferior vena cava. Serum was obtained after centrifugation at 3,000 g for 20 min, and then used for isolation of major lipoproteins by density gradient ultra centrifugation (Chapman et al., 1981). Briefly, ultra centrifugation was carried out at 40,000 rpm (288,000 g) for 18 h at 15°C. Lipoprotein layers were removed using glass Pasteur
pipettes, and the TG fraction of the isolated lipoproteins (VLDL and chylomicrons) were analyzed for individual FFAs.

**Fatty Acid Profile.**

Total TG from circulating lipoproteins and the heart were separated from phospholipids and cholesterol esters using thin-layer chromatography (Hrboticky et al., 1990). FA in the free form and the FA from the TG fractions were converted to their respective methyl esters, and separated by gas chromatography (Varian 3400 gas chromatograph) using a SP-2560 capillary column (100 mm x 0.25 mm internal diameter, 20-μm film thickness) (Supelco, Bellefonte, PA) (Innis and Dyer, 2002). Value of each individual FFA was expressed as a percentage of the total FFA present in the free form or the TG fraction of lipoproteins and heart.

**Preparation of Cardiac Myocytes**

For fluorescence measurements in the GSH study, calcium-tolerant myocytes were made from hearts (ventricles) by a previously described procedure (Rodrigues et al., 1992). In brief, hearts were removed from anesthetized rats and digested by perfusing Joklik buffer containing collagenase (228 U/ml), 0.5% BSA, and 50 mM CaCl₂ retrogradely through the heart. Myocytes were made calcium-tolerant by successive exposure to increasing concentrations of calcium. Our method of isolation yields a highly enriched population of calcium-tolerant myocytes that are rod shaped with clear cross striations in the presence of 1 mM Ca²⁺. Intolerant cells are intact, but hypercontract into vesiculated spheres. Yield of myocytes (cell number) was determined microscopically using a Neubauer haemocytometer. Myocyte viability (generally between 75-85%) was assessed as the percentage of elongated cells with clear cross striations that excluded 0.2% trypan blue. Cardiac myocytes, after isolation
were plated in laminin coated 6 well or 24 well culture plates using Media-199 (Sigma) to a cell density of 0.4 x 10^6 cells/ml and incubated at 37°C under an atmosphere of 95% O_2-5% CO_2 for 16-24 hours.

**Visualization and Estimation of ROS**

A redox sensitive dye, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) was used to assess the presence of free radicals. CM-H_2DCFDA is a cell-permeant indicator for ROS and is nonfluorescent until removal of the acetate groups by intracellular esterases, after which the probe is trapped inside the cells. ROS like H_2O_2, react with the probe, oxidize it and show green fluorescence. In brief, cells were isolated as described above. Thereafter, the cells were plated in NUNC™ 4-well chambered cover slips at a density of 0.1 x 10^6 cells/well. The cells were incubated in M-199 for 16 hours, after which a lane of wells was treated with 50 μM Staurosporine as a positive control for 2 hours (Pong et al., 2001). A vial (50μg) of dry CM-H_2DCFDA was taken and promptly mixed with 0.5 ml dimethyl sulfoxide (DMSO) and then diluted in 3.5 ml PBS. The media in the well was discarded and the cells were washed in PBS (37°C). Finally, each well was incubated with 0.5 ml of the diluted CM-H_2DCFDA probe. A set of wells was incubated with DMSO (diluent for CM-H_2DCFDA) only as a negative control. The cells were incubated for 30 minutes in an incubator at 37°C. The chambers were then directly put under a confocal scanning microscope (Biorad MRC 600) and excited at 480 nm. Micrographs were taken at 530nm at 400X magnification. Control and diabetic myocytes were done on same day to prevent inter-day variations in experimental setup.

For the quantative estimation of ROS, cells were plated on 48 well cell culture plates and incubated with 0.5 ml PBS and CM-H_2DCFDA per well. An increase in green
fluorescence was monitored over the next 120 minutes at 530nm fluorometer. Data was expressed as the percentage increase of fluorescence over time in each well, to negate the differences in cell numbers per well. A positive control, comprised of antimycin A (100 ng/ml), which blocks Complex III and increases ROS and a negative control, rotenone (5\(\mu\)g/ml), that blocks Complex I and decreases ROS formation, was used (Kulisz et al., 2002a). N-acetylcysteine (NAC, 5 mM) which can decrease ROS production by increasing the intracellular GSH concentration, was used to monitor the effect of GSH supplementation in vitro.

Visualisation and Estimation of Mitochondrial Membrane Potential

A novel carbocyanine dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) was utilized to assess changes in mitochondrial membrane potential. The dye exists as a monomer at low concentrations in cytoplasm and yields green fluorescence at 530 nm. Being a cationic dye, it accumulates in the living mitochondria and forms dimers (J-aggregates) and exhibits red fluorescence at 590 nm depending on the membrane potential. Formation of these J-aggregates is directly proportional to the mitochondrial membrane potential. Thus JC-1 is a sensitive marker for mitochondrial membrane potential, and its characteristics superior to other mitochondrial membrane potential markers (Mathur et al., 2000). In brief, cells were isolated as described above. Thereafter, the cells were plated in 24-well culture plate at a density of 0.1 \(\times\) 10\(^6\) cells/well. The cells were incubated in M-199 for 16 hours, after which a lane of wells were treated with 100 \(\mu\)M of the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (mCICCP) as a negative control 30 minutes prior to experimentation. The media from the wells was discarded and the wells were loaded with 10 \(\mu\)g/ml of JC-1 in PBS. The cells were subsequently
incubated for 10 minutes. Finally the cells were twice washed in ice-cold PBS. A lane of the well was kept free of cells and treated exactly the same (blank). Subsequently, the plates were scanned with the CYTOFLUOR™ fluorimeter at 530/580nm wavelength for J-aggregates and 485/530nm for the monomeric JC-1. Subsequently the red: green fluorescence was calculated by subtracting the blank from each lane (Srinivasan et al., 2000). Being a ratiometric probe, there was usually no need for the calculation of cell number in each well.

For confocal microscopy experiments, NUNC™ chambered coverslips were coated with laminin and the cells were plated at a density of $0.1 \times 10^6$ cells/well. The cells were incubated in M-199 for 16 hours, after which a lane of wells is treated with 100 μM of the mitochondrial uncoupler mCICCP for 30 minutes. The old media in the well was discarded and the cells were washed in PBS (37°C). Thereafter, 10 μg/ml of JC-1 in PBS were added to the cells for 10 minutes. Finally, the cells were twice washed in ice-cold PBS. The chambers were then directly put under confocal scanning microscope (Biorad MRC 600) and visualized at 530/580nm wavelength at 600X magnification for J-aggregates and 485/530nm for the monomeric JC-1. A single scan was performed to prevent mitochondrial damage during the experiment.

**Cardiac apoptosis.**

In the Lipotoxicity study, following Langendorff perfusion, part of the left ventricle was fixed in 10% neutral buffered formalin, embedded in paraffin, and 5 μm sections prepared. In the GSH study, the hearts were immediately fixed after isolation. The TdT mediated dUTP nick end labeling (TUNEL) assay was carried out on these sections using a Fluorescein-Fragel™ DNA fragmentation detection kit. During apoptosis, nuclear DNA fragmentation results in 3'-OH overhangs to which fluorescein
(FITC) labeled nucleotides were bound using terminal deoxynucleotidyl transferase (TdT) (with the emission of green fluorescence). All other nuclei were counterstained with red propidium iodide (Scarabelli et al., 1999). Slides were visualized using FITC and Texas Red filters under a Biorad 600 Confocal Microscope at 200x magnification. Four random sections of each heart were observed and quantified using the imaging software ‘Northern Eclipse’. Values are expressed as the number of TdT labeled nuclei per 10⁶ nuclei.

