

**CARDIAC CELL DEATH IN EARLY DIABETES:
MODULATION BY GLUTATHIONE AND
POLYUNSATURATED FATTY ACIDS**

by

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ABSTRACT

Diabetes is a growing problem in Canada. Cardiovascular disease (CVD) remains the major cause of morbidity and mortality among diabetic patients. Although the majority of CVD are attributed to coronary vascular disease, an increasing body of evidence supports the existence of an additional heart muscle defect, in the absence of any vascular pathology. Among other factors, this heart disease is associated with excessive cell death in both human and animal models of diabetes. It is possible that this loss of cells progressively leads to interstitial fibrosis, myocardial hypertrophy and eventual loss of contractile function.

Previous studies had established that oxidative stress due to excessive reactive oxygen species (ROS) generation and/or inadequate ROS neutralization, may be a key player in causing cardiac cell death via apoptosis in early diabetes. We used streptozotocin (STZ, 55 mg/kg) to make Wistar rats acutely diabetic and after 4 days examined the different processes that regulate cardiac glutathione (GSH), an important endogenous antioxidant. Reduction in myocyte GSH in diabetic rats was accompanied by increased oxidative stress, excessive reactive oxygen species, and an elevated apoptotic cell death. In this model, loss of GSH was not associated with any change in either biosynthesis or recycling of GSH, but with increased efflux of GSH out of diabetic cardiomyocytes. Gene expression of multidrug resistance protein 1, a transporter implicated in effluxing GSH during oxidative stress, was also elevated in these hearts. In the next study, we reported a loss of GSH specifically from the cardiac mitochondria (mitochondrial GSH, mGSH), the chief source of ROS. Such loss in mGSH could explain increased mitochondrial oxidative damage, and apoptosis via increases in caspase-9 and 3 activities in these hearts. Finally, the central role for mGSH loss in cardiac apoptosis was validated as exogenous GSH supplementation induced a robust amplification in mGSH and prevented apoptosis in diabetic rat hearts.

Apart from antioxidant levels, changes in cellular lipid homeostasis have also been linked to cell death in early diabetes. However, most of the existing studies on the cytotoxicity of fatty acids are on vascular cell systems and with saturated fatty acids like palmitic acid. In the last few decades, saturated fats from the human diet have been replaced by ω -6 PUFA in form of vegetable oils. Thus, the effect of the more relevant ω -6 PUFA on the heart, especially during diabetes remains unknown. Male Wistar rats were first fed a diet rich in ω -6 PUFA [20% (wt/wt) sunflower oil] for 4 wk followed by streptozotocin to induce diabetes. After a brief period of hyperglycemia (4 days), hearts were excised for functional, morphological, and biochemical analysis. Interestingly, in diabetic rats, ω -6 PUFA decreased caspase-3 activity, crucial for myocardial apoptosis. However, cardiac necrosis, an alternative mode of cell death, increased. Interestingly, depletion of cardiac ATP stores could explain this "switch" to necrotic cell death in these animals. This was supported by mitochondrial abnormalities, impaired substrate utilization, and enhanced triglyceride accumulation in ω -6 PUFA fed diabetic hearts. Further analysis revealed that even in the absence of diabetes, a pro-inflammatory response is initiated in the heart following ω -6 PUFA feeding via augmented phospholipase A₂ activity and breakdown of cardiolipin, a mitochondrial phospholipid. ω -6 PUFA fed hearts also demonstrated elevated inducible nitric oxide synthase expression, loss of Mn superoxide dismutase, and increased mitochondrial nitrotyrosine levels. In these hearts, oxidative damage to mitochondrial DNA (mDNA) was demonstrated by 8-hydroxyguanosine immunopositivity, overexpression of DNA repair enzymes, and a decrease in the mRNA expression of specific respiratory subunits encoded by the mDNA. Thus, although promoted as being beneficial, excess dietary ω -6 PUFA, with its predisposition to cause mitochondrial nitrosative damage and necrosis following hyperglycemia, could accelerate myocardial abnormalities in diabetic patients.

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LIST OF ABBREVIATIONS

$\Delta\psi_m$	Mitochondrial membrane potential
γ -GCS	Gamma glutamylcysteine synthase
μ l	Microliter
8-OHG	8-hydroxyguanosine
AGE	Advanced glycation end products
AIN	American institutes of Nutrition
ANOVA	Analysis of variance
BSO	Buthionine sulfoximine
CAD	Coronary artery disease
cGSH	Cytosolic GSH
CM-H ₂ DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester
COX-1	cytochrome-c-oxidase subunit 1
Cu/Zn SOD	Copper-zinc superoxide dismutase
CYTb	cytochrome b
DEM	Diethyl maleate
DHA	docosahexaenoic acid
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
EPA	Eicosapentaenoic acid
ETC	Electron transport chain
F.U.	Fluorescence units
FA	Fatty acids
FADH ₂	Reduced flavin adenine dinucleotide
GAD	Glutamic acid decarboxylase
GLC	Gas liquid chromatography
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulphide, oxidized glutathione

HNE	Hydroxynonenal
HPLC	High pressure liquid chromatography
i.v.	Intravenous
IAA	Insulin auto antibody
ICA	Islet cell auto antibodies
IGF-1	Insulin like growth factor 1
iNOS	Inducible nitric oxide synthase
PO	Palm oil
ip	Intraperitoneal
Kb	Kilobase
KH solution	Krebs-Henseleit solution
LADA	Latent autoimmune diabetes in adults
LDH	Lactate dehydrogenase
LPC	Lysophosphatidylcholine
LPL	Lipoprotein lipase
mCIB	Monochlorobimane
MDA	Malondialdehyde
mDNA	Mitochondrial DNA
mGSH	Mitochondrial GSH
mM	Millimolar
Mn-SOD	Manganese-superoxide dismutase
mRNA	Messenger ribonucleic acid
MRP	Multi drug resistance protein
MT	Metallothionein
MUFA	Monounsaturated fatty acids
NAC	N-acetyl cysteine
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
ND-1	NADH dehydrogenase subunit 1
NF- κ B	Nuclear factor kappa B
NIDDM	Non-insulin-dependent diabetes mellitus

nM	Nanomolar
NO	Nitric oxide
OGG-1	8-oxoG-DNA glycosylase
OxPhos,	
OXPHOS	Oxidative phosphorylation
PC	Protein carbonyls
PGC-1	Peroxisome proliferator-activated receptor gamma coactivator 1
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLA ₂	Phospholipase A ₂
pNA	Para-nitroaniline
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Real time-polymerase chain reaction
SDH	Succinate dehydrogenase
SEM	Standard error of means
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TBARS	Thiobarbituric acid reactive substances
TBS-T	Tris buffered saline-Tween
TCA	Trichloroacetic acid
TCA cycle	Tricarboxylic acid cycle, Kreb's cycle
TdT	Terminal deoxynucleotidyl transferase
TG	Triglyceride
TNF- α	Tumor necrosis factor alpha
TUNEL	TdT mediated dUTP nick end labeling
ZDF	Zucker diabetic fatty
ω -3 PUFA	Omega-3 polyunsaturated fatty acids
ω -6 PUFA	Omega-6 polyunsaturated fatty acids

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DEDICATION

This thesis is dedicated to my family. I miss you and thanks for being there for me.

1. Introduction

1.1. Historical View On 'Diabetes'

A disease of "frequent urination" was initially reported in ancient Egypt around 3,500 years ago and was named 'diabetes' by the Greek physician Aretaeus in 100 A.D. Von Mering and Minowski first proposed the role of the pancreas in development of diabetes in 1889. Owing to the seminal discovery of Banting, Best, Collip and McLeod (1921; University of Toronto), it was revealed that diabetes was a consequence of a lack of insulin (Type 1 diabetes), a discovery for which they received the Nobel Prize in 1923. With subsequent advancements in glucose management and an increased longevity, complications of the eyes, heart, blood vessels, kidneys, limbs and nerves also increased. The Diabetes Control and Complications Trial (DCCT) [1] and the UK Prospective Diabetes Study (UKPDS) [2] have demonstrated that lack of adequate control over blood glucose is the main culprit behind these complications, and aggressive therapy with either insulin or oral glucose lowering agents could bring down their incidence in diabetic patients.

1.2. An Overview Of Diabetes Mellitus

Diabetes is fast taking the form of a pandemic. An estimated 30 million people had diabetes in 1985. By the year 2000, the figure jumped to 150 million, worldwide. In the next 25 years, this figure has been projected to be as high as 300 million. As a developed nation, diabetes is a major health concern in Canada. Currently, more than two million Canadians have diabetes. More alarming is the fact that in the next few years, another 1 million new cases of diabetes would be diagnosed, taking the total up to 3 million, by 2010 [3]. Even in 1998, the total economic burden of diabetes and its chronic complications in Canada was between US\$4.76

and \$5.23 billion [4]. In the same year, the direct costs for managing just the disease without any associated complications was US \$573 million [4].

Diabetes mellitus has been defined by an expert committee [5] as “a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both.” Based on clinical etiology, diabetes is classified into two major types: a) Type 1 diabetes (T1D) (previously termed as insulin-dependent diabetes mellitus or IDDM) characterized by a total loss of circulating insulin and therefore an obligatory requirement for exogenous insulin and b) Type 2 diabetes (T2D), caused by a combination of impaired insulin action and an inadequate compensatory insulin secretion. At a very early age, in T1D patients (5-15% of all diabetes cases), the pancreatic β cells are destroyed, leading to a total loss of insulin. Other than unknown factors that cause such a destruction (idiopathic T1D), several autoantibodies may be responsible in eliciting an autoimmune response within pancreatic β cells [6]. In this regard, islet cell auto antibodies (ICAs), insulin auto antibody (IAAs), glutamic acid decarboxylase auto antibodies (GAD 65), and tyrosine phosphatase auto antibodies (IA-2 and IA-2 β) have been identified in the serum of T1D patients [6].

T2D patients comprise over 85-95% of all diabetic cases and are usually diagnosed by fasting hyperglycemia and by oral glucose tolerance tests. T2D (previously known as non-insulin-dependent diabetes mellitus or NIDDM) is often asymptomatic and characterized by insulin resistance and relative insulin insufficiency. The development of hyperglycemia is gradual over years after the demonstration of insulin resistance. A positive family history of T2D increases the probability of acquiring this disease by 2.4 fold [7]. Further confirmation of the role of heredity in the development of T2D comes from studying monozygotic twins (with 100% concordant genes) and dizygotic twins (with 50% concordant genes) where the incidence of both having T2D are 35-58% and only 17-20% respectively [8, 9]. Inactive lifestyle,

abdominal fat, and overeating has been also been positively correlated with both obesity and the development of T2D. With advancing disease, as oral hypoglycemic therapy fails, T2D patients may require exogenous insulin, a factor that made the older terms of IDDM and NIDDM, invalid.

Unfortunately the epidemiology of diabetes is also changing worldwide. In recent years, there has been a huge upsurge in the cases of obesity, insulin resistance and T2D in both adults and the pediatric population [10, 11]. More advanced detection techniques are identifying low titers of autoantibodies in the adult population with diabetes (latent autoimmune diabetes in adults, or LADA). These cases were previously believed to have T2D, but are now being reclassified as the new 'Type 1.5' diabetes (5-10% of all diabetic cases) [12]. These patients are characterized with a low body mass index like T1D patients but with high circulating triglycerides and insulin resistance comparable to classical T2D patients.

1.3. Diabetes is a Cardiovascular Disease

In patients with diabetes, cardiovascular disease is the leading cause of death [13]. In fact, the key role that diabetes plays in the development and progression of heart disease has led the American Heart Association to classify diabetic patients as having 'cardiovascular disease' [14]. Diabetes in an otherwise healthy individual increases the risk of death due to cardiac causes to levels comparable to nondiabetic patients with previous myocardial infarction [15]. The increase in cardiovascular events in diabetes is mainly attributed to coronary artery disease (CAD) [16, 17], with fatal and non-fatal CAD events being two- to four-fold higher in these patients [17]. Diabetes also alters the outcome of CAD events, and up to a seven-fold increase in mortality among patients with CAD is observed with co-existing diabetes [18, 19]. Although the majority of these patients are prone to aggressive macrovascular atherosclerotic disease [20], an increased susceptibility to heart failure is also evident [21]. The Framingham Heart

Study concluded that heart failure in men (2-fold) and women (5-fold) with diabetes was greater than non-diabetic individuals [22]. This conclusion was verified in subsequent trials [23-25].

CAD does not entirely account for the increased morbidity and mortality from heart failure in diabetes [26]. In 1972, autopsies on four diabetic patients with congestive heart failure and nephropathy demonstrated an absence of coronary atherosclerosis [27]. In other studies with diabetic patients, scarring and interstitial collagen deposition was observed without arterial obstruction in hearts, suggesting the presence of a heart muscle specific disease or 'cardiomyopathy' [28]. In the following years, the notion of a diabetic cardiomyopathy has been widely debated [29] as a simultaneous presence of hypertension and CAD in many diabetic patients makes it difficult to isolate this diabetes specific 'heart-muscle' disease. Nevertheless, an increasing body of evidence supports the existence of metabolic, functional, and structural changes in the diabetic heart, in the absence of any vascular pathology and has been termed as 'diabetic cardiomyopathy' [16, 30, 31].

Cardiomyopathy is defined as diseases of the myocardium associated with cardiac dysfunction [32]. Among the various types of cardiomyopathies, diabetic cardiomyopathy is classified as a 'specific cardiomyopathy' characterized by impaired diastolic function [33]. In spite of intense research, its etiology remains poorly understood [28]. Defects in the sarcoplasmic reticulum, mitochondria, and/or metabolic pathways seem to be responsible for the development of diabetic 'cardiomyopathy' [16].

Early diastolic changes and a late systolic alteration characterize diabetic cardiomyopathy. These changes can directly arise from alterations in the regulation of intracellular cations; which in turn regulate the contractile properties of the heart [28]. In spite of considerable research, 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 [34]. In addition, the $\text{Na}^+/\text{Ca}^{++}$ exchanger mechanism is impaired together with a decreased myofibrillar Ca^{++} ATPase activity [35]. Decreased Ca^{++} binding to the sarcolemma and decreased Ca^{++} intake in the sarcoplasmic reticulum are characteristics of rodent models of diabetes [28, 35]. 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 [36]. Diabetic hearts also demonstrate reduced sensitivity to norepinephrine and β -adrenoceptor mediated stimulation [37]. This may result from a decreased number of receptors or a defect in signaling [38]. Lately, oxidative stress has also been implicated in the etiology of diabetic cardiomyopathy [28, 39] along with other diabetic complications [40]. 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 [41]. Lately, oxidative stress induced cardiac cell death in the form of both apoptosis and necrosis has also been correlated with diabetic cardiomyopathy, both in human patients and rodent models of diabetes [42-44].

1.4. Basic Mechanisms Of Oxidative Stress

In the first step of glucose oxidation, intracellular glucose is converted to pyruvate via glycolysis. Glycolysis also generates reduced nicotinamide adenine dinucleotide (NADH). NADH can either reduce pyruvate to lactate, or can donate reducing equivalents to the mitochondrial electron transport chain (ETC). Pyruvate derived from glycolysis can also be utilized by the tricarboxylic acid (TCA) cycle, to yield carbon dioxide, water, 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 respiratory 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 complex I, that transfers the electron to ubiquinone. Ubiquinone can also accept electrons from complex II. Electrons from reduced ubiquinone are then diverted, first to complex III and subsequently to 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 complexes, activates ATP synthase and generates ATP. However the system is not perfect and even under normal conditions, 1% of all oxygen consumed by the mitochondria is converted to free radicals [45]. With hyperglycemia, increased levels of electron donors from the TCA cycle in the form of NADH and FADH₂ causes an increased efflux of protons outside the mitochondria and leads to increased mitochondrial membrane potential [40]. Augmented proton electrochemical gradient generated by the ETC, leads to transfer of electrons to molecular oxygen to generate superoxide (O₂^{•-}) radicals [40], the precursor for all major reactive oxygen species (ROS).

It has been recently shown that overexpression of manganese-superoxide dismutase, (Mn-SOD) which dismutates superoxide 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 [40, 46]. In the heart, overexpression of Mn-SOD prevents the development of diabetic cardiomyopathy [47]. As superoxide radicals can also be generated de novo via the action of cytosolic enzymes like NADPH oxidase and xanthine oxidase or may leak from the mitochondria itself, the cytoplasmic SOD (Cu⁺⁺/Zn⁺⁺-SOD) also demonstrates beneficial effects in preventing diabetes induced glomerular injury [48].

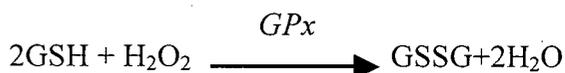
Superoxide *per se* is not a good oxidizing agent due to its short half-life. However, excess superoxide radicals may generate hydrogen peroxide, which in turn may generate hydroxyl radicals (OH[•]) via Fenton chemistry with the help of Fe⁺⁺ [49].



Hydroxyl radical is a very potent oxidant and causes changes in membrane fluidity and permeability, mitochondrial respiration and various other biochemical functions, leading to cellular dysfunction and death [49]. Thus, to prevent the deleterious effects of hydroxyl radicals, two endogenous antioxidant systems are present to neutralize its precursor, hydrogen peroxide [50]. Firstly, catalase present in the peroxisomes, can neutralize hydrogen peroxide to water and oxygen.



Secondly, glutathione peroxidase (GPx) can also reduce hydrogen peroxide to water with the help of reduced glutathione (GSH) which gets oxidized to glutathione disulfide (GSSG) in the process [51].



1.5. Synthesis, Metabolism And Action Of Glutathione (GSH)

Due to small amounts of catalase in the heart, GSH, a tripeptide thiol (L- γ -glutamyl-L-cysteinyl glycine) remains the most effective antioxidant against hydrogen peroxide [52, 53]. Its primary role is in the protection of cells and tissues against oxidative and nitrosative stress and against reactive electrophiles [54]. It has a low molecular weight and is synthesized *de novo* from three amino acids- cysteine, glycine and glutamate, by the action of two ATP-consuming enzymes- γ -glutamylcysteine synthase (γ -GCS) (Reaction a) and GSH synthase (Reaction b) [51]. The γ -GCS step is rate limiting for the synthesis of GSH.

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

Reaction b: L- γ -glutamyl-L-cysteine + glycine + ATP \Rightarrow GSH + ADP + Pi

As mentioned previously, GSH is oxidized to GSH disulfide (GSSG) by GPx, which catalyzes the reduction of H₂O₂. Normally, GSSG can be reduced back to GSH in an enzymatic recycling reaction by GSH reductase and NADPH. Adequate NADPH levels and GSH reductase activity maintains a GSH/GSSG ratio of > 100:1 (Figure 1.1). 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 redox-sensitive transcription factors like NF- κ B [55, 56].

Both GSH and GSSG can also be effluxed out of the cell by transporters and multidrug resistance proteins (MRPs) [57, 58]. GSH extrusion from the cell is irreversibly related to the apoptotic process [59]. In this regard, inhibition of GSH efflux has also been shown to rescue cells from apoptosis and cause them to undergo regeneration [60]. In the heart, during reperfusion after ischemia, the heart effluxes high levels of GSSG, which persists long after the generation of ROS has ceased [61]. Such a loss in the GSH pool may be prevented by preadministration of N-acetyl cysteine (NAC), a source of cysteine, which increases the cardiac GSH, and is beneficial in preventing changes in the ischemic-reperfused hearts [62, 63].

In recent years, the importance of mitochondrial GSH in preventing oxidative stress has come to light [64-66]. Being the major site of oxygen consumption, mitochondria are also the primary sites for the generation of ROS. Thus, although most of the cellular GSH is in the cytoplasm, a distinctly regulated pool is present in mitochondria. As mitochondria lack γ -GCS mRNA, it depends exclusively on the transport of cytosolic GSH into the matrix, and accounts for 10-15% of the total GSH pool [64]. Being negatively charged, GSH is actively transported

and conserved into the negatively charged mitochondria, and resists depletion even under oxidative stress [67] (Figure 1.1). 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 [68]. Further, treatment with buthionine sulfoximine (BSO), an inhibitor of γ -GCS, decreases 90% of the intracellular GSH but is non-toxic in most cells. This may be due to the inability of BSO to deplete mitochondrial GSH, as under cytosolic GSH depletion, the mitochondria actively accumulates the remaining GSH within the matrix as a protective mechanism [69]. However, diethyl maleate (DEM), which can permeate into the mitochondria and bind irreversibly to all preformed GSH is more cytotoxic [69]. Interestingly, treatment with DEM can in turn trigger γ -GCS activity [70]. Thus, GSH levels return to normal levels within 8-12 hours after DEM administration in vivo [71]. However, co administration of BSO and GSH in vivo has been shown to achieve chronic GSH depletion in the liver [72].