**Caspase-3 activity**

In the Lipotoxicity study, to substantiate the incidence of apoptosis, caspase-3 (whose activity increases preceding apoptosis) was estimated using a kit. Heart tissue (200 mg) was placed in cell-lysis buffer and homogenized (4°C). Subsequently, the suspension was centrifuged at 12,000 g for 3 min, the supernatant separated, and caspase-3 activity measured. ‘Active’ caspase-3 can remove the chromophore pNA (para-nitroaniline) from the labeled substrate DEVD-pNA; measuring free pNA in the reaction mixture is diagnostic for caspase activity (Gurtu et al., 1997). Negative controls were obtained using DEVD-fmk, an irreversible inhibitor of caspase-3. Values are expressed as O.D.U. (optical density units) per gram wet weight.

**Lipid peroxidation**

Oxidative stress is an outcome of imbalance between manufacture of reactive oxygen species and tissue antioxidant protection. Oxidative stress was determined in both the studies by the appearance of thiobarbituric acid-reactive substances (TBARS) like malondialdehyde. Frozen heart tissue was ground under liquid nitrogen, and homogenized. After acidification of the homogenate with 1% phosphoric acid and treatment with 0.6 % TBA solution, the mixture was heated in boiling water for 1 h in
the presence of 0.4% butylated hydroxy toluene to prevent further oxidation of the tissue (Mihara and Uchiyama, 1978). After cooling, the adduct was extracted into 4 ml n-butanol, and the absorbency measured at 540 nm against 1,1,3,3-tetramethoxypropane (utilized as the standard).

**Estimation of Myocardial Glutathione (GSH)**

Myocardial GSH was measured using a commercially available kit (Cayman Chemicals) for both the studies. Tissue GSH was reacted with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to produce a yellow colored 5-thio-2-nitrobenzoic acid (TNB). GS-TNB, produced in parallel, is recycled back to GSH by glutathione reductase to produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample. Values are expressed as μM GSH per gram wet weight.

**Serum Measurements**

Blood samples were collected at the time of sacrifice and were immediately centrifuged (7000 x g for 10 min at 4 °C). Serum was collected and stored at -20 °C until assayed. Commercially available kits were used to measure glucose, TG (Sigma Chemical) and FFA (Wako). Serum insulin was measured using a double-antibody radioimmunoassay kit from Linco Research (St. Louis, MO).

**Materials**

Joklik minimal essential medium was obtained from Gibco Canada (Burlington, ON, Canada); [3H] triolein was purchased from Amersham Canada (Oakville, ON, Canada), [3H]-oleic acid from NEN Life Sciences Products (Boston, MA, USA), heparin sodium injection (Hapalean; 1000 U.S.P. U/ml) from Organon Teknika
(Toronto, ON, Canada). CGS-21680, Research Biochemicals International (RBI-Sigma-Aldrich, Ont., Canada), and Collagenase (class-2, 325 U/mg) was obtained from Worthington Biochemical Corporation (Freehold, NJ, USA). NEFA-C kit (WAKO chemicals GmbH, Neuss, Germany), triolein standard (Sigma Chemicals Co, St. Louis, MO, USA), Insulin RIA kit (Linco Research Inc., MO, USA). Fluorescent probes JC-1 and CM-H$_2$DCFDA were obtained from Molecular Probes. Glutathione and Caspase-3 assay kits were obtained from Cayman Chemicals and Oncogene respectively.

**Statistical Analysis**

Values are means ±SE. One-way ANOVA followed by the Bonferroni tests or the unpaired Student's t-test was used to determine differences between group mean values. The level of statistical significance was set at $P < 0.05$. 
TABLE 1  
Composition of experimental high fat diets

<table>
<thead>
<tr>
<th>INGREDIENTS</th>
<th>SUNFLOWER OIL DIET</th>
<th>PALM OIL DIET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g%     kcal%</td>
<td>g%     kcal%</td>
</tr>
<tr>
<td>Protein</td>
<td>24.1   21</td>
<td>24.1   21</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>44.9   39</td>
<td>44.9   39</td>
</tr>
<tr>
<td>Fat</td>
<td>20.7   40</td>
<td>20.7   40</td>
</tr>
<tr>
<td>Total</td>
<td>100    100</td>
<td>100    100</td>
</tr>
<tr>
<td>kcal/g</td>
<td>4.62   4.62</td>
<td>4.62   4.62</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>INGREDIENTS</th>
<th>g   kcal</th>
<th>g   kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200   800</td>
<td>200   800</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>3     12</td>
<td>3     12</td>
</tr>
<tr>
<td>LoDex 10</td>
<td>75    300</td>
<td>75    300</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>75    300</td>
<td>75    300</td>
</tr>
<tr>
<td>Sucrose</td>
<td>218.8 875</td>
<td>218.8 875</td>
</tr>
<tr>
<td>Cellulose, BW200</td>
<td>50    -</td>
<td>50    -</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>10    90</td>
<td>10    90</td>
</tr>
<tr>
<td>Sunflower Oil</td>
<td>165   1485</td>
<td>-     -</td>
</tr>
<tr>
<td>Palm Oil</td>
<td>-     -</td>
<td>165   1485</td>
</tr>
<tr>
<td>Mineral Mix †</td>
<td>35    -</td>
<td>35    -</td>
</tr>
<tr>
<td>Vitamin Mix ‡</td>
<td>10    40</td>
<td>10    40</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2     -</td>
<td>2     -</td>
</tr>
<tr>
<td>FD&amp;C Dye #40</td>
<td>0.1   -</td>
<td>-     -</td>
</tr>
<tr>
<td>FD&amp;C Dye #1</td>
<td>-     -</td>
<td>0.1   -</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>843.9</strong> 3902</td>
<td><strong>843.9</strong> 3902</td>
</tr>
</tbody>
</table>

†Mineral Mix (mg/g): D i-Calcium P hosphate 50 0; M agnesium O xide 24; P otassium c itrate 220; Potassium Sulfate 52, Sodium Chloride 74, Chromium KSO₄. 12H₂O 0.55, Cupric Carbonate 0.3, Potassium Iodate 0.01, Ferric Citrate 6, Manganous Carbonate 3.5, Sodium Selenite 0.01, Zinc Carbonate 1.6, Sucrose 118.03
‡Vitamin Mix (mg/g): Vitamin A 0.8, Vitamin D₃ 1, Vitamin E 10, Menadione Sodium bisulfite 0.08, Biotin1% 2, Cyanocobalamin 0.1% 1, Folic Acid 0.2, Nicotinic Acid 3, Calcium Pantothenate 1.6, Pyridoxine–HCl 0.7, Riboflavin 0.6, Thiamin HCl 0.6, Sucrose 978.42
TABLE 2:  
Characteristics of diabetes in standard chow fed rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight, g</strong></td>
<td>381 ± 9</td>
<td>359 ± 11</td>
</tr>
<tr>
<td><strong>Serum Parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>8.6 ± 0.2</td>
<td>22.1 ± 0.4*</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>2.8 ± 0.4</td>
<td>0.8 ± 0.3*</td>
</tr>
<tr>
<td>Triglyceride, mmol/l</td>
<td>0.8 ± 0.1</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>FFA, mmol/l</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td><strong>Cardiac parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-3 activity, O.D.U/ g.w.w</td>
<td>1.6 ± 0.1</td>
<td>3.3 ± 0.3*</td>
</tr>
<tr>
<td>TUNEL positive nuclei / 10⁶ nuclei</td>
<td>101 ± 15</td>
<td>495 ± 20*</td>
</tr>
</tbody>
</table>

Values are means ± SE for 6 animals in each group. Animals were maintained on standard chow diets (5% corn oil) for 4 weeks prior to diabetes induction (55 mg/kg STZ). Diabetic animals were kept for 4 days before being killed. *Significantly different from controls, P < 0.05. O.D.U, optical density units; g.w.w, gram wet weight; TUNEL, TdT-mediated dUTP nick end labelling.
### TABLE 3

Characteristics of diabetes in high fat fed rats

<table>
<thead>
<tr>
<th></th>
<th>SUNFLOWER OIL</th>
<th></th>
<th>PALM OIL</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td><strong>Body Weight, g</strong></td>
<td>485 ± 14</td>
<td>393 ± 11*</td>
<td>473 ± 8</td>
<td>389 ± 11*</td>
</tr>
<tr>
<td><strong>Serum FFA, mmol/l</strong></td>
<td>0.4 ± 0.0</td>
<td>1.8 ± 0.8*</td>
<td>0.4 ± 0.0</td>
<td>1.1 ± 0.2*</td>
</tr>
<tr>
<td><strong>Serum Glucose, mmol/l</strong></td>
<td>11.1 ± 0.5</td>
<td>23.8 ± 0.5*</td>
<td>13.4 ± 0.6</td>
<td>23.1 ± 0.3*</td>
</tr>
<tr>
<td><strong>Serum Insulin, ng/ml</strong></td>
<td>5.5 ± 1.1</td>
<td>1.5 ± 0.6*</td>
<td>5.1 ± 1.8</td>
<td>0.8 ± 0.3*</td>
</tr>
<tr>
<td><strong>Total Cardiac FFA, μg/mg protein</strong></td>
<td>1.6 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE for 6 animals in each group. Animals were maintained on the two different high fat diets (20% w/w) for 4 weeks prior to diabetes induction (55 mg/kg STZ). Diabetic animals were kept for 4 days before being killed. Values are those obtained before death and were from fed animals. *Significantly different from their respective controls, P < 0.05.
TABLE 4.
Lipid profile in normal chow fed animals

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Lipoprotein</th>
<th>Cardiac TG</th>
<th>Cardiac FFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic Acid</td>
<td>20.9 ± 0.9</td>
<td>24.7 ± 1.2</td>
<td>14.8 ± 0.5</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>26.0 ± 1.3</td>
<td>29.3 ± 1.1</td>
<td>25.1 ± 1.6</td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>26.4 ± 1.5</td>
<td>18.3 ± 1.6</td>
<td>20.2 ± 0.7</td>
</tr>
<tr>
<td>Docosahexaenoic Acid</td>
<td>7.5 ± 0.6</td>
<td>3.8 ± 0.6</td>
<td>6.6 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4 animals in each group. Animals were maintained on the two different high fat diets for 4 weeks prior to diabetes induction. Plasma lipoproteins of density ≤1.006 g/ml and cardiac TG were separated. FFA from TG were converted to methyl esters and separated by gas chromatography; of the total TG-FA that were estimated, only the relevant FA are listed.
### TABLE 5
Triglyceride-FA profile in control and diabetic rats fed high fat diets

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th><strong>SUNFLOWER OIL</strong></th>
<th><strong>PALM OIL</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td><strong>A. Lipoproteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>10.9 ± 0.6</td>
<td>12.7 ± 1.2</td>
</tr>
<tr>
<td>C16:1</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>C18:0</td>
<td>4.4 ± 0.9</td>
<td>4.5 ± 1.0</td>
</tr>
<tr>
<td>C18:1</td>
<td>20.1 ± 0.9</td>
<td>13.5 ± 1.2</td>
</tr>
<tr>
<td>C18:2n6</td>
<td>46.4 ± 2.5</td>
<td>40.8 ± 3.3</td>
</tr>
<tr>
<td>C20:4n6</td>
<td>8.6 ± 1.8</td>
<td>14.3 ± 4.0</td>
</tr>
<tr>
<td>C22:6n3</td>
<td>0.9 ± 0.3</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td><strong>B. Heart</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>10.4 ± 0.4</td>
<td>9.7 ± 0.8</td>
</tr>
<tr>
<td>C16:1</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>C18:0</td>
<td>5.2 ± 0.1</td>
<td>8.3 ± 1.5</td>
</tr>
<tr>
<td>C18:1</td>
<td>26.0 ± 0.6</td>
<td>24.8 ± 0.8</td>
</tr>
<tr>
<td>C18:2n6</td>
<td>48.6 ± 0.6</td>
<td>42.5 ± 1.5</td>
</tr>
<tr>
<td>C20:4n6</td>
<td>3.3 ± 0.3</td>
<td>5.9 ± 1.5</td>
</tr>
<tr>
<td>C22:1</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>C22:6n3</td>
<td>0.9 ± 0.2</td>
<td>4.8 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4 animals in each group. Animals were maintained on the two high fat diets for 4 weeks prior to diabetes induction. Serum lipoproteins and cardiac TG were separated. FFA from TG were separated by GC; only those FA demonstrating values greater than 1% of the total TG-FA are listed. *Significantly different from sunflower oil fed diabetic; †Significantly different from sunflower oil fed control, P < 0.05.

C16:0, palmitic acid; C16:1, palmitoleic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2n6, linoleic acid; C20:4n6, arachidonic acid; C22:1, erucic acid; C22:6n3, docosahexaenoic acid
TABLE 6
Oxidative Stress parameters in rats fed high fat diets.

<table>
<thead>
<tr>
<th></th>
<th>SUNFLOWER OIL</th>
<th>PALM OIL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td>MDA (nmol/g.w.w)</td>
<td>49.3 ± 5.8</td>
<td>61.8 ± 5.3</td>
</tr>
<tr>
<td>GSH (μM/g.w.w)</td>
<td>1.2 ± 0.1</td>
<td>0.7 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE for 6 animals in each group. Animals were maintained on the two different high fat diets (20% w/w) for 4 weeks prior to diabetes induction (55 mg/kg STZ). Diabetic animals were kept for 4 days before being killed. The hearts were isolated, homogenized and estimated for different oxidative stress related parameters. *Significantly different from their respective controls, \( P < 0.05 \).
# Table 7

General Characteristics of rats in GSH study

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>DIA</th>
<th>DIA+ BSO</th>
<th>DIA+ DEM</th>
<th>DIA+BSO+ DEM</th>
<th>DIA +GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight, (g)</td>
<td>384± 5</td>
<td>366± 3</td>
<td>361± 6</td>
<td>367± 12</td>
<td>355± 12</td>
<td>358 ± 18</td>
</tr>
<tr>
<td>Serum Glucose</td>
<td>8.3 ± 0.2</td>
<td>22.9± 0.3*</td>
<td>25.1± 0.5*</td>
<td>27.7± 0.7*</td>
<td>26.1±0.7*</td>
<td>25.7± 0.1*</td>
</tr>
<tr>
<td>(mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Glutathione</td>
<td>2.5± 0.4</td>
<td>2.9± 0.5</td>
<td>0.6± 0.4†</td>
<td>NE</td>
<td>NE</td>
<td>4.3± 0.2†</td>
</tr>
<tr>
<td>(μM/g.w.w)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Reduced Glutathione</td>
<td>2.2± 0.4</td>
<td>2.0± 0.4</td>
<td>0.4± 0.3†</td>
<td>NE</td>
<td>NE</td>
<td>3.8± 0.4†</td>
</tr>
<tr>
<td>(μM/g.w.w)</td>
<td></td>
<td></td>
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</tbody>
</table>