Other than its redox functions, GSH can form thioester or thiolester conjugates of different compounds including xenobiotics with the help of glutathione-S-transferases to promote their detoxification and elimination in Phase II metabolism [73]. This pathway promotes biliary excretion, and interorgan transport for final renal excretion via the mercapturate pathway. GSH has also a prominent role in signal transduction and gene expression. Protein glutathionylation of enzymes such as phosphorylase, creatine kinase, RAS, carbonic anhydrase control their intracellular activity [73]. The formation of disulfide bonds with the help of GSH has also been suggested to dictate protein-folding, and enzyme action. S-nitrosoglutathione (GS-NO) has been suggested as a storage and transfer form of nitric oxide [74]. Presence of GSH also helps to maintain the cysteine groups in the promoter regions of genes like NF- κ B and AP-1, and allow their expression and biological activity [75].

1.6 Figures

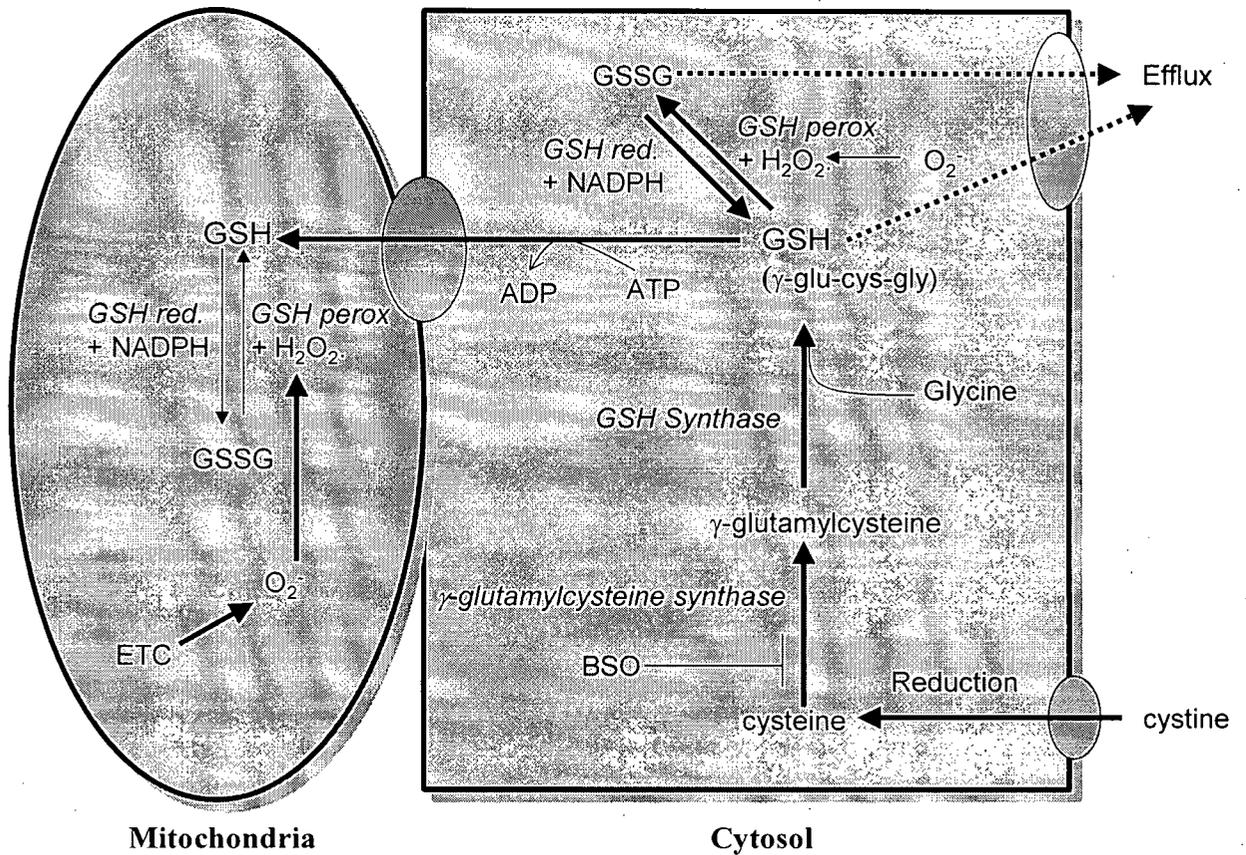


Fig.1.1 Pathways of intracellular glutathione (GSH) metabolism Extracellular cystine gets reduced intracellularly to cysteine and is utilized by γ -glutamylcysteine synthase to produce γ -glutamylcysteine. This step is the rate-limiting pathway for de novo GSH biosynthesis and can be blocked by buthionine sulfoximine (BSO). In the next step, γ -glutamylcysteine is utilized by glutathione synthase to produce GSH (γ -glutamyl-L-cysteinyl glycine). GSH can then either be transported to the mitochondria or remain in the cytosol, where they are utilized by GSH peroxidase to neutralize hydrogen peroxide (H_2O_2), and gets converted to oxidized glutathione (GSSG). GSH reductase present within both mitochondria and cytosol can recycle GSSG to 'active' GSH with the help of NADPH. Both GSSG and GSH can also be effluxed out of the cell with the help of membrane-bound transporters. red., reductase; perox., peroxidase; ETC, electron transport chain

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2. Cardiac Cell Death In Early Diabetes

2.1 Hyperglycemia And Cellular Lipids: Common Pathways To Oxidative Stress

ROS generation in the diabetic heart is a combined effect of both hyperglycemia and hyperlipidemia. Glucose oxidation generates NADH and FADH₂ in the electron transport chain (ETC) of the mitochondria, to produce energy [1]. Increased proton electrochemical gradient generated by the ETC leads to transfer of electrons to molecular oxygen to generate superoxide radicals and hydrogen peroxide [2]. Hence, with onset of hyperglycemia, glucose auto-oxidation is increased [3]. Glucose auto oxidation involves the process of formation of glyoxal via the process of degradation by retroaldol condensation reactions [4]. Concurrent oxidative modifications by hydrogen peroxide also can convert glucose to glucosone and glycoaldehyde to glyoxal [4, 5]. Glyoxal may be later converted to 3-deoxyglucosone and methyl glyoxal and contribute to the formation of early glycation products and oxidative stress. Free radical damage of the mitochondrial membrane can precipitate a further rise in ROS generation in the heart (ROS induced ROS release) [6]. Augmented superoxides can also increase diacylglycerol synthesis or phosphatidylcholine hydrolysis, thereby activating PKC [7]. Hyperglycemia also provokes excessive production of AGE [2]. In this process, glucose reacts non-enzymatically with amino acids in proteins to form early glycation products (Schiff bases) and intermediate glycation products (Amadori bases). These compounds are then degraded to AGEs like glyoxal, methylglyoxal, and carboxymethyllysine [8]. Binding of AGEs to their receptors leads to alterations in cell signaling, and further production of free radicals [9]. Finally, hyperglycemia activates the polyol pathway, which converts excess glucose to sorbitol via aldose reductase. Increased activation of aldose reductase leads to rapid depletion of NADPH, its cofactor [10]. As NADPH is also required for the activity of

glutathione reductase for the regeneration of active glutathione (GSH) from its 'oxidized form (GSSG), intracellular levels of this critical antioxidant decreases rapidly in diabetes [2]. Depletion of GSH allows oxidants to go unchecked, causing irreversible cellular damage.

In addition to hyperglycemia, diabetes is also characterized by dyslipidemia and 'lipid overload' in non-adipose tissues like the heart [11]. Independent of hyperglycemia, excess lipids can also precipitate oxidative stress, as observed in the Zucker diabetic fatty rat (ZDF), a model of Type 2 diabetes [12, 13]. Fatty acids (FA) can directly impair mitochondrial function via uncoupling of oxidative phosphorylation [14]. During uncoupling, electron transfer through ETC is blocked, resulting in buildup of a voltage gradient across the mitochondrial membrane. As a result, excess electrons are transferred to molecular oxygen to form ROS like superoxide [14, 15]. Due to its short half-life, superoxide *per se* is not a good oxidizing agent. However, excess superoxide radicals may generate hydrogen peroxide, that in turn may generate cytotoxic hydroxyl radicals (OH^\cdot) with the help of iron (Fe^{++}) [16]. Recently, FA were also reported to transport iron to the mitochondria, thus further strengthening oxidative stress [17]. Although FA can also deplete intracellular GSH levels [18, 19], and activate PKC [20], the *de novo* production of ceramide, a proapoptotic sphingolipid appears to be the most damaging of all non-oxidative consequences of excess FA in the heart. Ceramides interact with cardiac mitochondria to induce the release of free radicals, that extensively damage cellular membranes and proteins [21, 22]. Overall, hyperglycemia and lipid overload are currently believed to be responsible for oxidative stress (Figure 2.1) and the development of heart muscle disease in diabetes.

2.2 The Relevance Of Early Diabetes

Till recent years, a number of clinical studies have demonstrated the incidence of heart disease only after chronic diabetes. In patients with Type 1 diabetes, heart disease is observed at least 7 years following the diagnosis of diabetes [23]. In Type 2 patients, the time course for the

development of heart disease has been difficult to predict. This is largely a consequence of under diagnosis of about 50% of the diabetic population [24]. Additionally, as obesity, hypertension and insulin resistance are beset with their own cardiovascular problems, the causal effect of hyperglycemia on heart disease in Type 2 diabetes is unresolved. In experimental studies, most of the existing literature on diabetic cardiomyopathy suggest the development of cardiac pathology only after chronic diabetes [25-27]. In recent years, with the help of advanced imaging techniques, an increasing prevalence of heart disease is being recognized in otherwise healthy diabetic patients. While older studies reported around 30% of Type 2 diabetic patients as having diastolic dysfunction based on conventional electrocardiography, more rigorous techniques like Doppler imaging revealed upto 60% of these subjects as having diastolic failure [28]. This early detection of heart disease points to fundamental biochemical changes during acute diabetes as the likely initiator of cardiovascular disease.

In animal models, there is a growing recognition that cardiac dysfunction can occur early following induction of diabetes [29], and slower myocyte shortening and relengthening were observed within 4-6 days of diabetes [30]. In fact, high glucose perfusion for 2 hours increased peroxynitrite and superoxide levels, leading to cell death in working rat hearts, effects that were prevented by the addition of GSH to the perfusion buffer [31]. In patients, Dr. Ceriello and his group have hypothesized that acute hyperglycemia, as observed following a meal or oral glucose loading, in an otherwise 'well-controlled' diabetic individual, leads to 'postprandial hyperglycemic spikes'. These acute surges in plasma glucose precipitated a variety of cardiovascular stress signals including impaired ventricular function, arrhythmias, and accumulation of pro-thrombotic factors [32, 33]. The role of oxidative stress in these disorders was supported by direct measurement of oxidative markers or the ameliorative effect of antioxidants [33, 34].

Early myocardial functional changes in diabetes are also related to acute changes in cardiac lipids. In our studies, following streptozotocin (STZ, a pancreatic β -cell toxin) injection, hypoinsulinemia and hyperglycemia ensues within 24 hours [35]. At this time point, cardiac glucose oxidation decreases, and cardiac energy provision is almost exclusively via β -oxidation of fatty acids [36]. To guarantee FA supply to the diabetic heart, lipoprotein lipase (LPL) activity at the coronary lumen is rapidly elevated within hours [37], and by hydrolyzing circulating lipoproteins, provides an augmented supply of FA to the heart [36, 38]. However, such adaptive mechanisms following acute hyperglycemia could eventually become counterproductive, and lead to 'lipotoxicity'. Increased FA oxidation can promote acidosis and generation of free radicals, making the diabetic heart more prone to injury [39]. As described, non-oxidative metabolism of FA can rapidly induce ceramide and nitric oxide formation, damage the mitochondrial membrane, and induce oxidative stress [40, 41]. Such diverse cytotoxic effects of FA on the mitochondria could also lead to respiratory chain malfunction and a lower cardiac efficiency within 2 weeks of diabetes [42]. In vitro studies have also demonstrated that addition of excess FA to cardiomyocytes can precipitate cell death via apoptosis within 16-24 hours [40, 41].

2.3 Basic Mechanisms Of Cell Death

Characteristics of the diabetic heart include myocardial hypertrophy and interstitial fibrosis [43]. It has been proposed that this hypertrophy may be an insufficient compensatory response to increased cell death in the diabetic heart. Indeed, hearts from patients with Type 2 diabetes are characterized by a 85-fold increase in apoptosis, and a 4-fold increase in necrosis [43]. Apoptosis is an energy dependent process of cell death characterized by nuclear condensation and formation of small apoptotic bodies that are engulfed by phagocytic cells (macrophages) without any rupture of the cell membrane [44, 45]. The molecular mechanisms

behind apoptotic cell death in the heart have only recently become apparent, and caspases have been proposed to be the 'central executioners' of this cellular demise (Figure 2.2). Caspases are a family of cysteine containing proteases that get activated following an apoptotic insult. Subsequently, caspases disintegrate intracellular proteins, cause nuclear DNA fragmentation and cell death [44]. Two major pathways exist to activate caspases from inactive procaspases in the cytosol. The first involves activation of downstream effector caspases like caspase-3 via 'death' receptor mediated activation of upstream caspases like caspase-8 ('extrinsic' pathway) [45]. However this pathway is likely not important in causing cell death in the acutely diabetic heart [46]. The second pathway known as the 'intrinsic' or the mitochondrial pathway of apoptosis [45], initially involves a loss of the mitochondrial membrane potential. This signals the translocation of pro-apoptotic Bcl-2 family members like Bax to the mitochondria, and the release of cytochrome c from the mitochondrial intramembrane space into the cytosol. As a protective mechanism, antiapoptotic Bcl-2 family members like Bcl-2 and Bcl-X_L interact with the proapoptotic members to prevent this release of cytochrome c. Cytochrome c once released, forms an 'apoptosome', and activates caspase-9. Caspase-9 initiates the caspase cascade, terminating in the activation of caspase-3, nuclear DNA damage and apoptotic demise [45]. Lately, it has been proposed that cardiac apoptosis may not proceed towards DNA degradation and cell death ('apoptosis interruptus') but may lead to the disintegration of myofibrils such that the contractile properties are lost ('zombie' myocytes) [47]. As caspase activation and subsequent proteolysis in apoptosis requires energy, adequate ATP levels are required. Thus, when ATP stores are depleted, the cells may be directed towards necrosis, by the same initiating steps [48, 49]. Necrosis or '*accidental*' cell death is an energy independent process characterized by cell membrane rupture [48]. Interestingly, at the earliest stage of diabetes, necrotic cell death is absent in rodent hearts [50, 51], and only appears after 15 days of hyperglycemia [52]. At this time, necrosis is temporally related to mitochondrial

dysfunction and ATP loss [53]. In chronic diabetes, cardiac necrosis is a common feature and is observed both in humans and animal models of the disease [26, 43]. Both apoptosis and necrosis in the diabetic heart is believed to be a result of oxidative stress. In this regard, ROS such as hydrogen peroxide (H_2O_2) and reactive nitrogen species (RNS) like peroxynitrite ($ONOO^-$) can induce membrane lipid peroxidation, alteration of cellular proteins, and initiation of diverse stress-signaling pathways, including cell death [54].

2.4 Oxidant Induced Cell Death In Experimental Diabetes

Development of Type 2 diabetes is a gradual process, with no clear demarcation between the 'pre-diabetic' and the hyperglycemic phase. Thus, most of the existing work on early diabetes and cell death is performed in insulin deficient STZ-diabetic rats. In these models, we and others have demonstrated that cardiomyocyte death proceeds via an apoptotic pathway, with an increase in caspase-3 activity and oxidative stress [35, 50, 51]. In vitro, exposing myoblast H9c2 cells to high glucose induced cytochrome c release into the cytosol followed by caspase-3 activation and apoptosis [55]. Increased prevalence of ROS can oxidatively damage DNA and increase p53. This transcription is factor responsible for increased production of angiotensinogen and ultimately angiotensin II [50, 56]. Binding of angiotensin II to its receptors promotes generation of free radicals like superoxide and peroxynitrite in the heart [50, 51]. Interestingly, both the angiotensin II blocker like losartan and IGF-1 diminishes oxidative stress and prevents cardiac apoptosis in diabetes [57]. Recently, taurine, an inhibitor of the angiotensin receptor also demonstrated a similar effect [58]. With hyperglycemia, upregulation of PKC δ can also precipitate cardiac apoptosis. PKC δ may exert its cytotoxicity either through enhanced generation of ROS or reduction of nuclear factor- κ B, a survival signal against apoptosis [59]. Endothelin, a potent vasoconstrictor, can also activate PKC and cause cardiac apoptosis during diabetes [60]. The cellular content of cardiac GSH, a crucial

endogenous antioxidant is also reduced following hyperglycemia in vitro [61] and exogenous supplementation with GSH can reverse cardiac apoptosis following diabetes [31]. In addition to GSH, metallothionein (MT), a potent antioxidant [62] has also been shown to decrease cardiac apoptosis. Additionally, administration of MT prevents hyperglycemia induced myocardial cell death and preserves cardiac function [63] whereas increasing biosynthesis of MT through exogenous administration of zinc also prevents the development of diabetic cardiomyopathy in rats [64].

Being a terminally differentiated organ with low regeneration capacity, even a low rate of cardiac cell death (ranging from 0.08-0.25% of total myocytes) over the long term could deplete a significant number cardiomyocytes, the units of cardiac contractility [65]. Thus, for the prevention of long-term cardiac problems in diabetes, early recognition and intervention of the deleterious pathways of cell death are required.

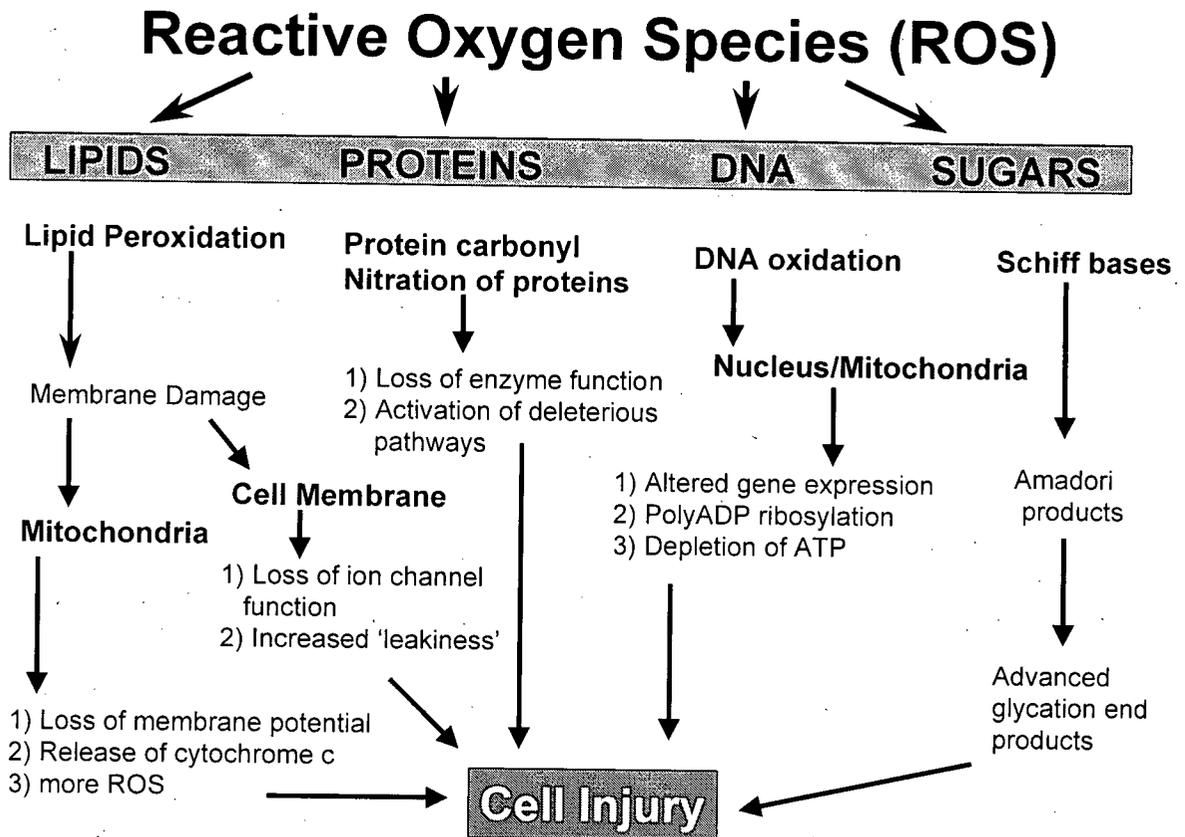


Fig. 2.1 *Mechanisms of ROS induced injury in diabetes* Both hyperglycemia and hyperlipidemia can increase ROS production in the diabetic heart. Once ROS is produced, it can have multiple effects on different biomolecules like lipids, proteins, DNA and sugars. The major pathways of injury are depicted in this schematic.