Values are means ± SE for 6 animals in each group. Animals were treated with either BSO and/or DEM subsequent to the induction of diabetes (55 mg/kg STZ). Diabetic animals were kept for 4 days before being killed. The hearts were isolated, homogenized and estimated for cardiac glutathione. Serum was separated for the estimation of glucose. g.w.w, gram wet weight; NE, could not be estimated. *Significantly different from untreated controls, † Significantly different from untreated diabetics. $P < 0.05$. 
Figure 1 Average food intake in animals on two different high fat diets for 4 weeks prior to diabetes induction. There was no significant difference in food intake among the two groups over the 4-week period.
Figure 2 Relative proportions of major FFA in the heart subsequent to HF feeding and diabetes. Animals were maintained on the two different high fat diets for 4 weeks prior to diabetes induction. Cardiac FFAs were extracted with chloroform:methanol:acetone:hexane solvent and were converted to their respective methyl esters and separated by gas chromatography. *Significantly different from sunflower oil fed diabetic. †Significantly different from sunflower oil fed control; $P < 0.05$. 

<table>
<thead>
<tr>
<th></th>
<th>Palmitic acid</th>
<th>Oleic acid</th>
<th>Linoleic acid</th>
<th>Docosahexaenoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUNFLOWER OIL</td>
<td></td>
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<tr>
<td>PALM OIL</td>
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</tbody>
</table>

CONTROL           DIABETIC        CONTROL           DIABETIC
Figure 3. Coronary lipoprotein lipase (LPL) and serum triglycerides (TG) in rats fed high fat diets. After 4 days of diabetes, rats were anesthetized, thoracic cavity was opened, and isolated hearts perfused. Coronary LPL was released with heparin (5 U/ml) over 5 minutes (Fig. 3A), and activity at varying time points determined using radiolabeled triolein. Total LPL activity released (area under the curve) was also plotted (Fig. 3B). Blood collected from the inferior vena cava was used for serum TG measurement using an appropriate kit (Fig. 3C). Results are the means ± SE of 6 rats in each group. *Significantly different from respective diabetic; †Significantly different from respective controls, $P < 0.05$. 
Figure 4 Effect of 4 days of diabetes on cardiac apoptosis in animals fed a high fat diet for 4 weeks. Following perfusion, fixing, and embedding in paraffin, control and diabetic heart sections were treated with TdT enzyme and fluorescein labeled nucleotide to visualize green/yellow apoptotic nuclei (as indicated by the arrows) in the representative confocal micrographs (Fig. 4A). All nuclei were counterstained red with propidium iodide. A. Sunflower oil fed control; B. Sunflower oil fed diabetic; C. Palm oil fed control; D. Palm oil fed diabetic.

Quantitative estimation was done using the imaging software 'Northern Eclipse' and the results were expressed per \(10^5\) nuclei (Fig. 4B). Results are the means ± SE of 6 rats in each group. *Significantly different from sunflower control, †Significantly different from all other groups, \(P < 0.05\).
Figure 5 Mitochondrial dysfunction after acute diabetes. Diabetic and control myocytes were isolated and incubated with the mitochondrial membrane potential sensitive fluorescent dye JC-1. Parallel controls were incubated with a mitochondrial uncoupler mCICCP. A. Live mitochondrial staining with JC-1 as visualized under confocal microscopy (magnification: 600x) under red channel. Diabetic myocytes demonstrated less of red fluorescence compared to the controls. B. JC-1 ratio (red: green) as estimated quantitatively using fluorimeter. Results are the means ± SEM of 3 rats in each group. *Significantly different from control. †Significantly different from untreated diabetic, P<0.05.
Figure 6 Oxidative stress in acute diabetes. Diabetic and control myocytes were isolated and incubated with the ROS sensitive dye CM-H$_2$DCFDA. Parallel controls were incubated with antimycin A (AA, positive control) and rotenone (ROT, negative control). Diabetic myocytes were also incubated with n-acetyl cysteine (NAC) to replenish intracellular glutathione levels. A. Live mitochondrial staining with CM-H$_2$DCFDA as visualized under confocal microscopy (magnification: 400x) under green channel. Diabetic myocytes demonstrated more fluorescence compared to the controls. B. Quantitative estimation of ROS over 120 mins using CM-H$_2$DCFDA as estimated quantitatively using fluorimeter. Results are the means ± SEM of 3 rats in each group. UTX, untreated. *Significantly different from control. †Significantly different from untreated diabetic, P<0.05.
Figure 7 Effect of diabetes on cardiac apoptosis in control, diabetic and diabetic treated with BSO, DEM, BSO+DEM and GSH. TUNEL assay was performed on paraffin-embedded heart sections in quadruplicate. Estimation was done using the imaging software 'Northern Eclipse' and the results were expressed per 10^6 nuclei. Results are the means ± SEM of 4 rats in each group. UTX, untreated. *Significantly different from control. †Significantly different from untreated diabetic, P<0.05.
Figure 8: Cardiac lipid peroxidation in control, diabetic and diabetic treated with BSO, DEM, BSO+DEM and GSH. TBARS assay was performed on isolated, frozen hearts and MDA levels estimated that is directly proportional to lipid peroxidation. Results are expressed as percentage increase to control and are the means ± SEM of 4 rats in each group. UTX, untreated. *Significantly different from control. †Significantly different from untreated diabetic, P<0.05.
5. RESULTS

1. Lipotoxicity Study

*Cardiac apoptosis in standard chow fed rats following diabetes.* Administration of STZ induces hyperglycemia and hypoinsulinemia within 24 h that persisted all through the 4-day period (Table 2). There was no significant change in body weight, serum FFA or serum TG at this early stage of diabetes. Based on previous reports of an augmented apoptosis in hearts from STZ diabetic rats and mice (Cai et al., 2002; Fiordaliso et al., 2000), we attempted to corroborate these results in our model of acute diabetes. TUNEL staining showed that diabetic hearts had an approximately 5-fold increase in apoptotic cells compared to control hearts (Table 2). There was a corresponding 2-fold increase in active caspase-3 activity in diabetic hearts (Table 2).

*STZ diabetes in high fat fed rats.* Having established the incidence of apoptosis in the acutely diabetic heart, we next tested the influence of HF feeding on this apoptotic process. There was no difference in food intake (Fig.1) or body weight (Table 3) between rats fed a semi-purified diet enriched with either palm oil or sunflower oil for 4 weeks. Although not the focus of this study, when compared to serum insulin and glucose values from standard chow fed rats (see Table 2), HF fed animals were hyperinsulinemic, with slightly elevated serum glucose (Table 3). Diabetes for 4 days reduced serum insulin and caused hyperglycemia. More importantly, induction of diabetes in HF fed animals caused a substantial loss of body weight and marked elevation of serum FFA (Table 3). These latter changes were more intense than changes observed following a comparable diabetic interval in standard chow fed animals (Table 2). When compared to standard chow fed rats (0.24±0.03 μg/mg
protein), cardiac FFA following HF feeding was 6-7 fold higher in the control groups (Table 3). Unlike serum FFA that changed dramatically in the HF groups following diabetes, FFA levels in the heart remained unchanged compared to the respective controls (Table 3).

**Serum lipoprotein and cardiac TG fatty acid composition.** Table 4 illustrates the lipoprotein and TG derived fatty acids of the normal chow fed animals. Table 5 demonstrates the TG derived fatty acids in circulating lipoproteins (Table 5A) and heart (Table 5B) in HF fed animals. Feeding palm oil successfully magnified the palmitic acid content within TG in both fractions when compared to sunflower oil fed controls (as a % of total fatty acids). Diabetes had no additional effect in modifying palmitic acid levels. Of the other major FFAs that changed in either lipoprotein or cardiac TG following HF feeding, oleic acid increased after palm oil whereas sunflower oil essentially increased linoleic acid. Arachidonic acid within lipoprotein TG increased after sunflower oil feeding and remained high after diabetes induction. These changes in arachidonate levels were not observed in cardiac TG within the different groups. Docosahexaenoic acid (DHA) was the only major FA that changed in the lipoprotein and cardiac TG following diabetes, with a greater increase observed in the sunflower oil diabetic group. In summary, palm oil feeding increased the percentage of saturated fatty acids whereas sunflower oil increased the polyunsaturated fatty acids (PUFA) content of the lipoprotein and cardiac TG.

**Cardiac free fatty acid composition.** Determination of cardiac FFA revealed a profile that was almost similar to that observed for FA within TG present in lipoproteins and the heart (Fig.2). Thus, palm oil feeding increased palmitic and oleic acid whereas sunflower oil increased linoleic acid. On induction of diabetes, oleic and linoleic acid
tended to increase whereas the proapoptotic palmitic acid did not change in both the HF groups. Interestingly, DHA levels were the lowest in palm oil fed diabetic hearts.

**Cardiac heparin-releasable LPL activity.** Perfusion of whole hearts from different groups with heparin resulted in the release of LPL activity into the coronary perfusate. The heparin-mediated LPL discharge was rapid, and peak activity, thought to represent LPL located at or near the endothelial surface, was observed within 1 min. Peak (Fig. 3A) and total (Fig. 3B) LPL activity released over 5 min in the coronary perfusate was significantly higher in the sunflower oil fed compared to palm oil fed rats. Interestingly, serum TG levels were also lower in palm oil fed controls compared to the sunflower oil group (Fig. 3C), suggesting that increased palmitic acid makes lipoproteins better substrates for LPL and hence limits the need for this enzyme at the coronary lumen. Subsequent to induction of diabetes, both HF fed animals demonstrated low cardiac LPL activity (Fig. 3A and B), with a substantial increase in serum TG (Fig. 3C).

**Cardiac apoptosis in high fat fed rats following diabetes.** To determine whether dietary manipulation of palmitic acid affects cardiac apoptosis in the rat following diabetes, animals were fed HF diets for 4 weeks prior to STZ injection, and cell death evaluated by TdT staining (Fig. 4A and B). When compared to standard chow fed controls (Table 2), apoptotic cell death increased just by feeding HF. Furthermore, palm oil augmented this apoptotic effect when compared to sunflower oil fed controls. Although diabetes increased apoptosis in both HF groups, palm oil fed diabetic groups demonstrated the highest amount of TdT labeled nuclei. This effect occurred independent of any further increase in either total FFA (Table 3) or free palmitic acid in the heart (Fig.2), and illustrates the synergistic effect of glucotoxicity in this process.
Oxidative stress and glutathione levels in HF fed diabetic rats. Palmitic acid provokes cardiomyocyte apoptosis through oxidative stress by way of excess production of free radicals. Both high fat feeding and hyperglycemia independently bring about oxidative damage in the myocardium, frequently as a consequence of oxidation of cellular lipids by free radicals like hydroxyl ions. As a by-product of this reaction, TBARS like MDA are generated and its measurement is a direct correlation to the extent of oxidative damage. To examine the collective effect of HF feeding and hyperglycemia on oxidative stress, MDA was measured in hearts from the different groups. When compared to standard chow fed (27.2±4.9 nmol/g wet weight) animals, all HF groups had high MDA levels, with the highest in hearts isolated from palm oil fed diabetic rats (Table 5). Intracardiac GSH is essential for neutralizing ROS in the heart (Giugliano et al., 1995). Compared to standard chow fed animals (2.1±0.2 μmol/g wet weight), GSH levels were depleted in all HF groups. Interestingly, diabetes further reduced GSH levels only in the sunflower oil fed animals without any increase in lipid peroxidation (Table 6).

2. GSH Study

Mitochondrial dysfunction following acute diabetes. Changes in mitochondrial membrane potential have been proposed to be a key player behind cytochrome c release and apoptosis. Hyperglycemia induced cardiac apoptosis has also been intimately linked with cytochrome c release. In order to test the mitochondrial membrane potential in our model of acutely diabetic STZ rats, we isolated cardiomyocytes from control and diabetic rats and investigated the mitochondrial membrane potential in these cells with the help of a dual fluorescent dye, JC-1. Under confocal microscopy the diabetic myocytes consistently demonstrated less red
fluorescence (Fig 5a) signifying less J-aggregate formation, which in turn is directly proportional to the mitochondrial membrane potential. Thus when quantified as a ratio to the green fluorescence with the help of a fluorometer, diabetic myocytes demonstrated less mitochondrial membrane potential with respect to controls (Fig 5b). Preincubation with a mitochondrial uncoupler MLCCP (negative control) profoundly decreased the mitochondrial membrane potential.

**Oxidative stress following acute diabetes.** Mitochondrial superoxide generation has been proposed as a prime contributor behind diabetes related complications (Nishikawa et al., 2000b). Hyperglycemia induced cardiac apoptosis has also been intimately linked with an increase in ROS production (Shizukuda et al., 2002; Kajstura et al., 2001). In order to test the incidence of oxidative stress in our model of acutely diabetic STZ rats, we isolated cardiomyocytes from control and diabetic rats and investigated the prevalence of free radicals like H$_2$O$_2$ and OH$^-$ in these cells with the help of a ROS sensitive dye, CM-H$_2$DCFDA. Under confocal microscopy the diabetic myocytes consistently demonstrated more green fluorescence (Fig 6a) signifying more oxidation of the dye or the occurrence of ROS. When quantified fluorimetrically, diabetic myocytes demonstrated more fluorescence over 120 minutes with respect to controls (Fig 6b). Incubation with antimycin A (positive control) induced a quantum increase in fluorescence with rotenone (negative control) demonstrating a decrease. Incubation with NAC decreased the fluorescence to less than control levels.

**Cardiac glutathione levels following STZ diabetes and various treatments.** STZ diabetes within 4 days did not cause any significant change in body weight. Hyperglycemia was pronounced after STZ administration in all diabetic groups. (Table 2 and Table 7). Four days of hyperglycemia was not associated with a decrease in
either myocardial total glutathione or the 'active' GSH levels. However, administration of BSO for 3 days reduced GSH to almost undetectable levels. Being so low with BSO alone, myocardial glutathione levels could not be estimated for either DEM or DEM+ BSO studies. Additionally, chronic administration of GSH was successful in increasing both total and 'active' GSH levels by almost 2 fold.