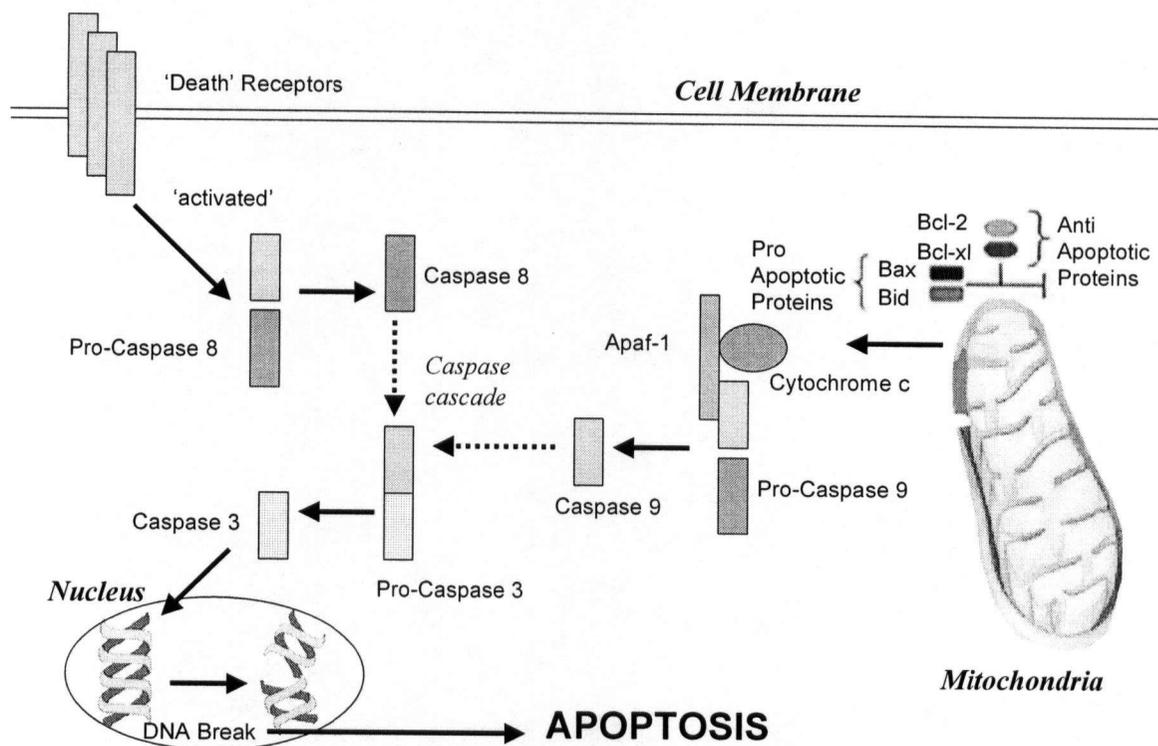


Fig. 2.2 Overview of the basic mechanisms of apoptosis Caspases, the most important enzymes in the apoptotic pathway, can be initiated by either of two mechanisms. The first involves activation of downstream effector caspases like caspase-3 via 'death' receptor mediated activation of upstream caspases like caspase-8 ('extrinsic' pathway). However this pathway probably plays a minor role in causing cell death in the acutely diabetic heart. The second pathway known as the 'intrinsic' or the mitochondrial pathway of apoptosis initially involves a loss of the mitochondrial membrane potential, followed by the release of mitochondrial cytochrome c. The release of cytochrome c depends on the relative levels of pro-apoptotic and anti-apoptotic Bcl-2 family members on the mitochondrial membrane. Cytochrome c once released, forms an 'apoptosome', with Apaf-1 and pro-caspase 9, and activates caspase-9. Both caspases 8 and 9 can lead to the caspase cascade, terminating in the activation of caspase-3, nuclear DNA damage and apoptosis.

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3. Effect Of Fatty Acids On Cardiac Cell Death In Diabetes

Although lipids have been acknowledged as a significant contributor to diabetic cardiac complications, most of the existing studies on the cytotoxicity of fatty acids are on cell systems like endothelial and smooth muscle cells [1-5]. It has been proposed that as these vascular cells are directly exposed to plasma fatty acids, deleterious effects on the heart are primarily mediated through this vascular component. However, in diabetes, with an increased influx of fatty acids for cardiac energy needs, the same fatty acids that injure endothelial and smooth muscle cells, also enter the cardiomyocytes and cause damage. One weakness with the traditional approach of examining fatty acid cytotoxicity has been the tendency to group 'fatty acids' as a single entity, with little attempt made to isolate the effects of individual fatty acid classes. As the cellular fate of each class of fatty acids is different with regard to their metabolism, special attention is required when assessing the effects of fatty acids on cell death.

3.1 Saturated fatty acids

Both humans and chronic models of diabetes demonstrate cardiac lipid overload, suggested to cause 'lipotoxicity' [6]. The causal effect of lipotoxicity in diabetic heart disease was strengthened in studies using Zucker diabetic fatty rats (ZDF) rats, a model of Type 2 diabetes. In these studies, amelioration of cardiac lipids by troglitazone decreased formation of ceramide, a proapoptotic sphingolipid, and reversed cardiac dysfunction [7, 8]. The first step in de novo ceramide biosynthesis is catalyzed by serine palmitoyl transferase, and involves the condensation of palmitoyl CoA and serine [9]. As the saturated fatty acid, palmitic acid (C16:0) is the precursor of palmitoyl CoA, most studies demonstrating detrimental effects of FA on myocytes have hence focused on using saturated fatty acids.

Like glucose and FA, ceramides also release cytotoxic free radicals and cytochrome c from the mitochondria, thus provoking apoptotic cell death [10]. Increased ceramide levels also upregulates the expression of inducible nitric oxide synthase (iNOS) by activating the transcription factor NF κ B [9]. Increase in iNOS expression leads to excess production of nitric oxide (NO), which can combine with superoxide to produce the highly damaging RNS, peroxynitrite (ONOO⁻) [11]. As hyperglycemia can independently augment cardiac NO production [12], a rise in ceramide levels can cause additive damage to cardiomyocytes, and a further increase in cardiac apoptosis [6].

One drawback with the conventional approach of examining lipotoxic effects on the heart is that most studies have used in vitro incubations with single fatty acids. Although this setting partly resembles fatty acid derived from plasma, it does not account for fatty acid obtained from other physiologically relevant sources like hydrolysis of lipoproteins by cardiac lipoprotein lipase (LPL). This is particularly important as: i) LPL increases in the diabetic heart [13, 14], ii) the molar concentrations of fatty acids in lipoproteins is approximately 10-fold higher than that of fatty acids bound to albumin, iii) LPL has a selective affinity towards palmitic acid containing lipoproteins (47.5% of total fatty acids released) [15] and iv) LPL derived palmitic acid causes a 8-fold greater cell death, than when it is used individually [1]. Thus, a true measure of the effects of palmitic acid on apoptosis should include an examination of its in vivo effects, and few studies have focused on the role of dietary fat on in vivo cardiac apoptosis.

In a recent study, we demonstrated that feeding 20% w/w palm oil (40% energy, rich in palmitic acid, PO) increased cardiac apoptosis and oxidative stress under normoglycemic conditions [16]. Superimposition of diabetes by STZ induced a further increase in the

cardiac apoptotic index, likely due to the additive effects of hyperglycemia. However, only a fraction (0.02-0.03%) of total cardiomyocytes underwent apoptosis in PO fed diabetic hearts. In a recent study by Okere *et al*, a high fat diet rich in saturated fatty acids (60% by calorie) promoted ceramide levels and caused apoptosis, again in only 0.03% of total cardiomyocytes [17]. This is in marked contrast to in vitro studies with palmitic acid, where up to 30% of the total cell population perished [18, 19]. It is likely that under in vitro conditions, energy requirement is reduced in isolated, non-beating, and quiescent cardiomyocytes in culture. Thus, exogenous fatty acids may undergo a greater flux towards non-oxidative pathways, provoking an exaggerated apoptotic response. It is also possible that in vivo, phagocytes rapidly clear apoptotic cells (within 24 h), and thus determination of apoptosis in the intact heart is underestimated [20]. As palmitic acid generates RNS like peroxynitrite, this highly reactive anion can induce nitration of tyrosine residues of proteins, leading to the formation of nitrotyrosine [11]. Protein expression of nitrotyrosine increased in PO fed diabetic hearts [16].

The apoptotic pathway in the heart is controlled by a number of proteins of the Bcl-2 gene family that can either promote (e.g., Bax) or prevent (e.g., Bcl-2) apoptosis [21, 22]. Upon apoptotic stimuli, oxidative stress increases Bax activity. Other members of the same family like Bcl-2 can heterodimerize with Bax, and prevent apoptosis. Thus, estimation of Bax/Bcl-2 ratio has been used to detect pro- or anti-apoptotic environments [21]. PO feeding augmented Bax mRNA in the heart. Superimposition of diabetes in PO fed hearts further increased Bax expression, but decreased mRNA levels of the protective Bcl-2, leading to an increased Bax/Bcl-2 ratio. An augmented Bax/Bcl-2 mRNA ratio has been previously linked to an increased lipoapoptosis in pancreatic islets of Zucker Diabetic Fatty (ZDF) rats [21].

3.2 Monounsaturated fatty acids

Monounsaturated fatty acids (MUFA) like palmitoleic acid (C16:1) and oleic acid (C18:1) are biosynthesized from saturated fatty acids like palmitic and stearic acid respectively [23]. Traditionally, MUFA has been used as a control to demonstrate the higher cytotoxicity of palmitic acid. In a study on rat pancreatic β cells, palmitoleic acid prevented palmitate induced cytochrome c release, DNA fragmentation and apoptosis [24]. In human pancreatic β cells, palmitic acid decreased the expression of antiapoptotic factor Bcl-2, both under normo and hyperglycemia [25]. In the same study, co incubation with palmitoleic and oleic acids conferred protection by increasing the expression of Bcl-2 [25]. In cardiomyocytes, oleic acid also inhibits the deleterious effects of palmitic acid and prevents or delays palmitate-induced apoptosis [26, 27]. The molecular mechanism behind the antiapoptotic potential of monounsaturated fatty acids comes from studies in the Chinese hamster ovary (CHO) cells [28]. Co-supplementation with 200 μ M oleate, prevents palmitate-induced apoptosis in CHO cells, by either preventing the formation or scavenging of excess ceramide and free radical generation. As intracellular fatty acids in the free form can promote the formation of both these detrimental molecules, monounsaturated fatty acids like palmitoleic acid or oleic acid have the unique ability of promoting palmitate incorporation into inert triglycerides and rescuing palmitate-induced apoptosis [25]. Interestingly, oleic acid, as well as palmitic acid, is toxic in cells with impaired triglyceride synthesis [29]. Thus, in the setting of a mismatch between lipid entry and utilization, oleic acid may facilitate triglyceride accumulation in non-adipocytes as the first line of defense against lipotoxicity [29].

In addition to its effects on triglyceride storage, feeding diets rich in oleic acid (42.7–43.9% of all fatty acids) for 30 days increases the activity of critical antioxidants like

catalase, superoxide dismutase and glutathione peroxidase [30]. This diet was also shown to be protective against loss of myocardial fibers following ischemia-reperfusion (IR) injury. As loss of myofibers may be a result of extensive cell death, increasing endogenous cardiac antioxidants may be a beneficial feature of a monounsaturated diet [30]. Alternative mechanisms explaining the beneficial effect of oleic acid against palmitate-induced apoptosis may be its involvement in the upregulation of phosphoinositide 3-kinase (PI3K) [31], an antiapoptotic factor for most cells. PI3K activation enhances cell survival and antagonizes apoptosis via protein kinase PKB in many cell types including cardiomyocytes [32]. As hypoinsulinemia in diabetes leads to a downregulation of PI3K activation, accumulation of oleic acid may indeed play an important role in the prevention of diabetic cardiac cell death [33, 34].

3.3 The Western Diet

Although majority of the work in the area of lipotoxicity, oxidative stress and diabetes has been attempted with saturated and monounsaturated fatty acids, the current western diet is richer in polyunsaturated fatty acids than either of the two previous classes of fatty acids [35-37]. As obesity and its attendant complications like diabetes and hypertension continues to grow, increased awareness among the population has led to an indiscriminate substitution of atherogenic saturated cooking fats with vegetable oils containing ω -6 PUFA [35]. Unfortunately, most of the coronary vascular benefits have been attributed to ω -3 PUFA like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and not ω -6 PUFA. As the Western diet is scarce in fish oils, the richest source of the beneficial ω -3 PUFA, dependence on the other kind of PUFA (i.e. ω -6 PUFA) has increased. In addition to the intentional modification of dietary fats, farmed animals are fed grains and seeds containing high amount

of ω -6 PUFA [35]. Consumption of these animals (and their products) has led to an even greater increase in the dietary ω -6 PUFA. Thus, although the total amount of dietary fat has not changed significantly, ω -6 PUFA has increased up to 5-10% of the total energy intake [38]. In the United Kingdom, the daily consumption of linoleic acid has gone up from 11 g/day in 1970 to 14 g/day in 1990 [39].

In summary, a high dietary intake of ω -6 PUFA has augmented our ω -3: ω -6 ratio to between 15:1 to 20:1, whereas during our evolution, the ratio was 1:1 or even less [40]. In our current society, the traditional diet of Greece (Crete) closely approximates the Paleolithic diet and is linked with long life expectancy and low cardiovascular disease. The traditional Greek diet contains a sizeable consumption of fish (approximately 30 times that of US population), a rich source of ω -3 PUFA, as well as a large amount of vegetables (including wild plants), fruits, nuts and legumes, all rich sources of folate, calcium, GSH, antioxidants, vitamins E and C, and minerals [41]. Olive oil (rich in oleic acid, a MUFA) is the chief cooking medium. In addition, because the meat comes from animals that grazed rather than being fed grain, it contains (n-3) polyunsaturated fatty acids (PUFA) as do milk and milk products [41]. Clinical studies like the Lyon Heart study examined the effects of a modified Greek diet with a ratio of 4:1 for linolenic acid:linoleic acid [42, 43]. After 2 years, the study reported a 70% decrease in mortality. The Singh and the GISSI study also corroborated the cardiovascular benefits of the Greek diet [44-46].

3.4 Biochemistry of PUFA

Naturally occurring dietary PUFAs can be divided into two major classes. Based on the position of the terminal double bond, the PUFAs are divided into ω -3 (the double bond 3 carbons away) and ω -6 (the double bond 6 carbons away) classes [39]. The ω -6 and ω -3

double bonds are entered into fatty acids by plant enzymes Δ 12 desaturase and Δ 15 desaturase. As these enzymes are absent in animals, dietary ω -6 and ω -3 PUFAs are termed as 'essential fatty acids'. The minimal essential requirement of these fatty acids was 0.2 to 1% of daily energy [47]. The parent fatty acids of these two groups are linoleic (18:2n-6) and linolenic (18:2n-3) acids. Plant oils are typically rich in linoleic acid, rather than linolenic acid. Interconversion between ω -3 and ω -6 PUFA is not possible in mammalian cells [48]. However, dietary linolenic and linoleic acids can be converted to different ω -3 and ω -6 PUFAs respectively within the cell via the process of desaturation (which adds double bonds) and elongation (which adds carbon atoms). Linoleic acid is converted via the combined action of desaturases and elongases to yield arachidonic acid (C20:4n-6). Using the same pathways, linolenic acid is converted to eicosapentaenoic (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) [39].

3.5 Probable effects of ω -6 PUFA in the diabetic heart

Multiple pathways have been attributed to ω -6 PUFA that can adversely affect cardiac health. Both diabetes and high ω -6 PUFA feeding has been shown to activate phospholipase A₂ (PLA₂) in the heart [49, 50]. This class of enzymes specifically cleaves the acyl group at the *sn*-2 position of most phospholipids, releasing constituent ω -6 PUFA and lysophosphatidylcholine (LPC) [51]. The released ω -6 PUFA can undergo lipid peroxidation by reacting with ROS to produce various toxic metabolites. The highly reactive hydroxyalkenal, 4-hydroxy-2-nonenal (HNE), is formed by the reaction of superoxide with membrane ω -6 PUFA [52]. HNE can induce apoptosis in various cell types including neuronal cells [53], colon carcinoma cells [54] and endothelial cells [55]. Recently it has been proposed that overproduction of HNE can directly modify mitochondrial respiratory

chain and affect mitochondrial respiration in the diabetic rat heart [56]. Further, a major route of HNE metabolism in the heart includes its conjugation with glutathione (GSH) and activation of the polyol pathway in the normoglycemic heart [57]. As diabetes causes a depletion of cardiac GSH, with an activation of the aldose reductase pathway, such clearance mechanism could further lower cardiac GSH, thus precipitating greater toxicity.

Cardiolipin, a mitochondrial membrane phospholipid, is absolutely essential for the activity of key OxPhos enzymes [58, 59]. We and others have shown that chronic ω -6 PUFA feeding causes a loss of cardiolipin in the rat heart [16, 60, 61]. High ω -6 PUFA feeding increases cardiac phospholipase A₂ (PLA₂) activity [50]. PLA₂ can hydrolyse cardiolipin to induce a loss in total cardiolipin mass, as seen in rat brain and liver [62, 63]. A drop in cardiolipin may damage the mitochondria and impair energy production by the heart. Indeed, ω -6 PUFA feeding can increase myocardial oxygen consumption and decrease cardiac efficiency [64], oxidize mitochondrial DNA, with structural alterations [65]. The mitochondrial damage is prevented by coenzyme Q₁₀, a mitochondrial antioxidant [66, 67].

Incubation of human endothelial cells with linoleic acid, a ω -6 PUFA selectively upregulates transcriptional activity of nuclear factor kappaB (NF-kappaB) and activator protein 1 (AP-1) [4]. Increase in activity of these factors stimulated increased mRNA levels of several inflammatory proteins including tumor necrosis factor alpha (TNF- α) in these endothelial cells [4]. TNF can stimulate neutrophils, monocytes, T cells and endothelial cells to generate free radicals, which in turn produce more damage to the myocardium [68]. As diabetes per se increases cardiac TNF- α levels within 2 weeks, additive effect of linoleic acid induced TNF- α release could precipitate increased cardiotoxicity [69].

3.6 Rationale And Objectives

In recent years, with the help of advanced imaging techniques, an increasing prevalence of heart disease is being recognized in early clinical diabetes [70]. In animal models, cardiac dysfunction can occur within hours of inducing hyperglycemia in vitro [12], or within 4-6 days of diabetes in vivo [71]. In most cases, oxidative stress is blamed for inducing such an acute dysfunction [12, 71-73]. Oxidative stress occurs as a result of an imbalance between the production of reactive oxygen species (ROS), and their neutralization by antioxidants [74]. In the diabetic heart, augmented ROS production can arise from multiple sources like defective mitochondria, glucose auto-oxidation, protein glycation, and increased activity of cytosolic xanthine oxidase [75]. Irrespective of its origin, ROS can induce cell death via mechanisms including lipid, protein and DNA oxidation and initiation of diverse stress-signaling pathways. In both human and experimental diabetes, loss of GSH, a crucial cardiac antioxidant has been correlated to increased cardiac oxidative stress [76-80]. Although unclear in diabetic hearts, recent studies propose a role of specific sub cellular GSH pools like that of mitochondria, in modulating oxidative stress and cell death [81-84].

Although lipids have been acknowledged as a significant contributor to diabetic cardiac complications, most of the existing studies on the cytotoxicity of fatty acids have been performed in vitro, with single saturated fatty acids like palmitic acid on quiescent cultured cardiomyocytes [5, 18, 19, 85, 86]. However, in the last few decades, saturated fats from the human diet have been replaced by ω -6 PUFA in form of vegetable oils [35-37, 48]. Thus, the effect of the more relevant ω -6 PUFA on the heart, especially during diabetes remains unknown.

My research proposal focused on identifying the role of GSH and ω -6 PUFA in modulating cardiac cell death during early diabetes. We had 3 objectives:

1. To determine the source of ROS and cardiac GSH status in acute diabetes.
2. To evaluate the role of sub cellular pools of GSH in modulating cardiac cell death in diabetes.
3. To examine the effect of ω -6 PUFA feeding on the myocardium, both during normoglycemic and hyperglycemic conditions.