*Cardiac apoptosis in rats under various treatments following diabetes.* Administration of STZ induces hyperglycemia that led to similar (4-5 fold) increase in apoptosis. Based on previous reports of an augmented apoptosis in hearts from glutathione depleted rats and mice, we attempted to corroborate these results in our model of acute diabetes. However administration of BSO or DEM alone was unsuccessful in further augmenting apoptosis. However, administration of both BSO and DEM led to a tremendous rise in apoptosis (Fig.7). Chronic administration of GSH completely reversed the hyperglycemia induced rise in apoptosis to its control levels (Fig.7).

*Cardiac lipid peroxidation in rats under various treatments following diabetes.* Based on previous reports of an augmented lipid peroxidation in hearts from glutathione-depleted rats and its intimate relation to apoptosis, we attempted to corroborate these results in our model of acute diabetes. Hyperglycemia per se induced increased lipid peroxidation within 4 days. However, similar to apoptosis, administration of BSO or DEM alone was unsuccessful in further augmenting MDA levels. However, administration of both BSO and DEM led to a tremendous rise in lipid peroxidation (Fig.8). Chronic administration of GSH completely reversed the hyperglycemia induced rise in oxidative stress to its control levels (Fig.8).
Diabetes is a significant risk factor for cardiovascular diseases with the majority of these complications being attributed to coronary vascular pathology (Ledet et al., 1979). However, both in humans and animal models of diabetes, an additional heart muscle specific disease in the absence of any vascular pathology has also been described, and termed “diabetic cardiomyopathy” (Rodrigues et al., 1995). Numerous studies have been done to explain the etiology of this multifactorial disorder. Probable candidates to explain this heart disease include autonomic abnormalities, metabolic disorders, Ca\textsuperscript{++} handling defects, abnormal contractile protein and enzyme function, interstitial fibrosis (Rodrigues et al., 1995) and more recently physiological cell death or apoptosis (Frustaci et al., 2000).

An abnormal increase in plasma glucose may predispose cardiomyocytes to death by apoptosis such that contractile function is ultimately altered. Changes in protein kinase C activity, generation of reactive oxygen species (ROS) (Shizukuda et al., 2002), mitochondrial dysfunction (Cai et al., 2002), and activation of p53 and the renin-angiotensin system (Fiordaliso et al., 2001) have all been put forward to explain this hyperglycemia induced cell death. More recently, it has also become clear that the development of cardiomyopathy in diabetes could also occur subsequent to changes in lipid homeostasis in the heart (‘lipotoxicity’) (Dyntar et al., 2001). In non adipose tissues, this term is used to describe an overload of fatty acids (FFA) exceeding their capacity for oxidation/utilization (Zhou et al., 2000).

During diabetes, as glucose transport and oxidation is defective, energy production in the heart is almost exclusively via β-oxidation of FFA. To guarantee FFA supply to the
diabetic heart, lipoprotein lipase (LPL) activity at the coronary luminal surface is elevated (within hours) (Sambandam et al., 1999; Rodrigues et al., 1997b; Sambandam et al., 2000). Interestingly, a) $V_{\text{max}}$ of LPL increases linearly with the increase in palmitic acid content of chylomicrons and very low density lipoprotein (VLDL) (Sato et al., 1999; Sato et al., 2002); b) incubation of VLDL with LPL predominantly releases palmitic acid (47.5% of total fatty acids released) (Gouni-Berthold et al., 2001), and c) following acute diabetes, the predominant FFA species that is enhanced within the myocardium is palmitic acid (Han et al., 2000). Thus, by hydrolyzing circulating lipoproteins (Sambandam et al., 1999; Rodrigues et al., 1997b; Sambandam et al., 2000), LPL may provide a continuous supply of palmitic acid to the diabetic heart, a preferred FFA for $\beta$-oxidation. Palmitic acid in excess causes intracellular accumulation of ceramide and ROS in various cell systems (Listenberger and Schaffer, 2002; Maedler et al., 2001). Additionally, in both neonatal and adult cardiomyocytes, palmitic acid induces apoptosis, either in the presence or absence of hyperglycemia (Sparagna et al., 2000; Dyntar et al., 2001). All of these experiments utilized free palmitic acid, and in vitro model systems. Although this setting partly resembles palmitic acid derived from plasma, it does not account for other FFA sources [e.g., provision of palmitic acid via breakdown of triglyceride (TG) within lipoproteins], nor does it take into account the effect of palmitic acid in a milieu of diverse FFA, under in vivo conditions. Moreover, regulatory mechanisms that control FFA homeostasis are compromised in isolated cells. The objective of the Lipotoxicity study was to determine the impact of dietary manipulation of palmitic acid on cardiomyocyte apoptosis and oxidative stress in the rat heart under normoglycemic and hyperglycemic conditions in vivo.
Figure 9. Schematic of the two pathways of apoptosis. Fas, a member of the death receptor family binds to its ligands, recruit FADD and activate BID. FLIP in turn inhibits the activation of caspase 8. Caspase 8 once activated propagates the caspase cascade and causes apoptosis. Antiapoptotic members of the BCI-2 family (including BCI-X) interact with proapoptotic members like BAX, to determine the release of cytochrome c (cyto c) and Smac/DIABLO from the mitochondria. Cytochrome c recruits apaf-1 and activates caspase 9, that like caspase 8 can initiate the caspase cascade. Additionally, Smac/DIABLO inhibit iAPs (inhibitor of apoptosis proteins) and propagates apoptosis.
As a number of issues like strain, dosage of STZ and duration of the diabetic state may influence apoptosis; we were concerned about the prevalence of this mode of cell death in our acute model of diabetes. We detected a 5-fold increase in apoptosis in the standard chow fed diabetic heart using TUNEL, and further validated this observation by identifying an increase in caspase-3 activity, the proteolytic enzyme involved in the execution of apoptosis. As both gluco- (Fiordaliso et al., 2001; Baumgartner-Parzer et al., 1995) and lipotoxicity (Sparagna et al., 2001; Sparagna et al., 2000) can be independently pro-apoptotic, serum levels of glucose, TG and FFA were measured. Four days after STZ, animals were hyperglycemic but not hyperlipidemic. Although our results point toward glucotoxicity being the key regulator of apoptosis at this time point, this may not be entirely accurate. Accordingly, given that coronary luminal LPL is elevated in our moderately diabetic hearts (Sambandam et al., 1999; Rodrigues et al., 1997b; Sambandam et al., 2000), these hearts may still be provided with excess palmitic acid among all other fatty acids (Gouni-Berthold et al., 2001). Interestingly, VLDL hydrolysis-derived palmitic acid (via LPL) causes an 8-fold greater vascular smooth muscle cell death than when it is used individually (Gouni-Berthold et al., 2001).

To underline the role of palmitic acid in eliciting apoptosis in vivo, we utilized a palm oil diet rich in palmitic acid. Sunflower oil, rich in linoleic acid, was used as an isocaloric control. Both HF diets were able to equally augment total cardiac FFA, and could explain the increase in apoptosis and oxidative stress in the control groups. However, feeding palm oil substantially increased palmitic acid in the free and esterified form in the heart, and could have directed the heart towards excessive apoptosis in these animals subsequent to diabetes (Listenberger and Schaffer, 2002). Oleic acid, a desaturation-elongation product of palmitic acid, also increased following palm oil.
Interestingly, oleic acid has either been shown to be ineffective (Sparagna et al., 2000) or decreases (Dyntar et al., 2001) cardiac apoptosis when incubated simultaneously with palmitic acid. More importantly, oleate may have an antiapoptotic effect in certain cell lines (Hardy et al., 2000). Whether oleic acid performs a similar function to prevent even greater apoptosis in palm oil diabetic hearts is yet to be determined.

Heart LPL plays a significant role in lipoprotein catabolism (Augustus et al., 2002). As $V_{\text{max}}$ of LPL for lipoproteins goes up linearly with increase in palmitic acid (Sato et al., 1999; Sato et al., 2002), but not with linoleic acid (Sato et al., 2002), an efficient lipolysis of lipoproteins is maintained with low LPL, and could substantiate the decrease in circulating TG in palm oil fed control animals. Interestingly, FFA in the heart remained unchanged following diabetes despite a dramatic increase in circulating FFA in both HF groups. This may imply that additional compensatory mechanisms must be working to limit the excessive delivery of FFA into the heart. In fact, unlike previous data demonstrating high cardiac LPL following diabetes (Sambandam et al., 1999; Rodrigues et al., 1997b; Sambandam et al., 2000), LPL activity declined in HF fed diabetic rats. Such control of cardiac LPL could be the limiting factor preventing disproportionate delivery of FFA into the heart, as suggested earlier (Fielding and Frayn, 1998), and given the importance of cardiac LPL in determining circulating TG levels (Levak-Frank et al., 1999), could also explain the high levels of serum TG following diabetes.

Oxidative stress has been implicated in the etiology of several lipotoxic (Zhou et al., 2000; Unger, 2002; Listenberger and Schaffer, 2002) and diabetic (Cai and Kang, 2001; Nishikawa et al., 2000a) complications. It can arise as a result of an imbalance between free radicals like superoxide and hydrogen peroxide and antioxidants like the GSH system and manganese superoxide dismutase (MnSOD) (Droge, 2002). Lipid
peroxidation increased significantly in hearts from all HF fed groups, with the maximum amplification occurring in palm oil fed diabetic rats. This exaggerated oxidative stress could explain the high cardiac apoptosis in the latter group, and supports previous suggestions linking oxidative stress to palmitate induced cardiac apoptosis (Listenberger and Schaffer, 2002; Zhou et al., 2000).

High fat feeding per se decreased cardiac GSH with the lowest levels being observed in sunflower oil fed diabetic hearts. Active glutathione is regenerated by GSH reductase using NADPH as a co-factor. PUFA like linoleic acid and DHA, which are high in this group, have to undergo β-oxidation via an NADPH-dependent 2,4-dienoyl-CoA reductase pathway (Smeland et al., 1992). Following diabetes and activation of the polyol pathway, glucose is shuttled to sorbitol via aldose reductase and NADPH (Nishikawa et al., 2000a). Thus, under conditions of high PUFA oxidation and hyperglycemia, NADPH may become rate limiting for glutathione regeneration, and could explain the drop in GSH (Nishikawa et al., 2000a). Regardless of this observation, there was no increase in lipid peroxidation in this group. Although the mechanism for this observation is unclear, other protective pathways could help in preventing this damage. Included among these are the relatively higher free DHA levels in sunflower diabetic compared to palm diabetic hearts. Moderately elevated DHA has been suggested to act as an antioxidant (Horrocks and Yeo, 1999). Another potential candidate is linoleic acid, which made up almost 40% of the total FA present in the TG fraction in heart and lipoproteins, and 27% of total FFA in sunflower fed diabetic hearts. Linoleic acid in both free and esterified forms have been shown to increase the activity of MnSOD and prevent oxidative stress (Chan et al., 1997; Phylactos et al., 1994). Finally, the higher alpha tocopherol content of sunflower oil could also directly block lipid peroxidation (Ferre et al., 2001).
**Figure 10 Pathways of lipotoxicity and oxidative stress.** FFA can be either esterified to TG or can be oxidized in the mitochondria. If TG synthesis increases or accumulation of FFA increases, it can lead to the formation of ceramide. Ceramide can increase ROS formation or can affect mitochondrial cytochrome c release. Cytochrome c, once released can activate caspase 9 and cause caspase-3 activation leading to DNA fragmentation and apoptosis. ROS can lead to oxidative stress within the cell and lead to lipid peroxidation, membrane damage, caspase activation and apoptosis. The arrow represents the pathways of ROS formation and biotransformation leading to lipid peroxidation.
In conclusion, our results provide direct evidence that feeding a palm oil rich diet in combination with hyperglycemia augments programmed cell death in vivo. Whether this augmented apoptosis, albeit low, has any function in the development of diabetic cardiomyopathy is unresolved (Schaper et al., 1999). It should be noted that cardiac cells undergoing apoptosis may not always end in DNA fragmentation, and can survive even though their contractile proteins may have been degraded by caspases ("zombie myocytes") (Communal et al., 2002; Narula et al., 2001). Additionally, phagocytes rapidly clear apoptotic cells (within 24 h) (Haunstetter and Izumo, 1998). Thus, although we may be underestimating the impact of apoptosis, it could be playing a significant role in the progression of cardiomyopathy in these animals. Following high fat feeding, compensatory mechanisms like a drop in cardiac LPL may limit excessive delivery of FFA to the heart. Under these conditions, the use of LPL activators to decrease hypertriglyceridemia (Tsutsumi et al., 1995) would have limited significance for cardioprotection. The use of a PUFA rich diet as an isocaloric control brought into focus some concerns, and questions its beneficial role in the cardiovascular system, especially following diabetes. In these circumstances, the drop in myocardial GSH, together with the reported increase in linoleic acid and potentially its various metabolites (Moran et al., 2000) could exert detrimental effects in the diabetic heart. Whether this latter phenomenon could explain preliminary observations of impaired cardiac morphology and function in these animals is presently being investigated. Alterations in mitochondrial morphology and function in both animal and human diabetes is well established with changes in mitochondrial membrane potential being proposed as the key player behind cytochrome c release and apoptosis following hyperglycemia (Cai et al., 2002). In our animals, only four days of hyperglycemia
induced a drop in the mitochondrial membrane potential. This was a novel observation, as a change in mitochondrial membrane potential has not been documented previously in such a short time following hyperglycemia. Additionally, incubating diabetic cells for 12-18 hours in normal glucose did not reverse the damage. It has been suggested that acute hyperglycemia can induce irreversible changes within cells that can persist even after the hyperglycemic insult is withdrawn ('hyperglycemic memory') (Nishikawa et al., 2000a; Brownlee, 2001b). Although the nature of the change/damage is not clear, there are several pieces of evidence that support the above observation. For example, in one study, diabetic dogs had normal eyes after 2.5 years of diabetes. Although these dogs were kept eu glycemic for another 2.5 years, they still developed severe retinopathy. In the DCCT trial it has become apparent that 'diabetic memory' is also observed in patients. Thus, following the DCCT trial, diabetic patients monitored over the next 4 years demonstrated higher incidences of nephropathy and retinopathy despite having normal Hb\textsubscript{A\textsubscript{1C}} values (Brownlee, 2001a). Overall, our results suggest that even short-term hyperglycemia is sufficient to induce 'hyperglycemic memory' in the mitochondria.