3.7 Figure

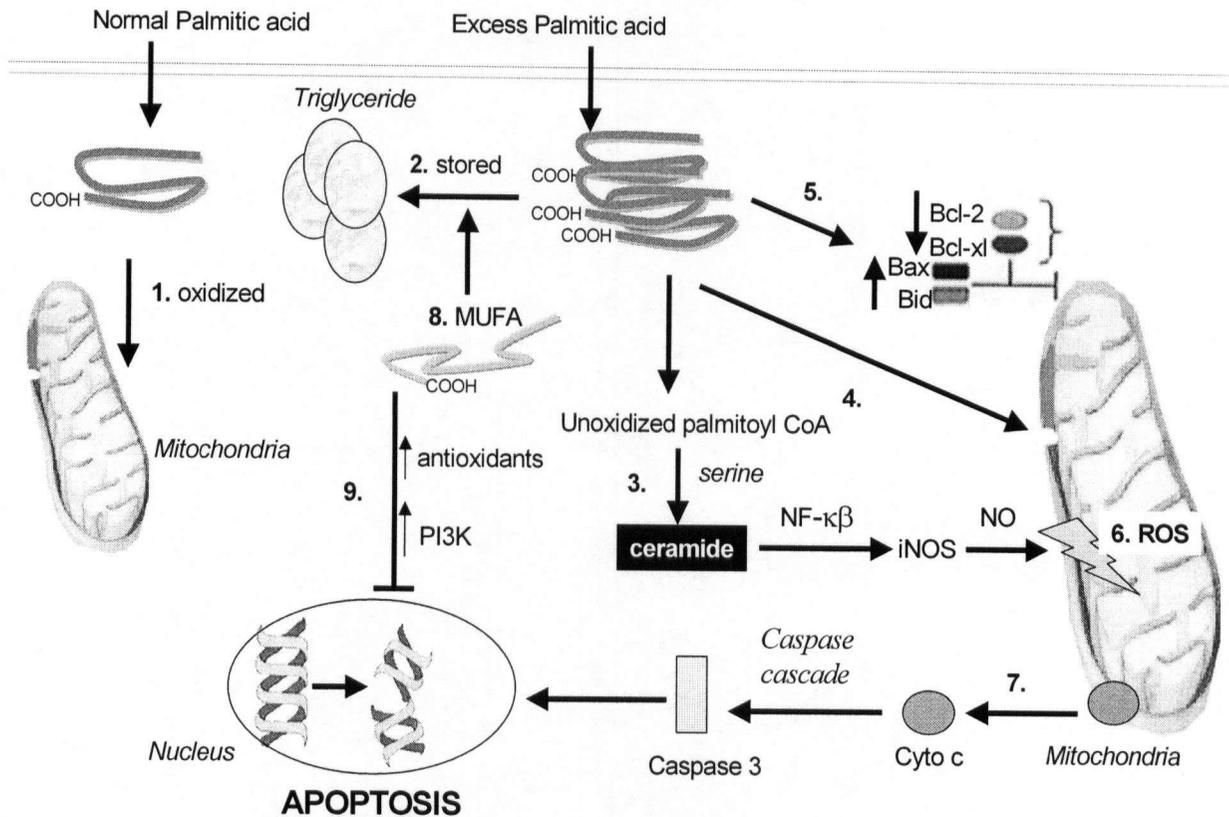


Fig. 3.1 *Modulation of cardiac cell death by saturated (palmitic acid) and monounsaturated fatty acids (MUFA)* Under normal conditions, moderate levels of palmitic acid is oxidized by the heart (1). However, under conditions of fatty acid excess (as during diabetes), cardiac oxidative capacity is superceded, leading to the storage of excess palmitic acid as triglycerides (TG) (2). As a non-adipose tissue, cardiac capacity of TG accumulation is low, and excess, unoxidized palmitoyl CoA is converted to ceramide, a pro-apoptotic sphingolipid (3). Ceramide, via upregulation of NF- κ B, increases the expression of inducible nitric oxide synthase (iNOS), and generates nitric oxide (NO). Excess palmitic acid can also directly damage mitochondrial membrane (4). Palmitic acid can upregulate the expression of pro-apoptotic factors like Bax and downregulate the expression of anti-apoptotic members of the Bcl-2 family, thus predisposing the heart to apoptosis (5). Increase in pro-apoptotic factors, damaged mitochondrial membrane and increased ceramide precipitates an increase in reactive oxygen species (ROS) (6). Increased mitochondrial ROS leads to the release of cytochrome c (cyto c) in the cytosol, which ultimately precipitates a caspase cascade, terminating in the activation of caspase 3, DNA damage and apoptosis (7).

MUFA (mainly oleic and palmitoleic acid) augments cardiac TG storage and removes free palmitic acid (8). Additionally, MUFA can also augment cardiac antioxidant levels and expression of PI3 kinase (PI3K), an important survival factor within the cardiomyocytes, and prevent or delay apoptosis (9).

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4. Increased Efflux Of Glutathione Conjugate In Acutely Diabetic Cardiomyocytes

4.1 Introduction

Diabetes is a significant risk factor for cardiovascular diseases with the majority of these complications being attributed to coronary vascular pathology. 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”[1]. Probable candidates to explain this heart disease include autonomic abnormalities, metabolic disorders, abnormal contractile protein and enzyme function, interstitial fibrosis [1], and more recently apoptosis, a regulated, energy dependent, cell suicide mechanism.[2, 3].

Reactive oxygen species (ROS) are highly reactive oxidants that cause tissue injury and cell death. Hyperglycemia induced increase in ROS, either due to an increased production or a decrease in the antioxidant capacity, has been implicated in inducing cardiomyocyte apoptosis [4]. Once increased, ROS like hydrogen peroxide (H_2O_2) initiates oxidative modification of structural and functional proteins and lipids leading to apoptotic cell death. With respect to antioxidants, glutathione is a tripeptide (L- γ -glutamyl-L-cysteinyl glycine, GSH) involved in the protection of cells and tissues against H_2O_2 induced damage [5]. γ -glutamylcysteine synthetase (γ -GCS) is the rate-limiting enzyme for the biosynthesis of GSH. Once formed, reduced GSH neutralizes H_2O_2 and is oxidized to GSH disulfide (GSSG). GSSG can be reduced back to GSH in an enzymatic recycling reaction catalyzed by GSH reductase (GR) and NADPH [5]. Another pathway contributing to GSH homeostasis is that of cellular GSH extrusion [6]. Under conditions of oxidative stress, glutathione binds to lipid peroxidation products that are then effluxed from the cell by transporters like P-glycoproteins and multidrug resistance proteins (MRPs) [7].

Although the regulation of γ -GCS and GSH reductase has been extensively studied in the chronically diabetic heart [8, 9], their contribution in modulating cardiac oxidative stress in the acutely diabetic heart is largely ignored. Additionally, the role of glutathione efflux in modulating cardiac oxidative stress during diabetes is unknown. Using acutely diabetic animals, the objective of the present study was to examine the glutathione status and to determine if myocyte GSH efflux contributes towards cardiac oxidative stress.

4.2 Materials And Methods

4.2.1 Experimental Animals

Animals were cared for in accordance with the principles promulgated by the Canadian Council of Animal Care and the University of British Columbia. Male Wistar rats (350-360 g) were obtained from the University of British Columbia Animal Care Unit. Animals were made diabetic with a single intravenous injection of streptozotocin (STZ, 55 mg/kg). STZ causes selective β -cell death with profound hyperglycemia developing within 24h. A commercially available kit (Sigma) was used to measure serum glucose obtained from tail vein blood samples. Diabetic rats were kept for 4 days, anesthetized with 65 mg/kg sodium pentobarbital i.p, the thoracic cavity opened, and hearts removed.

4.2.2 Cardiac Apoptosis

Part of the left ventricle was fixed in 10% formalin, embedded in paraffin, and 5 μ m sections prepared. TdT mediated dUTP nick end labeling (TUNEL) assay was carried out on these sections using a TUNEL kit (Oncogene) as described previously [10]. At least six random sections of each heart were quantified using the software 'Northern Eclipse'. Values are expressed as number of TdT labeled nuclei per 10^6 nuclei. To substantiate the incidence of apoptosis, caspase-3 (whose activity increases preceding apoptosis) was estimated using a kit (Clontech). Briefly, heart tissue (200 mg) was placed in ice-cold cell-lysis buffer (10 mM HEPES, 2 mM EDTA, 0.1 % CHAPS / NP40, 5 mM DTT, 1 mM PMSF, 10 μ g/ml pepstatin A, 20 μ g/ml leupeptin, 10 μ g/ml aprotinin) and homogenized. Subsequently, the suspension was centrifuged at 12,000 x 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 [11]. Values are expressed as O.D.U. (optical density units) per 100 mg wet weight.

4.2.3 Cardiac Lipid Peroxidation

Oxidative stress was determined by the appearance of thiobarbituric acid-reactive substances like malondialdehyde (MDA) [12]. 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 the presence of 0.4% butylated hydroxytoluene to prevent further tissue oxidation. After cooling, the adduct was extracted into n-butanol, and absorbance measured at 540 nm against 1,1,3,3-tetramethoxypropane as the standard.

4.2.4 Preparation of Cardiac Myocytes

Hearts were digested by perfusing modified Joklik minimal essential medium containing collagenase (228 U/ml), 0.5% BSA, and 50 mM CaCl₂ retrogradely through the heart [13]. Myocytes were made calcium-tolerant by successive exposure to increasing concentrations of calcium upto 1 mM Ca²⁺. Yield of cardiomyocytes (90-95% of total 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, were either used fresh for estimation of GSH or GSH efflux or were plated in laminin coated 6 well culture plates using colorless Media-199 (Sigma) to a cell density of 75,000 cells/well, and incubated at 37°C under an atmosphere of 95% O₂-5%CO₂ for fluorimetric estimation of ROS.

4.2.5 Estimation of GSH

GSH content of freshly isolated cardiomyocytes was measured using a commercially available kit (Cayman Chemicals). 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. Rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the total concentration of GSH in the sample. Oxidized glutathione (GSSG) was estimated using 2-Vinylpyridine[14]. The difference between total GSH and GSSG gave the value of the reduced GSH. Protein assay was performed according to the Bradford Method. Values are expressed as nM GSH per mg protein.

4.2.6 Cardiac Gene Expression

Cardiac gene expression was measured using semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) as described previously [10]. PCR primers were designed from rat sequences available in GenBank, and are as follows: gamma glutamylcysteine synthase heavy chain (γ -GCS_{HC}); 5'-CAAGAGAAGGGGGAGAGGAC-3' (forward) and 5'-TTATCGTGCAAAGAGCCTGA-3' (reverse) (accession # NM012815); multidrug resistance protein 1 (MRP-1); 5'-CCTTTTCCTGTGCAATCATGTA-3' (forward) and 5'-AGAACCTCTGCACAAAGAAGTA -3' (reverse) (accession # NM022281); β -actin; 5'-CGTAAAGACCTCTATGCCAA-3' (forward) and 5'-AGCCATGCCAAATGTGTCAT-3' (reverse) (accession # J00691). Levels of expression were indicated as the ratio of signal intensity for the mRNA of interest relative to that for β -actin mRNA.

4.2.7 Glutathione Reductase Activity

Ventricular tissue was powdered under liquid nitrogen, homogenized in cold buffer (50 mM potassium phosphate, pH 7.4, 1mM EDTA), and centrifuged at 7000g for 15 minutes at 4°C. 100 μ l of supernatant was removed for protein analysis (Bradford Assay). For the assay, GSSG and 20 μ l of samples were added to a cuvette. The reaction was initiated by the addition of 2 mM NADPH and absorbance recorded at 340 nm. Glutathione reductase (GR) activity was

measured by the decrease in absorbance, which accompanies oxidation of NADPH to NADP⁺ [15]. Values were expressed as nmol of NADPH/min/ml.

4.2.8 GSH Efflux

The bimanes are heterocyclic compounds that form fluorescent conjugates with thiols [16, 17]. Within the cell, monochlorobimane [syn-(ClCH₂,CH₃)-1,5-diazabicyclo-[3,3,0]-octa-3,6-dione-2,8-dione; mCIB), a freely permeable non-fluorescent dye, that conjugates preferably with reduced GSH [18]. Once complexed, mCIB emits a strong fluorescence (excitation, 380 nm; emission 485 nm). Briefly, freshly isolated cardiomyocytes (0.5×10^6) were transferred in duplicate to pre-warmed 10 ml test tubes. The cells were spun lightly to remove all media ($1000 \times g \times 1 \text{ min}$) and 2 ml of loading media (50 μ M mCIB, 10 μ M GSH, 1g/ml BSA, prepared in M-199) added. After 15 mins incubation, cells were spun again to remove loading media and 2 ml of the efflux media was added (loading media lacking mCIB). 50 μ l of the media was taken out immediately for estimation of the initial value. Cells were then incubated at 37°C under an atmosphere of 95% O₂-5%CO₂ either in the presence or absence of MK-571 (10 μ M) or verapamil (10 μ M). The cells were periodically shaken and 50 μ l of media aspirated at 15, 30 and 45 minutes for estimation of mCIB-GSH complex (fluorimetry) and lactate dehydrogenase (LDH; spectrophotometry). Finally, the cells were separated from the media by centrifugation for final estimation of cellular and media mCIB-GSH complex (at 45 minutes). Cellular content of the complex was estimated by sonication and precipitation of proteins with 10 μ l of 60% perchloric acid as described previously [16]. The precipitate was separated by centrifugation and 50 μ l of the resultant supernatant was estimated by fluorimetry. Total (media+cell) GSH was estimated by adding the fluorescence of the media at 45 minutes to the value obtained from the cellular mCIB-GSH complex (from 0.5×10^6 cells) at this time. Negative controls were generated using diethyl maleate (DEM) that complexes with GSH and

prevents formation of the mCIB-GSH complex. DEM (0.02%) was added 15 minutes before the start of the experiment.

4.2.9 LDH Release

To ascertain that the release of mCIB-GSH is not due to cell membrane leakage, lactate dehydrogenase (LDH) release into the media was estimated using an in vitro toxicology assay kit (Sigma). In brief, LDH reduces nicotinamide adenine dinucleotide (NAD⁺) that then converts a tetrazolium dye to a soluble, colored formazan derivative that was measured using a spectrophotometer (490 nm).

4.2.10 Measurement of ROS

A redox sensitive dye, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) was used to assess the presence of ROS [19]. CM-H₂DCFDA is a cell-permeant indicator for ROS like H₂O₂ that reacts with the probe, oxidizes it and emits green fluorescence. For the quantitative estimation of ROS, plated cells (65-75,000 myocytes/ per well) were either left untreated or incubated with MK-571 (10µM), verapamil (10µM), or N-acetylcysteine (NAC, 5mM) for 2 hours. Subsequently, 0.5 ml colourless M-199 and CM-H₂DCFDA (final concentration, 5 µM) were added per well. The increase in green fluorescence was monitored in a fluorimeter over the next 90 minutes (excitation: 480nm; emission: 530nm). Background was identified in wells without cells. Data was expressed per 75,000 cells as fluorescence units (F.U.) after subtracting the background at 90 minutes.

4.2.11 Statistical Analysis

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

4.3 Results

4.3.1 Cardiac apoptosis and oxidative stress in STZ diabetes

Table 4.1 depicts the general characteristics of control and 4-day diabetic animals. STZ injection induced hyperglycemia and a loss of body weight. Based on previous reports of increased apoptosis in hearts from STZ diabetic rats and mice, we attempted to corroborate these results in our model of diabetes. TUNEL staining showed that diabetic hearts had an approximately 5-fold increase in apoptotic cells compared to controls with a corresponding 2-fold increase in active caspase-3 activity. As hyperglycemia-induced cardiac apoptosis has been intimately linked to an increase in ROS production [19], cardiac oxidative stress (TBARS like MDA) was also determined and found to be elevated in 4 day STZ-diabetic rats (Fig. 4.1, top panel).

4.3.2 Glutathione status in STZ-diabetic hearts

Acute diabetes was associated with a significant decrease in cardiomyocyte GSH levels (Fig. 4.1, lower panel). Given that GSH can decrease, either due to changes in glutathione biosynthesis or a decrease in GSH regeneration [20], we measured gene expression of the heavy subunit of γ -glutamylcysteine synthetase (γ -GCS_H) and the activity of glutathione reductase, and determined that both remained unchanged following diabetes (Table 4.2). Interestingly, evaluation of a third pathway by which GSH can fall revealed that diabetic cardiomyocytes demonstrate a higher rate of GSH efflux, as measured by extrusion of mCIB-GSH into the incubation media (Fig. 4.2). This increase in GSH efflux was not a result of a difference in cell loading of the mCIB-GSH complex, as both control and diabetic cardiomyocytes had nearly identical total GSH-mCIB fluorescence (Fig. 4.2; right inset). Isolation of cardiomyocytes with collagenase leads to membrane damage and associated LDH release. As LDH release between control and diabetic cardiomyocytes were similar, it is

unlikely that the increase in GSH efflux in diabetic cardiomyocytes is a result of non-specific changes in membrane permeability (Fig. 4.2; left inset).

4.3.3 MRP like activity in diabetic cardiomyocytes

As MRP-1 has been associated with glutathione efflux under conditions of oxidative stress, its contribution towards efflux of mCIB-GSH in diabetic cardiomyocytes was investigated [7, 21]. 4 days of hyperglycemia led to an increase in cardiac MRP-1 gene expression (Fig. 4.3). Additionally, incubation of these myocytes with MK-571, a specific blocker of MRP-1 [22] reduced mCIB-GSH efflux to control levels [i.e., similar to cells in which mCIB-GSH efflux is completely inhibited using DEM, (Fig.4.4)]. To investigate the contribution of transporters other than MRP-1, diabetic cardiomyocytes were also incubated with verapamil, an inhibitor of P-glycoproteins [16]. Although verapamil lowered mCIB-GSH release into the incubation media, efflux was still higher than that observed with MK-571 (Fig.4.4).

4.3.4 Decrease in cardiomyocyte ROS following inhibition of GSH efflux

Following isolation of cardiomyocytes and plating for 2 hours, measurement of ROS using H₂DCFDA fluorescence revealed higher ROS levels in diabetic cardiomyocytes (Fig. 4.5). Given the association between GSH and ROS, cardiomyocytes were treated with either verapamil or MK-571 for 2 hours, and ROS levels measured. Unlike verapamil, MK-571, an efficient blocker of GSH extrusion in diabetic cardiomyocytes, was more effective in reducing ROS levels. Additionally, incubation of diabetic cardiomyocytes with NAC (that increases intracellular GSH levels) [23, 24] also decreased ROS.

4.4 Discussion

Heart disease in patients with diabetes can occur due to intrinsic defects in the heart muscle [1]. Of the many proposed mechanisms contributing towards this cardiac dysfunction, regulated cell death is a more recent addition to the list. In this regard, an increase in apoptosis was observed in hearts from diabetic patients and STZ-diabetic animals [2, 4]. 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. This is important as in strains like Wistar-Kyoto (WKY) rats, 55mg/kg STZ does not induce diabetic cardiomyopathy [25]. This could be related either to the lower incidence of hypertriglyceridemia, or an inherent resistance to the diabetogenic action of STZ, resulting in greater pancreatic insulin content in these rats [25]. However, with Wistar rats, we detected a 5-fold increase in apoptosis in the diabetic heart using TUNEL assay, and further validated this observation by identifying an increase in caspase-3 activity, the proteolytic enzyme involved in the execution of apoptosis [26]. Given that the STZ-diabetic rat demonstrates cardiomyopathy 4-6 weeks after STZ injection, it is possible that this initial loss of cells progressively leads to interstitial fibrosis, myocardial hypertrophy, and eventual contractile impairment [27].

Oxidative stress due to excessive ROS generation and/or inadequate ROS neutralization is believed to be a key player in inducing apoptosis. Once generated, ROS can cause cell death via lipid peroxidation reactions, alterations of cellular proteins and initiating diverse signaling pathways [28, 29]. Both ROS and lipid peroxidation were elevated in the diabetic heart and could be implicated in explaining cardiac apoptosis in these animals.

In an attempt to elucidate the mechanisms behind the increase in ROS, we measured GSH, the main antioxidant responsible for detoxification of ROS like H_2O_2 in the heart [30]. At least in chronic human and experimental diabetes, GSH levels decrease in various cells and organs like platelets [31], polymorphonuclear cells [32], liver, kidney, neurons and heart [33-35]. Our

results suggest that cardiac GSH levels are also reduced following acute hyperglycemia. In chronically diabetic rats (2-8 weeks), fall in GSH is often linked to decrease in biosynthesis and/or altered enzymatic recycling as a consequence of reduced gene expression [36] or activity [8] of γ -GCS, and decreased activity of glutathione reductase [8]. Interestingly, in our acute model of diabetes, fall in cardiomyocyte GSH could not be explained by either of the above mechanisms and indicated participation of GSH extrusion behind this drop. Indeed diabetic cardiomyocytes demonstrated higher GSH conjugate efflux compared to controls. Similar to our results, ischemia-reperfusion leads to loss of GSH, not by changes in glutathione reductase activity but by active GSH efflux [37]. Overall, our data suggests that at least at this early time point, GSH extrusion may be a major contributor in the fall of cardiac GSH.