Changes in mitochondrial membrane potential, as observed during hyperglycemia can lead to generation of ROS via alterations in the ETC. High levels of glucose can also generate glycated proteins that can donate electrons to molecular oxygen to produce \( \text{H}_2\text{O}_2 \) that has been intimately linked to the initiation of cardiac apoptosis (Khechail et al., 1997; Nakamura et al., 1997). In order to verify the incidence of oxidative stress in our model of acute diabetes, we isolated cardiomyocytes from control and diabetic rats and determined the presence of free radicals like \( \text{H}_2\text{O}_2 \) and \( \text{OH}^- \) in these cells using a ROS sensitive dye, CM-H\textsubscript{2}DCFDA. With antimycin A, a blocker of Complex III that increases leakage of electrons from the ETC, we observed a 7-fold increase in ROS.
Conversely, using rotenone, an inhibitor of complex I that blocks ETC at its initiation, ROS production decreased (Kulisz et al., 2002b). Hence we were assured that using CM-H$_2$DCFDA was an effective method for evaluating ROS. When measured 4 days following hyperglycemia, an increase in cardiac ROS was observed. As this rise in free radicals was inhibited by N-acetyl cysteine (NAC), which augments intracellular GSH in vitro, our data suggests that GSH could have beneficial effects in preventing ROS induced cytotoxicity (Higuchi et al., 2002; Catherwood et al., 2002).

In both humans and animals, GSH together with $\gamma$-GCS, the enzyme responsible for synthesizing GSH, are decreased in erythrocytes and the heart following chronic diabetes (Meister and Anderson, 1983; Xu et al., 2002). In diabetes, GSH can also decrease independent of a drop in $\gamma$-GCS expression. In this circumstance, hyperglycemia activates the polyol pathway. The first enzyme in this pathway is aldose reductase (AR) which mediates the reduction of glucose to sorbitol; during this process NADPH is depleted (Nishikawa et al., 2000a). As NADPH is a common cofactor for AR and GSH reductase (responsible for the regeneration of GSH from GSSG), the net effect can also decrease total cellular GSH levels (Lee and Chung, 1999). Irrespective of the mechanism, treatment with NAC can replenish cardiac glutathione stores and prevent the development of cardiac oxidative stress in chronic diabetic rats (Doi et al., 2001a). Additionally, apoptosis and oxidative stress in the isolated hearts perfused with high glucose was attenuated by exogenous administration of GSH in the buffer (Ceriello et al., 2002). Although much is known about GSH in chronically diabetic humans and animals, the regulation of myocardial GSH in acute STZ-diabetic animals is unknown. The acute regulation of cardiac GSH may be important, given its role in regulating oxidative stress and apoptosis, both of
which were augmented within 4 days of diabetes. Interestingly, we were unable to
detect any changes in myocardial GSH (both total and reduced) at this early time
point. It should be noted that the GSH measured represented the total cardiac GSH
and did not discriminate between the cytoplasmic and mitochondrial fractions. In this
regard, mitochondrial GSH has been suggested to be more crucial than the total
cellular GSH pool in controlling apoptosis and oxidative stress (Chen et al., 2001; Hall,
1999c). Indeed, antimycin A is more cytotoxic with GSH depleted in the mitochondria
compared to cytosolic GSH depletion alone (Garcia-Ruiz et al., 1995a). As
mitochondria lack \( \gamma \)-GCS mRNA, it depends totally on the transport of cytosolic GSH
into the matrix, and accounts for 10-15% of the total GSH pool (Griffith and Meister,
1985a). As GSH is negatively charged, it is actively transported and conserved into the
negatively charged mitochondria. Thus it is possible that a loss of mitochondrial GSH,
and not total GSH could be partly responsible for the increase in apoptosis and
oxidative stress in our study (Fernandez-Checa et al., 1998).

In order to investigate the importance of mitochondrial and cytosolic glutathione, we
attempted to deplete the acutely diabetic heart of glutathione and determine if
oxidative stress and apoptosis are amplified. BSO and DEM are frequently used to
deplete intracellular GSH by different mechanisms. BSO by blocking \( \gamma \)-GCS activity
can deplete cytosolic GSH levels but is unable to affect the mitochondrial GSH pool as
mitochondria can actively accumulate the remaining GSH. Thus, BSO is non-toxic
under most in vivo conditions, despite an overall decrease in cellular GSH. In our
study, although treatment with BSO decreased myocardial GSH levels by almost 80%,
apoptosis and oxidative stress remained unchanged. DEM can permeate into the cell
and mitochondria and bind irreversibly to all preformed GSH and is more cytotoxic
than BSO in vitro (Griffith and Meister, 1985c). However, it should be noted that in vivo, treatment with DEM can trigger a compensatory increase in \( \gamma \)-GCS activity that normalizes GSH levels within 8-12 hours after administration (Cai et al., 1997b; Gerard-Monnier et al., 1992b). Treatment of diabetic rats with DEM was also ineffective in augmenting either oxidative stress or apoptosis in vivo. Although unclear, it is possible that the compensatory rise in de novo GSH synthesis was sufficient in conferring protection against oxidative damage but was too low to be estimated by our conventional GSH recycling assay. Overall, using BSO and DEM independently, we were unable to influence either oxidative stress or apoptosis in the diabetic heart. In liver, co administration of BSO and DEM has been shown to achieve chronic glutathione depletion. When this strategy was used in diabetic animals, a significant increase in both oxidative stress and apoptosis ensued suggesting that both a drop in GSH (by DEM) and prevention of a compensatory increase in \( \gamma \)-GCS activity (by BSO) are necessary to exacerbate oxidative damage and cell death. Finally the importance of GSH in preventing regulated cell death is underlined in our study using exogenous supplementation of preformed GSH. Chronic treatment with GSH before and after the induction of diabetes increased both total and reduced GSH levels and reduced both lipid peroxidation and apoptosis in these acutely diabetic hearts.

In summary, although further studies are warranted, it appears that oxidative stress in diabetes may be linked to a loss of mitochondrial GSH, rather than total GSH at this early time point. Furthermore, attempts to replenish GSH by exogenous treatment could be an effective tool to prevent apoptosis and free radical damage in these animals.
Figure 11 Pathways of GSH metabolism. Extracellular cystine gets reduced to cysteine intracellularly to cysteine and is utilized by γ-glutamyl cysteine synthetase to yield γ-glutamyl-cysteine (γ-glu-cys). γ-glu-cys is acted upon by GSH synthetase to lead to GSH. GSH can then be either transported to the mitochondria, or converted to GSSG in the cytosol by glutathione peroxidase (GPX). GSSG can be converted back to GSH by GSH reductase and NADPH. Similar reactions also take place in the mitochondrial compartment (in the rectangle). Cytosolic GSH can also be effluxed out of the cell. The extracellular GSH can be acted upon by γ-glutamyl transpeptidase to γ-glu-cystine, which is reduced intracellularly to γ-glu-cysteine and cysteine. (Adapted from Pullar and Hampton, JBC 277:19402; 2002)
CONCLUSIONS

1. Superimposition of diabetes in palm oil fed animals resulted in the highest level of myocardial apoptosis and lipid peroxidation. Our data suggest that during diabetes, intake of saturated fatty acids like palmitic acid should be limited due to their potential role in accelerating ongoing heart disease.

2. Our data, for the first time, suggests that sunflower oil rich in n-6 PUFA when coupled with diabetes lowers cardiac glutathione. Additionally, enhancement of linoleic acid and its various metabolites could also exert detrimental effects on the diabetic heart.

3. Oxidative stress and mitochondrial dysfunction is apparent within 4 days of diabetes, and persists even after the hyperglycemic insult is withdrawn. This oxidative stress in the acutely diabetic heart may be due to loss of mitochondrial rather than total glutathione.
Reference List


Ref Type: Generic


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