MRP-1, a 190kDa membrane glycoprotein and member of the ATP-binding cassette (ABC) transporter family is well recognized for conferring multidrug resistance by effluxing anti-cancer drugs [16]. However, its affinity is highest for endogenous GSH conjugates [38], and can transport GSH out of astrocytes and cancer cell lines in response to oxidative stress [7, 21]. MRP-1 has ubiquitous distribution, including expression in the heart [39, 40]. In our study, cardiac MRP-1 gene expression increased following diabetes, potentially increasing GSH efflux with subsequent decrease in cardiomyocyte GSH. MK-571, a leukotriene D₄ receptor antagonist and an MRP-1 selective inhibitor [22] blocked mClB-GSH efflux in diabetic cardiomyocytes. As both MK-571 and NAC (which increases the intracellular concentration of glutathione) decreased ROS, our data suggests that MRP-1 has an important role in GSH and ROS homeostasis in the acutely diabetic heart.

In the heart, other ABC transporters like P-glycoproteins could potentially efflux GSH [41-43]. Verapamil, a P-glycoprotein blocker, partially decreased mClB-GSH efflux, and suggested a role for this transporter in diabetic cardiomyocytes. However, as verapamil can also influence MRP-1 when used along with GSH, the influence of P-glycoproteins in our

efflux experiments is likely minimal. Additionally, whether MRPs other than MRP-1 could explain this partial drop of mCIB-GSH efflux with verapamil is yet to be determined [17]. At present, the purpose for this enhanced MRP-1 expression in the diabetic heart is unknown. It is possible that MRP-1 through its efforts to reduce 4-hydroxynonenal (4-HNE; a toxic aldehyde responsible for the modification of proteins), inadvertently reduces GSH to which 4-HNE is conjugated [7, 44, 45]. Alternatively, for successful induction and maintenance of the apoptotic pathway in diabetic cardiomyocytes, it may be necessary for dying cells to lose their reducing capacity in favor of a pro-oxidizing environment, a goal that can be achieved easily through export of GSH, the most important cytosolic antioxidant [6]. It should be noted that MRP-1 has also been implicated in exporting the oxidized form of glutathione (GSSG). [46]. However, loss of GSSG also translates into net loss of GSH.

In conclusion, our findings demonstrate that the acutely diabetic heart is characterized by increased expression of MRP-1, reduction in GSH, increased oxidative stress and an elevated apoptotic index. Mechanisms responsible for the increase in MRP-1 expression in the diabetic heart are unknown and are currently being investigated.

4.5 Tables And Figures

Table 4.1 General characteristics of the experimental animals

	CONTROL	DIABETIC
Body Weight (g)	384 ± 5	366 ± 3*
Serum Glucose (mmol/L)	8.3 ± 0.2	22.9 ± 0.3 *
TdT Labeled Cardiomyocytes (per 10 ⁶ nuclei)	101 ± 16	495 ± 20*
Caspase-3 activity (O.D.U./per 100 mg w.w)	0.16 ± 0.01	0.39 ± 0.01*

Values are means ± SE for 4-6 animals in each group. Animals were made diabetic by STZ administration (55 mg/kg; i.v.). Diabetic animals were kept for 4 days before being killed. Values are those obtained before death and were from fed animals. *Significantly different from controls. $P < 0.05$. TdT, terminal deoxynucleotidyl transferase; O.D.U, optical density unit; w.w., wet weight.

Table 4.2 Factors Regulating Intracellular GSH Status

	CONTROL	DIABETIC
γ -GCS heavy chain/ β -actin mRNA expression	0.66 \pm 0.08	0.70 \pm 0.03
GR activity (nmol NADPH/min/ml/mg protein)	594 \pm 29	492 \pm 99

Values are means \pm SE for 4-6 animals in each group. Animals were made diabetic by STZ administration (55 mg/kg; i.v.). Diabetic animals were kept for 4 days before being killed. Values are those obtained before death and were from fed animals. *Significantly different from controls. $P < 0.05$. γ -GCS, gamma glutamylcysteine synthase; GR, glutathione reductase.

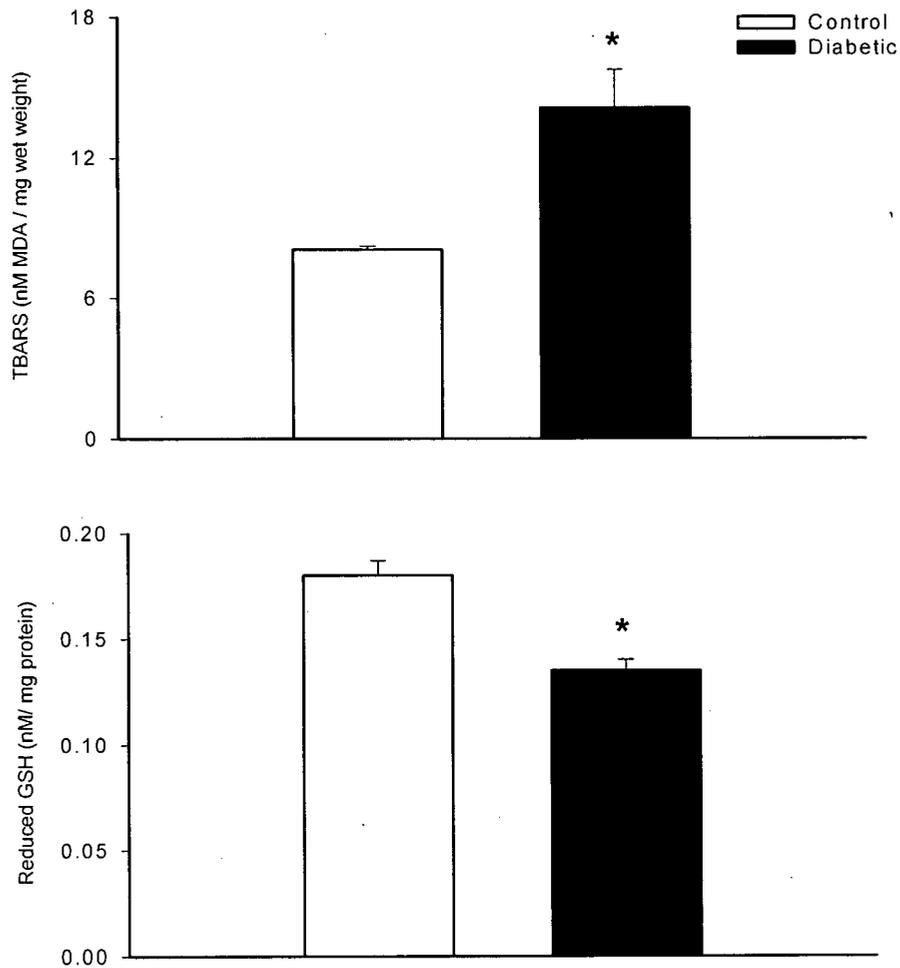


Fig. 4.1 *Oxidative stress in cardiac myocytes isolated from control and STZ-diabetic rats*
Lipid peroxidation was estimated with a thiobarbituric acid-reactive substance (TBARS) assay, with malondialdehyde (MDA) levels depicted in the upper panel. The lower panel shows cardiomyocyte GSH levels that were determined using an enzyme-recycling assay. *Significantly different from control, $P < 0.05$.

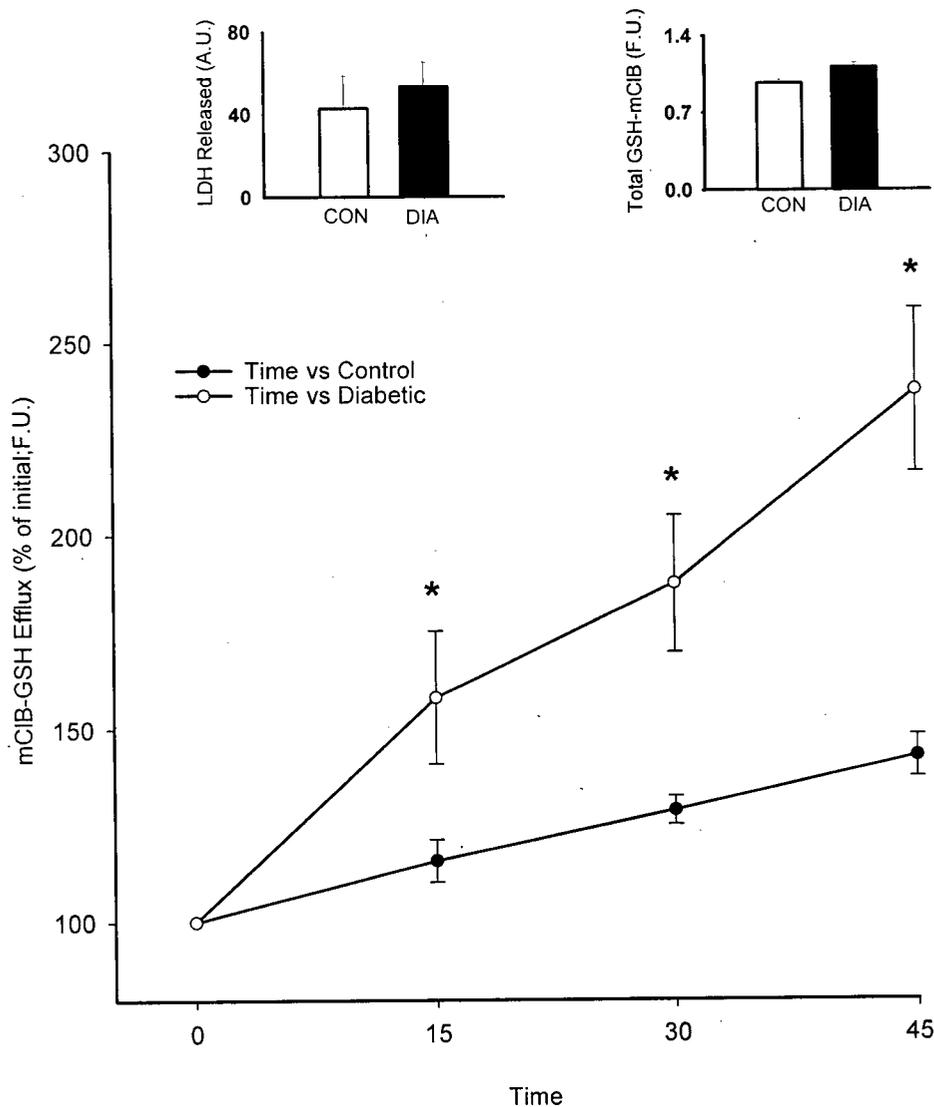


Fig. 4.2 *mCIB-GSH efflux from isolated cardiomyocytes* Control and diabetic cardiomyocytes were isolated, and incubated with 50 μ M mCIB and 10 μ M GSH. At time 0, the medium was removed and replaced with medium lacking mCIB, and the amount of mCIB-GSH release measured in the media over 45 minutes. The results are expressed per 0.5×10^6 cells in fluorescence units (F.U.) as a percentage of the initial value (time 0). Inset (left): Total LDH released into the media at termination of the experiment (45 minutes). Inset (right): Total mCIB-GSH fluorescence calculated by adding the fluorescence from cell lysates and the media at termination.. *Significantly different from control, $P < 0.05$. CON, Control; DIA, Diabetic; A.U., arbitrary units; F.U.; fluorescence units

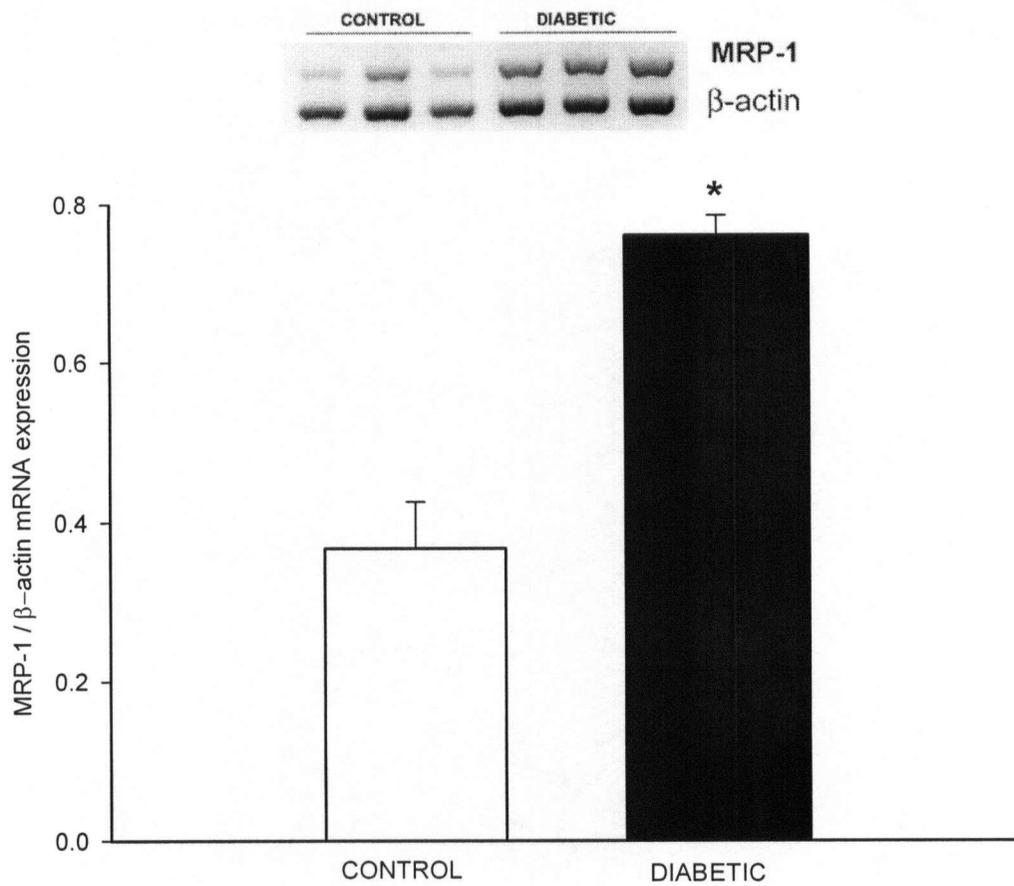


Fig. 4.3 *MRP-1 gene expression in hearts from control and diabetic rats* Bars represent the mean \pm SEM (n=3) of MRP-1/ β -actin ratio. The inset is a representative blot of MRP-1 RT-PCR products. *Significantly different from control, $P < 0.05$.

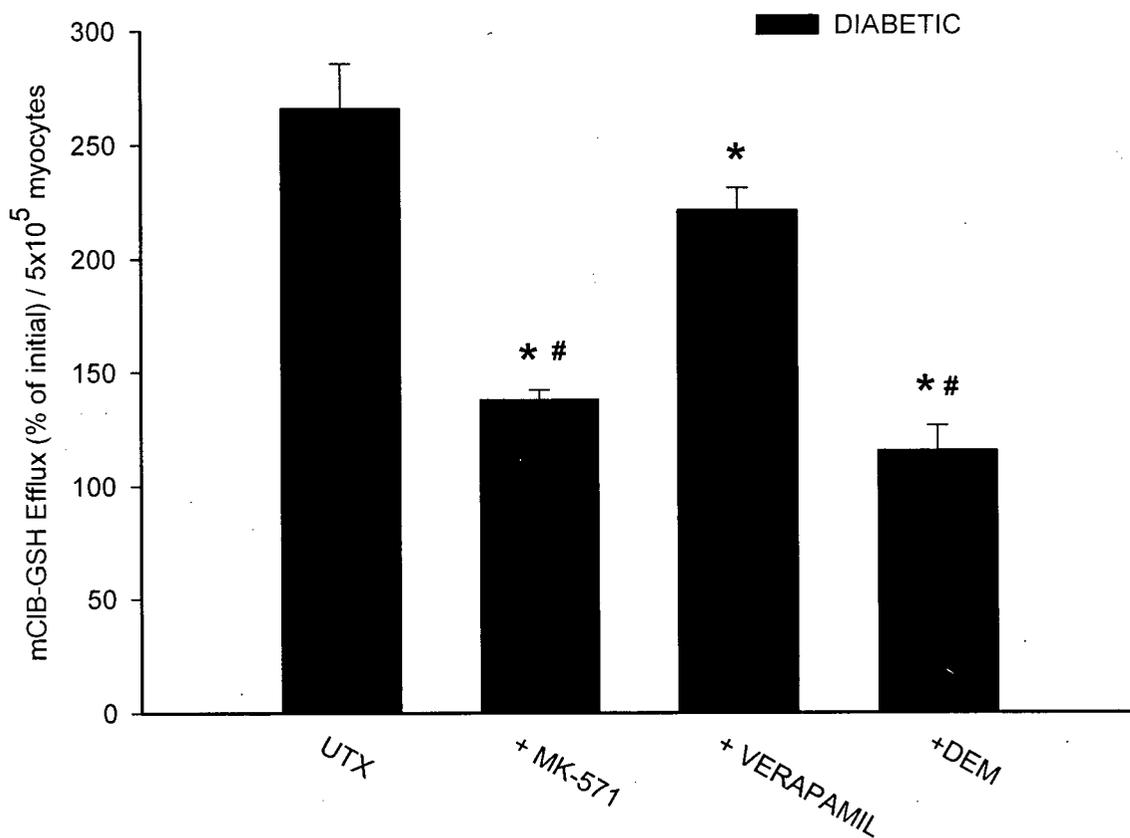


Fig. 4.4 *Effect of different inhibitors on the efflux of mCIB-GSH from diabetic cardiomyocytes* Diabetic cardiomyocytes were first incubated with media containing 50 μM mCIB and 10 μM GSH. After 15 minutes, it was replaced with medium lacking mCIB and media fluorescence calculated as percentage of the initial value (time 0). mCIB-GSH release in the media in the presence or absence of either MK-571 (10 μM) or verapamil (10 μM) was measured over the next 45 minutes. DEM (0.02%) was added 15 minutes before the start of the experiment and used as the negative control. * Significantly different from untreated diabetic myocytes. # Significantly different from verapamil treated myocytes, $P < 0.05$. UTX, Untreated

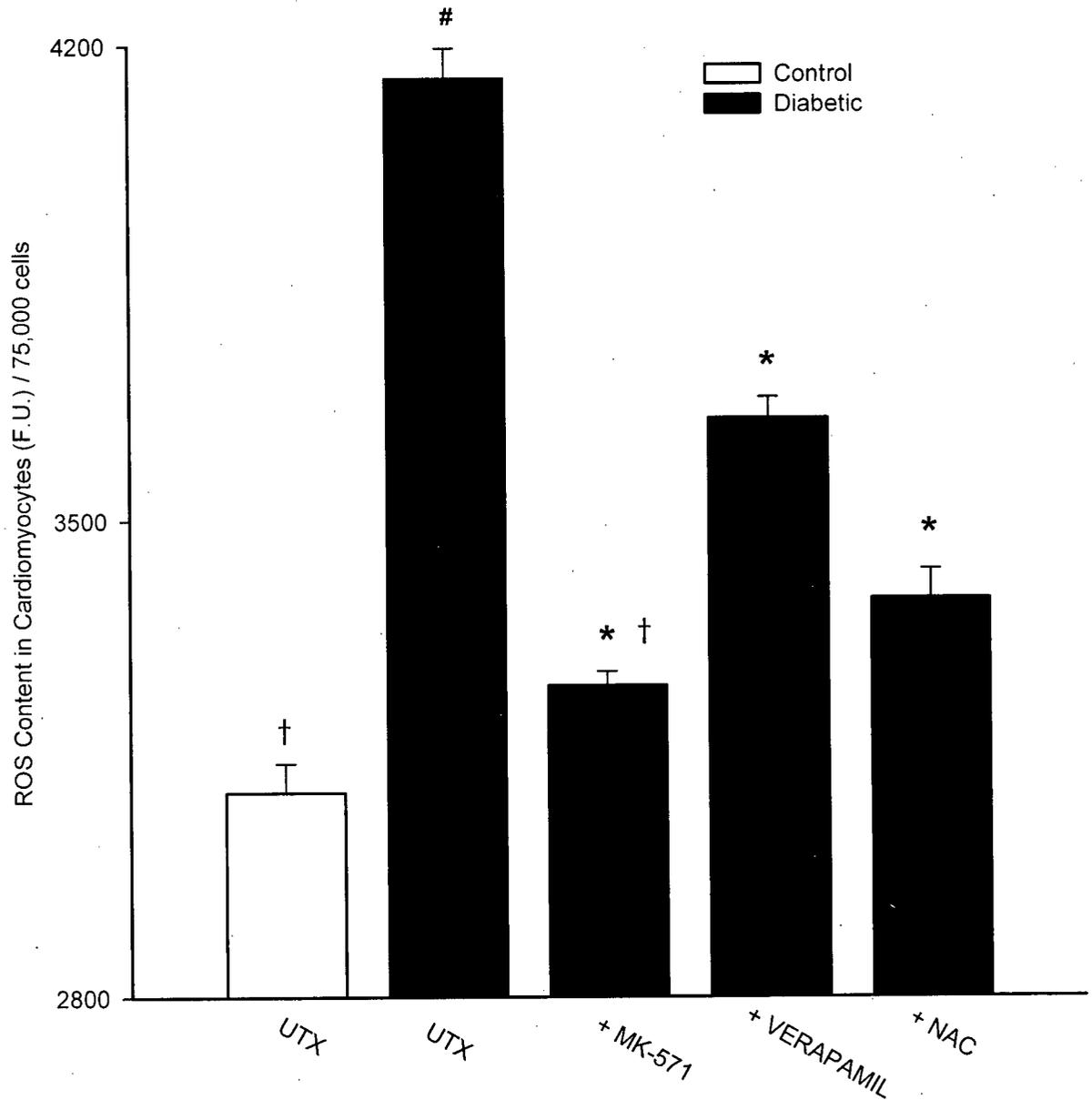


Fig. 4.5 *ROS status in control and diabetic cardiomyocytes* For the quantitative estimation of ROS, plated cells were either left untreated or incubated with MK-571 (10 μ M), verapamil (10 μ M), or N-acetylcysteine (NAC, 5 mM) for 2 hours. Subsequently, media with CM-H₂DCFDA (final concentration, 5 μ M) was added to each well. The increase in fluorescence was monitored in a fluorometer over the next 90 minutes (excitation: 480nm; emission: 530nm). The bars represent H₂DCFDA fluorescence (i.e. ROS content) in cardiomyocytes expressed in fluorescence units (F.U.) per 7.5×10^4 cells at termination of the experiment (45 minutes). *Significantly different from untreated diabetic myocytes; [#]Significantly different from verapamil treated myocytes. $P < 0.05$. UTX, Untreated

4.6 References

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5. Cardiomyocyte Apoptosis Induced By Short-Term Diabetes Requires Mitochondrial GSH Depletion

5.1 Introduction

Diabetes is a significant risk factor for cardiovascular diseases with the majority of these complications being attributed to coronary vascular pathology. 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 [1]. Probable candidates to explain this heart disease include autonomic abnormalities, metabolic disorders, abnormal contractile protein and enzyme function, interstitial fibrosis [1], and more recently apoptosis, a regulated, energy dependent, cell suicide mechanism [2-4].

In both human and animal models of diabetes, heart disease is associated with excessive apoptotic cell death [2, 3]. It is possible that this loss of cells progressively leads to interstitial fibrosis, myocardial hypertrophy, contractile impairment and eventual heart disease [5, 6]. Various factors including protein kinase C activation [7], upregulation of the renin-angiotensin system [2], p53 mediated gene transcription [8], and pro-apoptotic free fatty acids [6] have been implicated in inducing myocardial apoptosis during diabetes. Interestingly, all of these different elements appear to trigger cell death by a common 'oxidative stress' mediated pathway [9].

Oxidative stress occurs as a result of an imbalance between the production of reactive oxygen species (ROS), and their neutralization by antioxidants. In the diabetic heart, augmented ROS production can arise from defective mitochondria, glucose auto-oxidation, protein glycation, and increased activity of cytosolic xanthine oxidase [10]. Irrespective of its

origin, ROS such as hydrogen peroxide (H_2O_2) can induce cell death via mechanisms including lipid peroxidation, alteration of cellular proteins, and initiation of diverse stress-signaling pathways [11]. Among antioxidants, glutathione (L- γ -glutamyl-L-cysteinyl glycine, GSH) is a non-protein thiol crucial for protecting the heart against H_2O_2 induced damage [11]. In both human and experimental diabetes, loss of GSH has been demonstrated and correlated to increased cardiac oxidative stress [12-14]. Reduced GSH, present in the cytosol (cGSH, 80% of total cellular GSH) and mitochondria (mGSH, 10-15% of total GSH), acts as a hydrogen donor in the neutralization of H_2O_2 , and is itself converted to the inactive glutathione disulfide (oxidized glutathione; GSSG) [15]. Despite the importance of mGSH in preventing mitochondrial oxidative stress, mitochondria are incapable of de novo synthesis of GSH and rely on the import of GSH from the cytosol where it is manufactured from constituent amino acids.

Recently, we have reported that short-term diabetes induces cardiac GSH depletion, increased ROS levels, and cardiac apoptosis as early as 4 days after streptozotocin (STZ) administration [16]. Interestingly, similar observations were reported when cardiomyocytes were exposed to 24 hours of high glucose in vitro [17]. In both these studies, neither the source of ROS nor the site of GSH loss was explored. This is important because cytosolic and mitochondrial sources of ROS may affect specific pools of GSH [i.e. cGSH or mGSH], and differentially regulate apoptosis [15, 18]. Using acutely diabetic rats, the objective of the present study was to: (i) determine the source of cardiac ROS, and (ii) investigate the contribution of the respective intracellular GSH pools to cardiac apoptosis. We report that in the diabetic heart, mitochondria may be the chief source of ROS, with mGSH depletion being crucial in the activation of the cell suicide program in vivo.

5.2 Materials And Methods

5.2.1 Experimental Animals

The investigation conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985), and the University of British Columbia. 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. All animals were fed standard laboratory chow diet (PMI Feeds, Richmond, VA). Water was provided ad libitum. In some rats, diabetes was induced with a moderate dose of streptozotocin (STZ; 55 mg/kg, i.v.), a selective beta cell toxin, and hyperglycemia verified after 24 hours with a glucometer (Advantage Comfort, Roche Diagnostics). All diabetic rats were kept for 4 days following STZ administration, anesthetized with sodium pentobarbital (65 mg/kg, i.p.), the thoracic cavity opened, and hearts removed [5, 6, 16]. Following termination, plasma insulin was measured using rat insulin standards by radioimmunoassay (Novo, Copenhagen, Denmark).

5.2.2 Treatments

To examine the influence of acute diabetes on specific pools of GSH, in addition to determining how manipulation of these pools of GSH could affect myocardial apoptosis, some rats were left untreated or injected with L-buthionine sulfoximine (BSO; 10 mmol/kg, i.p.; dissolved in saline) and/or diethyl maleate (DEM; 4 mmol/kg, i.p.; dissolved in corn oil) for 4 days following STZ-administration. A control group with both BSO and DEM treatments was utilized to negate the possibility of non-specific toxic effects of these agents. BSO inhibits the activity of γ -glutamyl cysteine synthetase (γ -GCS), and blocks the de novo synthesis of GSH [11], whereas DEM conjugates and inactivates all GSH within the cell [18-20]. In contrast, to

augment cardiac GSH, some rats were treated with daily injections of GSH (400 mg/kg; pH 7.4; i.p.) for 10 days, prior to induction of diabetes. Administration of GSH was continued for 4 days following STZ-administration.

5.2.3 Preparation Of Cardiac Myocytes

Hearts were digested by perfusing modified Joklik minimal essential medium 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 (up to 1 mM). Yield of cardiomyocytes (90-95% of total 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. There was no difference in myocyte viability (percent of live cells) or yield (total number of cells x 10⁶) between control and diabetic rats at 4 days after the induction of diabetes. Immediately after isolation, fractions of cardiomyocytes were snap frozen in liquid nitrogen for estimation of various caspases. The remaining cardiomyocytes were either fractionated for determination of sub cellular pools of GSH, or plated on laminin coated culture plates as described previously [16].

5.2.4 Estimation of ROS

A redox sensitive dye, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) was used to assess the presence of ROS [4, 16]. CM-H₂DCFDA is a cell-permeant indicator that is oxidized in the presence of ROS such as H₂O₂, emitting green fluorescence. Myocytes were isolated and plated for 16 hours. Thereafter, the media was discarded and wells loaded with CM-H₂DCFDA (5 μM, 30 min, 37°C). Subsequently, cells were washed and incubated for a further 45 mins, after which green fluorescence was monitored in a fluorimeter at 485/530 nm. To identify the sources of ROS production, some

cardiomyocytes were incubated with rotenone (5 μ g/ml) or antimycin A (100 ng/ml), for 1 hour [21]. Rotenone blocks Complex I of the mitochondrial electron transport chain (ETC) and decreases mitochondrial ROS generation [21]. Conversely, incubation with antimycin A blocks Complex III and increases mitochondrial ROS [22]. Finally, N-acetylcysteine (NAC, 5 mM) which can decrease ROS production by increasing the intracellular GSH concentration [23], was used to monitor the effect of GSH supplementation in vitro. The emission was calculated per 75,000 cells in fluorescence units. For confocal microscopic images, chambered coverslips were utilized as described above. Myocytes were visualized using a confocal scanning microscope at 485/530 nm wavelength at 300x magnification for visualization of green fluorescence [16].

5.2.5 Determination Of Mitochondrial Membrane Potential

JC-1, a carbocyanine dye was utilized to assess changes in mitochondrial membrane potential ($\Delta\psi_m$) [24]. At low concentrations, this dye exists as a monomer in the cytosol, yielding green fluorescence at 530 nm [25]. Being cationic in nature, it accumulates in mitochondria and forms dimers, exhibiting red fluorescence at 590 nm. Formation of these dimers is directly proportional to $\Delta\psi_m$ [24]. Cells were isolated, plated and cultured for 16 hours. Thereafter, the media was discarded and the wells loaded with 10 μ g/ml JC-1 for 10 minutes, washed in ice-cold phosphate buffered saline, and scanned immediately with a CYTOFLUOR™ fluorimeter at 530/580 nm wavelength for dimers, and 485/530 nm for the monomeric JC-1. The emission was estimated per 75,000 cells in fluorescence units, and the red to green fluorescence ratio calculated. For confocal microscopic images, chambered coverslips were utilized instead of culture plates. Following procedures described above, myocytes were visualized using a confocal scanning microscope (Biorad MRC 600) at 530/580 nm wavelength, and 600x magnification. A single scan was performed to prevent mitochondrial damage during the

experiment. 100 μ M of the mitochondrial uncoupler, mCICCP was utilized as a negative control [25].

5.2.6 Separation Of Cytosol And Mitochondrial Fractions

Separation of cytosol and mitochondria was achieved by digitonin fractionation [26]. In brief, digitonin (0.24 mg/ml) was added to the cardiomyocyte pellet (2×10^6 cells) to disrupt the plasma membrane. After 2 mins, the mixture was transferred to eppendorf tubes containing 40% glycerol-silicone-oil mixture, and buffer containing 19.8 mM EDTA, 250 mM mannitol and 17 mM HEPES, and centrifuged at 13,000g for 3 mins. The top oil layer was separated and discarded without perturbing the middle buffer layer (cytosolic fraction). Triton X was added to the bottom glycerol layer (mitochondrial fraction), and sonicated for 15-20 secs. To determine fractionation efficiency, the glycerol and buffer samples were each assayed spectrophotometrically for succinate dehydrogenase (SDH; mitochondrial marker), and lactate dehydrogenase (LDH, cytosolic marker) activities. Since only ~5% of the total SDH was released into the buffer layer, mitochondria were recovered intact in the glycerol layer [26] before sonication. Additionally, LDH activity within the glycerol layer was also minimal (~5%).

5.2.7 Estimation Of Glutathione

Intracellular fractions were immediately subjected to deproteination with 5% metaphosphoric acid to remove protein thiol groups, snap frozen in liquid nitrogen and stored at -80°C . Determination of glutathione was performed within the next 48 hours. Cardiac GSH content was measured using a commercially available kit (Trevigen). 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. Rate of TNB production is directly proportional to this recycling reaction,

which is in turn directly proportional to the total concentration of GSH in the sample. Oxidized glutathione (GSSG) was estimated using 2-Vinylpyridine [27]. The difference between total GSH and GSSG gave the value of the reduced GSH. Protein assay was performed according to the Bradford method using a non-deproteinated portion of the samples. Values are expressed as nM GSH per mg protein.

5.2.8 Cardiomyocyte Apoptosis

Where indicated, part of the left ventricle was fixed in 10% formalin, embedded in paraffin, and 5 μ m sections prepared. To estimate cardiomyocyte apoptosis, TdT mediated dUTP nick end labeling (TUNEL) assay with cardiomyocytes specific labeling was carried out as described previously [5, 16]. At least six random sections of each heart were quantified using the software 'Northern Eclipse'. Values are expressed as number of TdT labeled nuclei per 10^6 nuclei.

5.2.9 Cardiac Caspase Activity

Activities of cardiac caspase-3, 8 and 9 were determined using the corresponding fluorescent caspase specific substrates DEVD-AMC (Molecular Probes), IETD-AMC (Biovision) and LEHD-AMC (Biosource), respectively. Briefly, 50-75 μ g of total cell protein was added to reaction buffer containing 50 μ M of the respective substrate, and incubated at 37°C for 1h. The enzyme-catalyzed release of AMC (aminomethylcoumarin) was quantified in a fluorimeter at 380/450 nm.

5.2.10 Lipid Peroxidation

As previously described, oxidative damage on lipids was determined by the appearance of thiobarbituric acid-reactive substances (TBARS) like malondialdehyde (MDA) in each of the subcellular fractions [6].

5.2.11 Protein Carbonyl Content

Protein carbonyls (PC) were assayed as an index of oxidative modification of proteins [28]. Briefly, 50 μ l of the fractions were added to an equal volume of 10% TCA, centrifuged at 6000g at 4°C for 5 min, and the supernatant discarded. The precipitated proteins were resuspended in 0.2% 2,4-dinitrophenyl hydrazine, and incubated for 1 h at 37°C. Subsequently, proteins were precipitated again with TCA, centrifuged, washed with ethanol:ethyl acetate, dissolved in 6 mM guanidine hydrochloride, and the absorbance measured spectrophotometrically at 370 nm.

5.2.12 Serum Lactate Dehydrogenase

As described previously, serum samples were isolated, kept at 4°C and processed within 24 hours for lactate dehydrogenase (LDH) using a commercially available kit (Sigma) [5].

5.2.13 Statistical Analysis

Values are means \pm SEM. One-way ANOVA followed by the Tukey or Holm-Sidak test was used to determine differences between group mean values. The level of statistical significance was set at $P < 0.05$.

5.3 Results

5.3.1 Mitochondrial Dysfunction Following Acute Diabetes

STZ administration increased blood glucose within 24 hours, which was sustained for the next 3 days (CON 7.1 ± 0.3 vs. DIA 23.4 ± 0.7 mM; $P < 0.05$), with a reduction in plasma insulin levels (CON 3.3 ± 0.4 vs. DIA 0.7 ± 0.1 , ng/ml; $P < 0.05$). One of the earliest diabetes-induced alterations is generation of ROS [4, 16]. To evaluate ROS levels, we utilized CM-H₂DCFDA, a dye that fluoresces green when oxidized by ROS (Fig. 5.1, upper panel). Using confocal microscopy (upper panel) and fluorimetry (Fig. 5.1, lower panel), diabetic myocytes revealed an increase in green fluorescence, suggesting increased prevalence of ROS within these cells. Treatment with rotenone reduced ROS production in diabetic myocytes to control levels. Diabetic cells were more sensitive towards antimycin A and demonstrated greater fold increases in ROS compared to control myocytes. Additionally, incubation with NAC decreased the fluorescence to less than control levels.

ROS generation in the diabetic heart may be regulated by changes in $\Delta\psi_m$ [29, 30]. Using confocal microscopy with JC-1, a dual fluorescent dye that dimerizes in the presence of intact $\Delta\psi_m$ (with emission of red fluorescence), diabetic myocytes consistently demonstrated less red fluorescence than control cells (Fig. 5.2, top panel), suggestive of inadequate dimer formation, and reduction in $\Delta\psi_m$ [24]. Using a fluorimeter, diabetic myocytes demonstrated a lower red to green fluorescence ratio for JC-1 as compared to controls (Fig. 5.2, lower panel), implying mitochondrial damage. Incubation of some cardiomyocytes with mClCCP demonstrated almost no red fluorescence (data not shown).

5.3.2 Cardiac GSH And Apoptosis Following Acute Diabetes

As reported previously, estimation of cardiac apoptosis revealed a nearly 4-fold rise in TUNEL positive nuclei in diabetic hearts (Fig 5.3, upper panel). As the most important cardiac antioxidant, in addition to being a key regulator of apoptosis [16, 17], reduced GSH was estimated in both the cytosolic and mitochondrial fractions. Interestingly, unlike the cytoplasmic pool, a depletion of mGSH (Fig 5.3, lower panel) was observed in 3-day diabetic rat hearts, implicating the importance of this specific pool of GSH in the cell suicide process.

5.3.3 GSH Modulation And Effects On Cardiac Apoptosis

To further investigate the relationship between GSH and cardiac apoptosis, we used different GSH-depleting agents and quantified TUNEL positive nuclei in diabetic hearts. BSO, known to inhibit *de novo* synthesis of GSH, decreased cytoplasmic (Fig 5.4, upper left panel) but not mitochondrial GSH (Fig 5.4, upper right panel) in diabetic hearts, likely due to the fact that mitochondria lack gamma glutamyl cysteine synthase, the target enzyme for BSO [31]. In an attempt to also influence mGSH, DEM, an agent that conjugates GSH and depletes both mGSH and cGSH in vitro, was used [18, 19]. Like BSO, DEM induced a substantial drop in cGSH (Fig 5.4, upper left panel), but was ineffective in reducing mGSH in vivo (Fig. 5.4, upper right panel). Interestingly, although both BSO and DEM treatments brought about substantial reductions in cGSH, they were ineffective in augmenting cardiomyocyte apoptosis in the diabetic heart (Fig 5.4, lower panel). Although co-administration of BSO and DEM did not further decrease cGSH levels compared to DEM only, mGSH was almost undetectable with this treatment (Fig 5.4, upper right panel). In this setting, cardiac apoptosis was further aggravated compared to the untreated diabetic group (Fig 5.4, lower panel). In control animals similar treatment resulted in the depletion of only cytosolic (UTX CON 18.7±3.9 vs CON+BSO+DEM 9.7±3.1, pmol./mg protein; $P<0.05$) but not mitochondrial GSH (UTX CON

7.2±1.9 vs CON+BSO+DEM 6.3±0.9, pmol./mg protein). Apoptotic rates remained unchanged compared to controls (data not shown).

In an attempt to prevent cardiac GSH loss, animals were pre-treated with exogenous GSH prior to the induction of diabetes, with the treatment continuing until termination. Unexpectedly, GSH supplementation decreased cGSH levels (Fig 5.4, upper left panel). However, there was a robust amplification in mGSH content in diabetic hearts (Fig 5.4, upper right panel), together with a substantial drop in the apoptotic index to control levels (Fig 5.4, lower panel).

5.3.4 Cardiac Oxidative Stress And Caspase Activity

Having implicated mitochondrial glutathione loss as a key participant in cardiac apoptosis, we investigated the outcome of GSH depletion on the initiation pathways of apoptosis. Within 3 days of diabetes, mitochondrial lipid (mTBARS) and protein (protein carbonyls, mPC) oxidation increased (Fig 5.5, upper panels), with no change in these parameters in the cytosol (data not shown). Estimation of caspase-9 (initiator caspase of the mitochondrial apoptotic pathway), caspase-8 (initiator caspase of the 'death receptor' mediated pathway) and caspase 3 (effector caspase of the above pathways) revealed an increase in caspase-9 and 3 activities only (Fig 5.5, lower panels), implicating predominance of the mitochondrial pathway in cardiac apoptosis at this early time point [32].

Treatment of diabetic rats with BSO or DEM increased cytosolic oxidative stress [TBARS (UTX DIA 23.8±2.5 vs DIA+BSO 36.9±4.9, DIA+DEM 36.2±3.6 nM MDA/mg protein; $P<0.05$), protein carbonyl content (UTX DIA 98.5±21.1 vs DIA+BSO 212.6±16.3, DIA+DEM 208±28.8 A.U./mg protein; $P<0.05$)] and caspase-8 activity (UTX DIA 19.7±2.5 vs DIA+BSO 33.1±2.7, DIA+DEM 36±3.2 F.U./mg protein; $P<0.05$) compared to the untreated diabetic group. These treatments were ineffective in further aggravating either mitochondrial oxidative

stress (Fig 5.5, upper panels) or caspase-9 and 3 activities (Fig 5.5, lower panels). Co-administration of both BSO and DEM led to a depletion of mGSH, specific increases in mitochondrial oxidative stress (Fig 5.5, upper panels) and caspase-9 and 3 activities (Fig 5.5, lower panels), and no change in caspase-8 (data not shown) compared to untreated diabetic rats. Co administration of BSO and DEM in control animals was unable to influence either oxidative stress or cardiac caspase activities (Fig.5.5), suggesting that it is not simply depletion of GSH, but the added effect of the prevailing diabetic environment that promotes cell death in diabetic hearts.

As severe glutathione depletion may precipitate necrotic death [5, 33], serum LDH was measured and found to be unchanged in all groups, suggesting that cell death was predominantly apoptotic in nature (data not shown). As GSH supplementation prevented cardiac apoptosis, reduced mitochondrial oxidative stress (Fig 5.5, upper panels) and activities of caspase 9 and 3 (Fig 5.5, lower panels), these data further confirm mitochondrial participation in cardiac apoptosis during acute diabetes [32].

5.4 Discussion

ROS are unstable oxygen containing molecules capable of modifying various cellular structures. Should the generation of ROS exceed their neutralization by antioxidants, oxidative 'stress' and premature cellular demise may occur [6, 16, 17]. In both clinical and experimental models of diabetes, oxidative stress is believed to be a key participant in causing cardiac injury [9]. In this study, using CM-H₂DCFDA, we confirmed the presence of ROS such as H₂O₂ and OH⁻ in cardiomyocytes isolated from acutely diabetic rats [34]. Given that under normal conditions, 1-2% of all oxygen consumed is converted to ROS by electron transfer in the mitochondrial ETC [35], we assessed the contribution of this pathway towards ROS generation in acutely diabetic rat hearts. In this regard, ubisemiquinone at complex III appears to be a major site for cardiac mitochondrial ROS generation [21]. Using rotenone, an inhibitor of the upstream complex I, we were able to decrease the excess ROS production in diabetic cardiomyocytes to control levels, likely as a consequence of inadequate electron transfer to the downstream complex III [21]. These results with rotenone precluded examination of the role of extra-mitochondrial-ROS-generating pathways in the acutely diabetic heart. As an alternate strategy, antimycin A, which inhibits electron transfer from complex III thereby prolonging the half-life of ubisemiquinone and increasing ROS, was used [21]. Antimycin A augmented ROS generation in both control and diabetic cardiomyocytes. However, the fold increase in ROS was greater in diabetic compared to control myocytes, supporting our conclusion that in diabetes, cardiac ROS is predominantly mitochondrial in origin.

Amplification of mitochondrial ROS may result from changes in $\Delta\psi_m$ (crucial in maintaining electron flux in the ETC thereby minimizing ROS generation). Using confocal microscopy and measurement of the red ($\Delta\psi_m < -100$ mV) to green ($\Delta\psi_m > -100$ mV) fluorescence ratio of the dye JC-1, a loss of negative $\Delta\psi_m$ was observed in diabetic

cardiomyocytes, suggesting that ROS generation may indeed be related to changes in $\Delta\psi_m$ [25]. Other than its role in ROS generation, the cell's commitment towards apoptosis may become irreversible following collapse of $\Delta\psi_m$ [29]. In this regard, changes in $\Delta\psi_m$ induces the opening of 'channels' in the mitochondrial membrane [permeability transition (PT) pore] leading to release of low-molecular-weight proapoptotic factors from the mitochondria like cytochrome c and apoptosis-inducing factor. These in turn can specifically activate initiator caspases like caspase-9 and effector caspases like caspase-3, leading to apoptosis [29]. As caspase-9 and 3 were both increased along with a substantial rise in cardiac apoptotic death following only 3 days of diabetes, our data suggests that the 'mitochondrial' pathway of apoptosis may predominate at this early time point.

In addition to changes in $\Delta\psi_m$, PT pore formation has also been linked to insufficiency in antioxidants like glutathione, and oxidative stress [30]. In this study, although cGSH remained unaltered, diabetic hearts were characterized by a decrease in mGSH, with a parallel increase in mitochondrial oxidative stress. Decrease in mGSH induces extensive ROS induced damage to mitochondrial membranes, which in turn causes a collapse of $\Delta\psi_m$ and activation of the mitochondrial death pathway [30, 36] (Fig. 5.6). As similar results have also been reported in chronically diabetic rodent hearts [12, 13], these data imply that loss of mGSH and the consequent ROS induced damage, may be an essential cause for the diabetes-induced early apoptosis in diabetic rat hearts.

To further examine the relationship between the different subcellular pools of GSH and apoptosis, we used BSO and DEM, agents that are known to deplete cellular GSH through different mechanisms. Administration of BSO, an inhibitor of γ -GCS activity, resulted in a 30% decline in cGSH, with no change in mGSH or apoptosis. To overcome the resistance of mGSH to change, DEM, an agent that irreversibly inactivates all pre-formed subcellular pools

of GSH, was used [18, 19]. Although DEM decreased cGSH (almost 70%), it was also incapable of either reducing mGSH or increasing cardiac apoptosis compared to untreated diabetic hearts. In other studies, DEM treatment prior to ischemia/reperfusion was also incapable of worsening damage in rat hearts [37]. It has been suggested that although DEM administration decreases mGSH in vitro, conjugation of cellular GSH with DEM in vivo triggers a compensatory increase in γ -GCS activity [38, 39], with newly formed GSH being transferred preferentially to the mitochondria. In this regard, during extensive cytosolic GSH depletion, the carrier system responsible for the transport of GSH may act to preserve mGSH levels [20, 31]. We speculated that abrogation of mGSH in vivo would only be possible if the compensatory rise in γ -GCS activity is prevented following DEM administration. To achieve this goal, BSO and DEM were co-administered over 3 days to STZ-diabetic rats. Using this novel approach, we were successful in depleting mGSH in diabetic rat hearts. Under these conditions, mitochondrial lipid peroxidation, protein oxidation and apoptosis were exaggerated, suggesting an obligatory role for mGSH in minimizing oxidative stress and apoptosis in acutely diabetic rat hearts. As cGSH levels was similar to that seen with DEM alone, it is unlikely that the increased cytotoxicity was due to cGSH depletion.

Exogenous GSH supplementation in pigs significantly increases myocardial GSH content and resistance to ischemia/reperfusion injury [40, 41]. Additionally, apoptosis and oxidative stress in isolated hearts perfused with high glucose were attenuated by addition of GSH to the perfusion buffer [42]. To prevent depletion of cardiac GSH following diabetes, rats were administered exogenous GSH. In acutely diabetic rat hearts, apoptosis and mitochondrial oxidative stress were averted by GSH supplementation. Measurement of GSH in the different pools revealed a selective increase in mGSH only, likely reflective of the need to protect the

diabetic mitochondria from oxidative damage. Unexpectedly, cGSH levels decreased, and the mechanism responsible for such an effect is presently unknown.

Two independent pathways can cause cardiac apoptosis, with cellular GSH levels influencing both the death-receptor-mediated 'extrinsic' and the mitochondrial 'intrinsic' pathways [43, 44]. Caspase-8 and 9 are initiator caspases for the extrinsic and intrinsic pathways respectively [32]. In our study, induction of diabetes was only associated with caspase-9 activation, an effect that was amplified using BSO and DEM in combination. As similar results have been reported in diabetic kidney and neuronal cells [45, 46], and as BSO and DEM increased caspase-8 without intensifying apoptosis, our data points towards a major role for the mitochondrial apoptotic pathway in the acutely diabetic heart.

In summary, at least following short-term diabetes, cardiac oxidative stress and apoptosis appear to have mitochondrial origins, with mitochondrial but not cytoplasmic GSH playing an essential role. As large-scale trials have failed to demonstrate any cardiovascular benefits using antioxidants like vitamin E and β -carotene in diabetic patients [47, 48], it is possible that: (i) the patients were in an advanced stage of the disease where damage reversal is difficult, (ii) agents like vitamin E only prevent lipid peroxidation, whereas ROS can affect a wide range of biomolecules, and (iii) the antioxidants were unable to reach therapeutic levels in the mitochondria where most of the ROS is generated. Thus, when using antioxidants, therapy should be initiated early, with scavenging of mitochondrial ROS being an essential property for preventing diabetic heart disease [35].

5.5 Figures

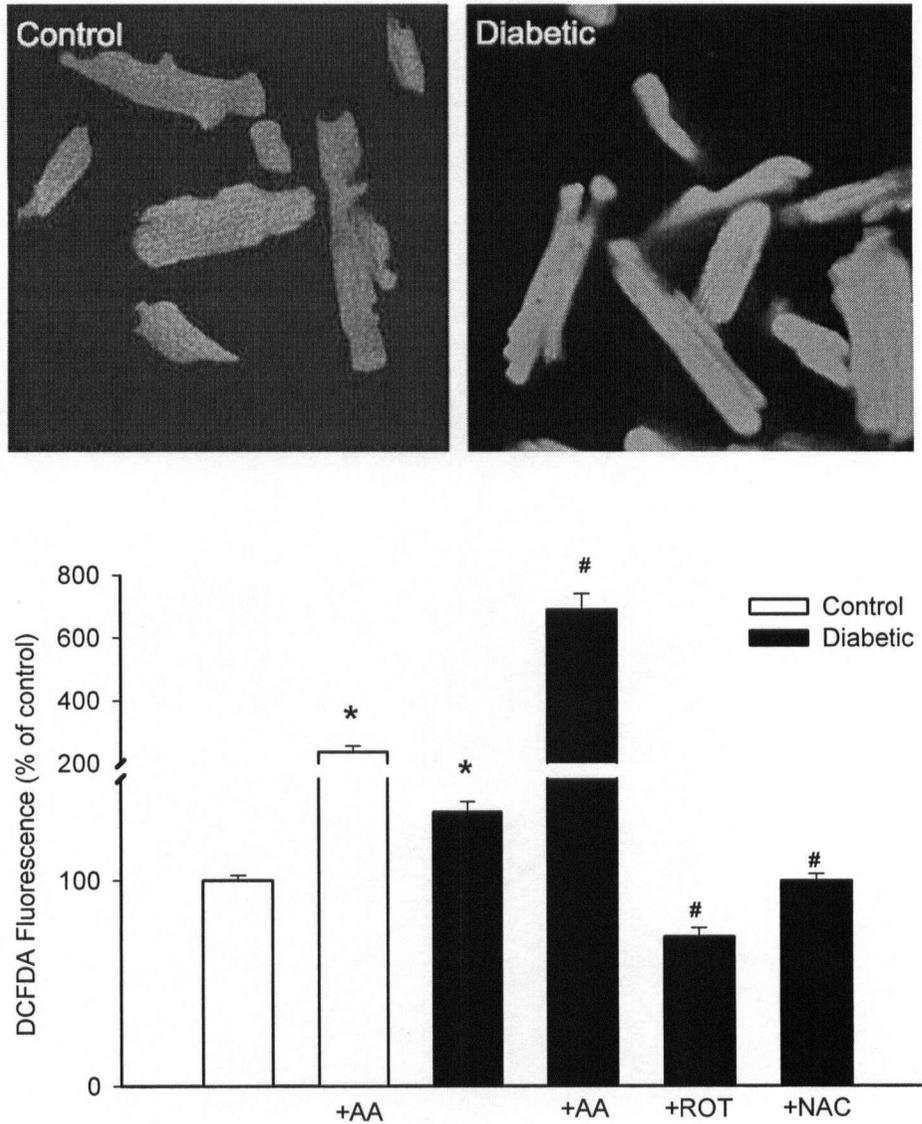


Fig. 5.1 *Reactive oxygen species in diabetic cardiomyocytes* To estimate ROS content, cardiomyocytes were incubated with CM-H₂DCFDA for 30 minutes, and visualized under confocal microscopy at 400x magnification (upper panel). To examine the origin of ROS, cardiomyocytes were either left untreated or pre-treated with rotenone (ROT, 5 µg/ml) or antimycin A (AA, 100 ng/ml) for 1 hour and subsequently, DCFDA fluorescence was monitored using a fluorimeter (excitation-480 nm; emission-530 nm) (lower panel). At termination (45 mins), DCFDA fluorescence was expressed in fluorescence units (F.U.) per 7.5×10^4 cells. Pre-incubation of diabetic myocytes with 5 mM NAC (1 h) was used to evaluate the relationship between GSH and ROS. Results are means±SEM of 4 rats per group. *Significantly different from untreated control myocytes; #Significantly different from untreated diabetic, $P < 0.05$.

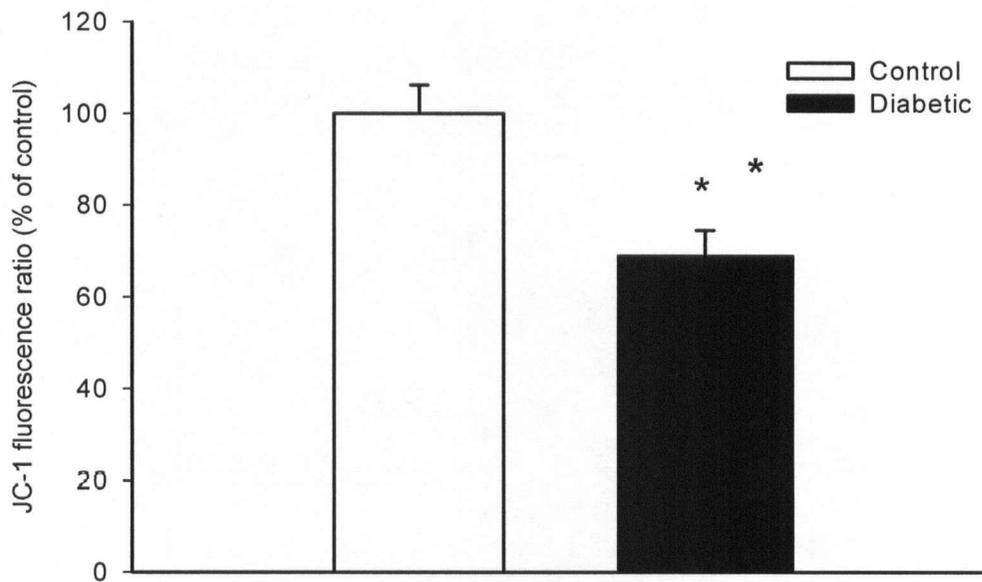
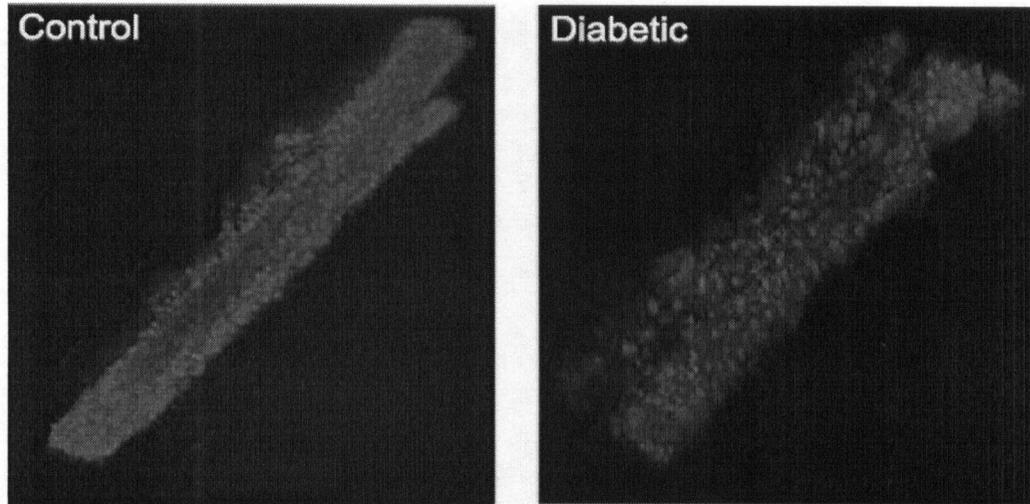


Fig.5.2 *Mitochondrial dysfunction following acute hyperglycemia.* Isolated control and diabetic myocytes were incubated for 10 mins with a mitochondrial membrane potential sensitive dye, JC-1. Red fluorescence due to JC-1 dimers was visualized under confocal microscopy at 600x magnification (upper panel). JC-1 fluorescence ratio (red to green) was quantitated using a fluorimeter (lower panel). Results are means \pm SEM of 4 rats in each group. *Significantly different from controls; $P < 0.05$.

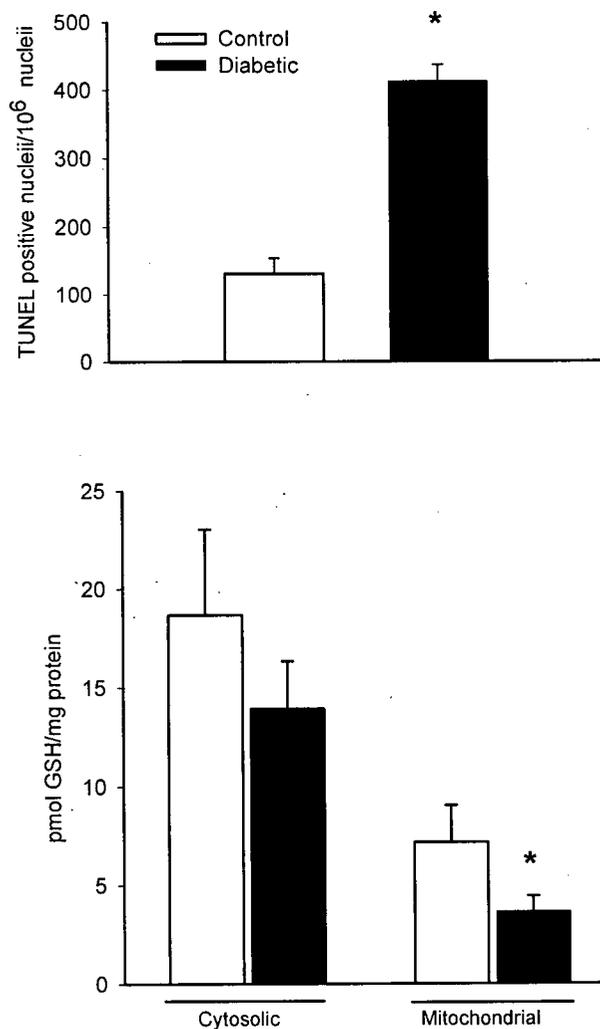


Fig. 5.3 *Effect of 4 days of diabetes on cardiac cell death and glutathione* TUNEL assay was performed on formalin-fixed rat heart sections and quantification of cardiac apoptosis was done using the imaging software 'Northern Eclipse.' The number of cardiomyocytes undergoing apoptosis is expressed per 10⁶ nuclei (upper panel). The lower panel describes the subcellular glutathione status in control and diabetic hearts. Cytosolic and mitochondrial fractions were separated using 'digitonin fractionation,' and assayed for GSH using an enzyme-recycling assay. Results are the means±SE of 6 rats in each group. *Significantly different from control; $P < 0.05$.

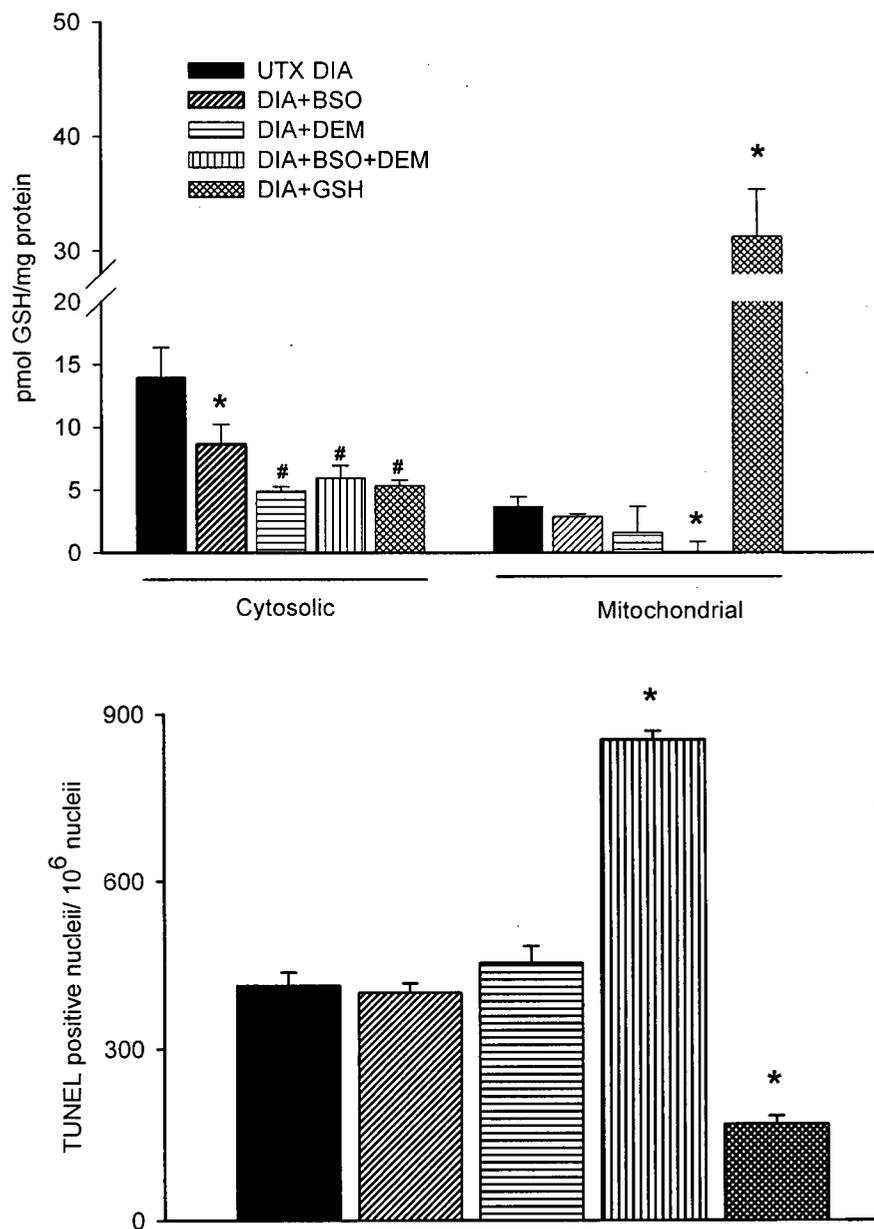


Fig. 5.4 *Effect of different treatments on GSH and cardiac apoptosis* In an effort to deplete cardiac GSH, daily doses of BSO (10 mmol/kg) and/or DEM (4 mmol/kg) were administered for 3 days. For exogenous GSH supplementation, control rats were administered GSH (400 mg/kg) for 10 days prior to STZ administration, and the treatment continued for a further 4 days. Following the above treatments, rats were killed, hearts isolated, and used either intact, or after isolation of cardiomyocytes. The upper panel depicts myocardial GSH in sub cellular fractions from diabetic hearts, whereas the lower panel represents cardiac apoptosis as determined by TUNEL staining. Results are the means \pm SE of 6 rats in each group. *Significantly different from untreated diabetic; #Significantly different from BSO; $P < 0.05$.

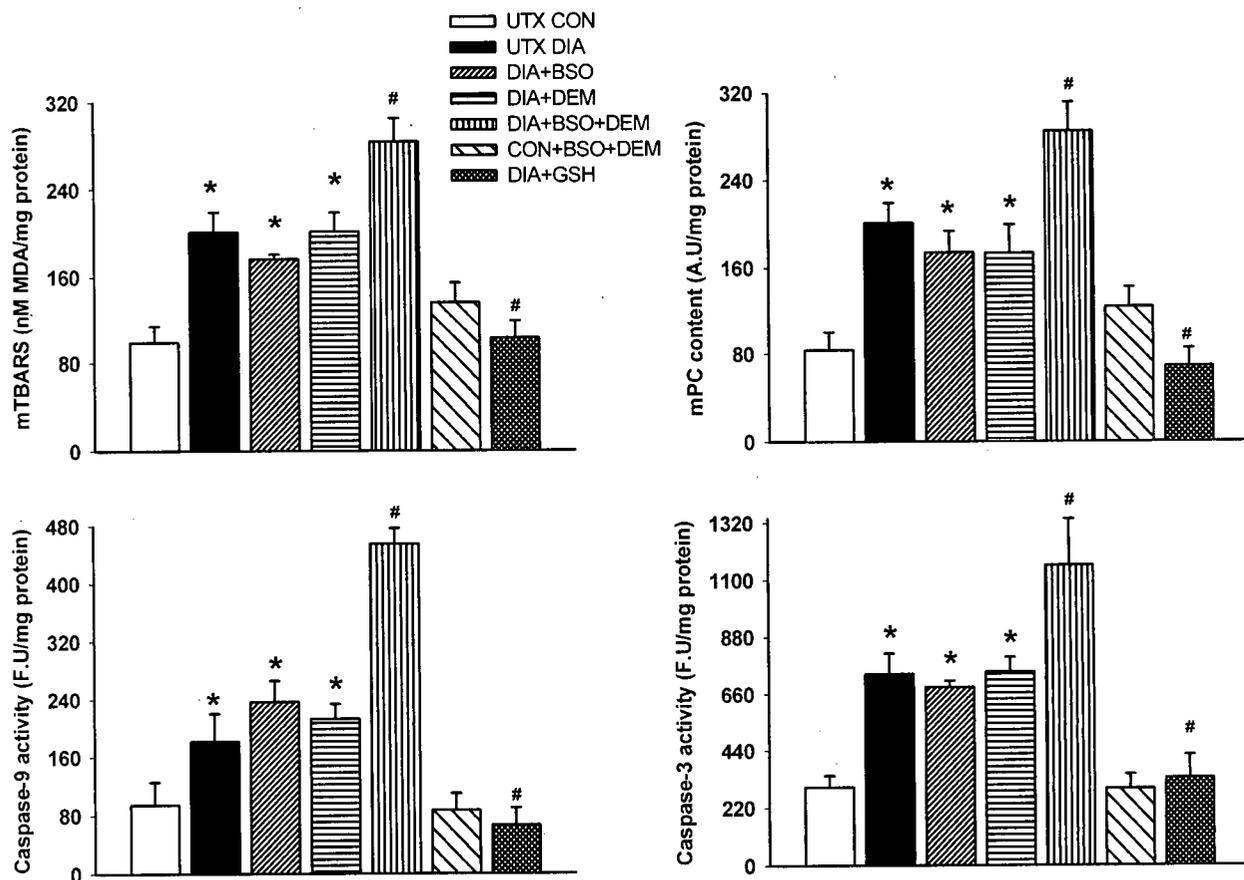


Fig. 5.5 Mitochondrial oxidative stress and caspase activities in control and diabetic hearts. Control hearts were either left untreated or treated with BSO+DEM. Diabetic rats were either left untreated, or treated with BSO, DEM, BSO+DEM or GSH. Mitochondrial oxidative stress was estimated by lipid peroxidation (mTBARS) and protein carbonyl content (mPC) (upper panels). The lower panels represent the quantitative estimation of cardiac caspase activities as determined using the corresponding fluorescent caspase specific substrates, DEVD-AMC (for caspase 3) and LEHD-AMC (caspase-9). Results are means \pm SE of 6 rats in each group. *Significantly different from control; #Significantly different from untreated diabetic. $P < 0.05$.

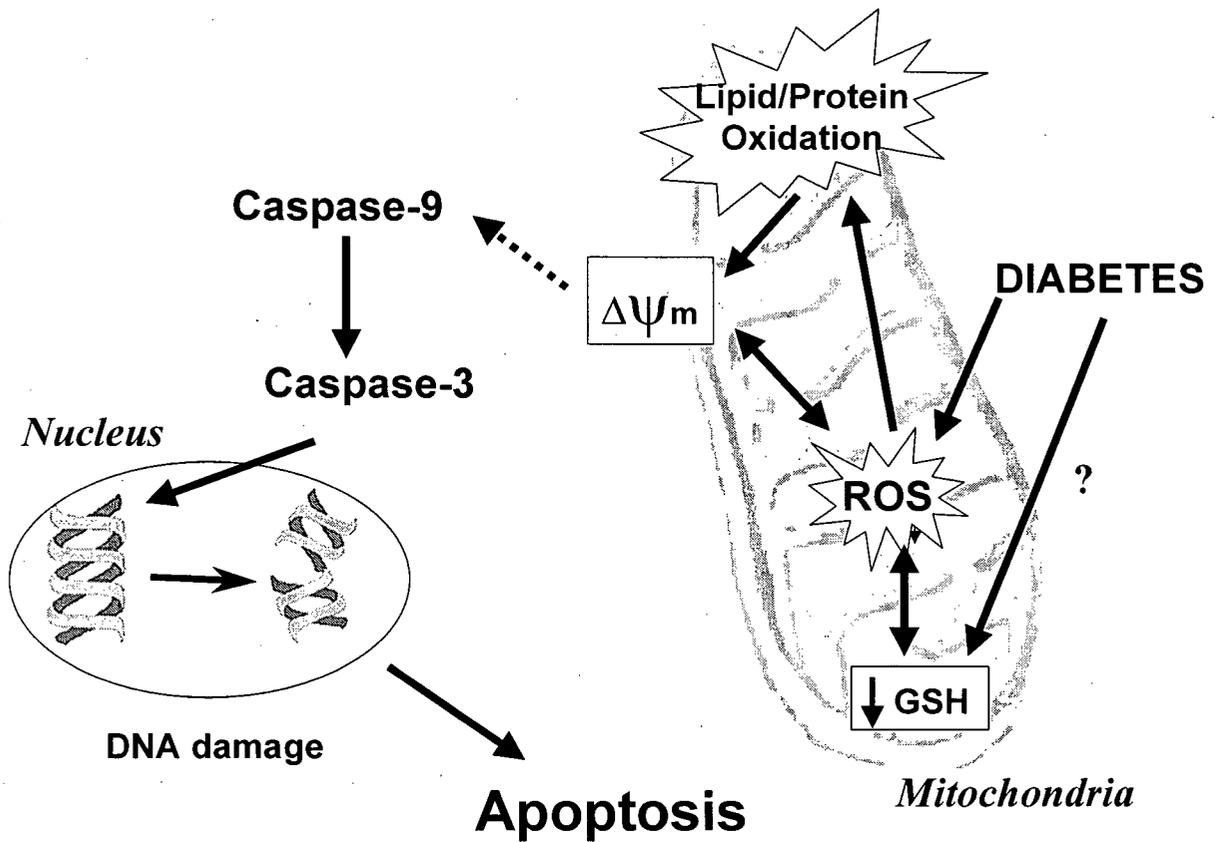


Fig. 5.6 *Proposed scheme of myocardial apoptosis in acute diabetes* Diabetes induces loss of mGSH and an increase in ROS. In turn, ROS can propagate lipid and protein oxidation leading to loss of mitochondrial membrane potential ($\Delta\Psi_m$). As a consequence, this loss of $\Delta\Psi_m$ may further exacerbate ROS generation, pore formation, activation of caspase-9 and 3, followed by apoptosis.

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6. Brief Episode Of STZ-Induced Hyperglycemia Produces Cardiac Abnormalities In Rats Fed A Diet Rich With ω -6 PUFA

6.1 Introduction

Obesity is a common nutritional condition in the Western world with attendant disorders like hypertension, insulin resistance and Type 2 diabetes. Over time, patients who develop diabetes are susceptible to heart disease, a leading cause of death in these patients [1]. Factors that largely account for this increased incidence of cardiovascular dysfunction include large and small coronary vessel disease [1]. However, a significant number of diabetic patients continue to suffer from left ventricular dysfunction and clinically overt congestive heart failure in the absence of vascular defects [1, 2]. Probable candidates to explain this heart disease include autonomic abnormalities, metabolic disorders, abnormal contractile protein and enzyme function, [1], and more recently apoptosis, a regulated, energy dependent, cell suicide mechanism [3, 4]. Given that animal models of diabetes [like the Zucker Diabetic Fatty rat [5] or the STZ diabetic rat [6] also display progressive myocardial abnormalities in the absence of vascular changes but in the presence of accelerated cell death, it is possible that this loss of cells progressively leads to interstitial fibrosis, myocardial hypertrophy, and eventual contractile impairment.

Increase in plasma glucose during diabetes has been suggested to predispose cardiomyocytes to death by apoptosis [3]. However, in diabetes, cardiac energy production is almost exclusively from fatty acids (FA), that are supplied in excess to the heart and have been implicated in causing cardiac lipid overload and cell death [7, 8]. Thus in vitro, palmitic acid, a saturated fatty acid causes intracellular accumulation of ceramide and reactive oxygen species

(ROS) in cardiomyocytes and can precipitate apoptotic cell death, either in the presence or absence of hyperglycemia [8, 9]. Recently, we have demonstrated that feeding a 20% w/w palm oil diet (rich in palmitic acid) to diabetic rats enhances cardiac apoptosis in vivo [10].

The majority of studies that have looked at the relationship between lipotoxicity and cardiovascular complications of diabetes have utilized lard or other sources of saturated dietary fat rich in palmitic acid [10-12]. However, in humans, increased awareness of obesity and its cardiovascular complications have led to an indiscriminate substitution of atherogenic saturated cooking fats with "heart-friendly" refined vegetable oils like sunflower oil, rich in ω -6 polyunsaturated fatty acids (ω -6 PUFA) [13]. In several studies, ω -6 PUFA conferred protection against arrhythmias [14] and coronary artery disease [15], and at least in human primary fibroblasts and Leydig cells, prevented apoptosis [16, 17].

Substantial data exists on the role of saturated FA in the development of cardiac apoptosis and ultimately cardiomyopathy in animal models of diabetes. However, in the light of current dietary recommendations, the impact of the more relevant ω -6 PUFA on cardiac function and morphology, especially during diabetes, is not clearly defined. We hypothesized that given the role of saturated fatty acids in accelerating cardiac apoptosis following diabetes, switching to a ω -6 PUFA rich diet may well be protective against cell death. Instead of preventing cardiac cell death, our data for the first time suggests that during diabetes, along with other morphological and functional abnormalities, ω -6 PUFA provision converts the mode of cellular demise to necrosis, an alternate form of cell death.

6.2 Materials And Methods

6.2.1 Experimental Animals

The investigation conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and the University of British Columbia. Previous studies have established that feeding of 20% w/w sunflower oil for 4 weeks increases the ω -6 PUFA content of circulating lipoproteins, [18] a major source of FA to the heart. To investigate potential beneficial effects of ω -6 PUFA, male Wistar rats (220-240 g) were first fed a ω -6 PUFA diet [(AIN)-76A supplemented with 20% w/w sunflower oil, Table 6.1] (Research Diets Inc.) for 4 weeks to increase cardiac PUFA content (PUFA Control; PC). Energy contribution from fats in this diet was 40% of the total energy intake, similar to human consumption [19]. Some PUFA fed animals were then given a moderate dose of streptozotocin (STZ; 55 mg/kg, i.v.), a selective beta cell toxin [20]. With this dose of STZ, there is only a partial destruction of β -cells with ~50% reduction in plasma insulin levels and stable hyperglycemia (PUFA Diabetic; PD) within 24 hours [21]. An acute time period (4 days) was chosen based on previous studies that demonstrate peak incidence of cardiac apoptosis after 3 days of STZ-diabetes in normal chow fed rats [4]. PUFA feeding was continued subsequent to STZ administration and after 4 days of diabetes, rats were anesthetized with sodium pentobarbital (65 mg/kg, i.p.), blood collected, and hearts excised for histological, morphological and biochemical analysis. Additional control (Normal Control, NC) and STZ (Normal Diabetic, ND) groups fed normal laboratory chow [5% corn oil; PMI Feeds, Table 1] were utilized to compare the effects of ω -6 PUFA feeding and diabetes per se.

6.2.2 Separation And Characterization Of Cardiac Lipids

Total cardiac lipids were extracted and solubilized in chloroform:methanol:acetone:hexane (4:6:1:1v/v/v/v). Separation of cardiolipin, triglycerides and FA was achieved using HPLC

(Waters 2690 Alliance HPLC, Milford, MA) equipped with an auto-sampler and column heater. FA were quantified as their respective methyl esters using heptadecaenoic acid (17:0) as the internal standard with a Varian 3400 GLC equipped with a flame ionization detector, a Varian Star data system, and a SP-2330 capillary column (30 m x 0.25 mm internal diameter) (Supelco, Bellefonte, PA) [22]. Value of cardiac FA, triglycerides and cardiolipin were expressed as $\mu\text{g}/\text{mg}$ protein.

6.2.3 Cardiac Caspase-3 Activity

To investigate the contribution of the apoptotic pathway, caspase-3 activity, the prime effector of cardiomyocyte apoptosis, was estimated (Molecular Probes). Caspase-3 is a cysteine containing protease crucial in the 'execution' phase of apoptosis and breaks down the contractile proteins in the heart [23]. Briefly, ventricular tissue (200 mg) was placed in cell-lysis buffer and homogenized (4°C). Subsequently, the suspension was centrifuged at $12,000g$ for 3 min, and the supernatant separated and reacted with aminomethylcoumarin (AMC)-derived substrate Z-DEVD-AMC. Caspase-3 activity is proportional to AMC released and is expressed as μM AMC released per mg protein.

6.2.4 Cardiac Necrosis

Necrosis leads to a loss of cell membrane integrity with release of lactate dehydrogenase (LDH) into the serum [24]. To verify necrosis, serum LDH was estimated using an appropriate kit (Sigma). However release of necrosis does not necessarily imply cardiac necrosis. Thus to verify cardiac necrosis, sections were evaluated histologically using Masson's Trichrome stain [25]. Briefly, ventricular tissue was fixed in 10% formalin, and sectioned at $5\ \mu\text{M}$. Sections were deparaffinized in xylene, rehydrated using various grades of ethanol and refixed in Bouins fluid for 1h at 56°C . Subsequently, sections were stained, dehydrated with acetone, cleared

with xylene, and mounted. Positive controls were generated by injection of isoproterenol (50 mg/kg; sc) to a normal chow fed rat, and the heart removed after 48 h [26].

6.2.5 Estimation Of GSH

Cardiac GSH content was measured from frozen, ground tissue using a commercially available kit (Cayman Chemicals). 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. Rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the total concentration of GSH in the sample. Oxidized glutathione (GSSG) was estimated using 2-Vinylpyridine [27]. The difference between total GSH and GSSG gave the value of the reduced GSH. Protein assay was performed according to the Bradford method. Values are expressed as nM GSH per mg protein.

6.2.6 ATP Assay

ATP from heart tissue was extracted using 2.5% TCA, pH adjusted to 7.8 by 1M KHCO₃ and ATP (nmol/mg protein) measured by a luciferase-luciferin assay kit (Sigma).

6.2.7 Electron Microscopy.

Morphological evaluation of hearts was carried out using transmission electron microscopy. Briefly, left ventricular tissue was fixed in 1.5% glutaraldehyde and paraformaldehyde, cut into small blocks (~1 mm³) and fixed for 8 h at 4°C. After washing, tissue was post fixed with 1% osmium tetroxide and further treated with 1% uranyl acetate and dehydrated using increasing concentrations of ethanol (50-100%). Blocks were embedded in molds, polymerized, and sectioned at ~100 nm. Sections were stained with 1% uranyl acetate and Reynolds lead citrate. Images of the longitudinal-sections were obtained with a Hitachi H7600 electron microscope.

6.2.8 Heart Function And Substrate Oxidation

Hearts from halothane (2-3%) anesthetized rats were perfused in the working mode with modified Krebs-Henseleit buffer (including 1.0 mM [9,10-³H] palmitate pre-bound to 3% BSA, 5.5 mM [U-¹⁴C] glucose, 2.0 mM calcium, and 100 U/L insulin.) at a preload of 11.5 mmHg as described previously [28]. An afterload of 80 mmHg was kept for the first 30 min. During this time, samples of perfusate and hyamine hydroxide were taken every 10 min for measurement of glucose and FA oxidation. Subsequently, the afterload is clamped off causing the peak systolic pressure to increase to ~135 mmHg, and the perfusion continued for another 20 minutes. Heart function [rate pressure product (RPP)-heart rate, [mmHg. min⁻¹. 10⁻³] was measured using a Direcwin physiograph (Raytech).

6.2.9 Serum Measurements

Serum samples were stored at -20°C until assayed. Diagnostic kits were used to measure glucose (Sigma), insulin and leptin (Linco) and non-esterified fatty acids (Wako).

6.2.10 Statistical Analysis.

Values are means ± SEM. One-way ANOVA followed by the Tukey or Holm-Sidak test was used to determine differences between group mean values. The level of statistical significance was set at $P < 0.05$. Statistical analysis

6.3 Results

6.3.1 General Characteristics Of Experimental Animals

Table 6.2 describes the general characteristics of the experimental groups. There was no difference in food intake (data not shown) between rats fed ω -6 PUFA diet or normal chow for 4 weeks. However, ω -6 PUFA feeding increased body weight compared to normal chow. When compared to fed NC rats, PC animals had higher circulating TG and increased serum insulin (likely as a consequence of insulin resistance), but no overt hyperglycemia. STZ precipitated loss of circulating insulin and caused overt hyperglycemia in both ND and PD groups. Interestingly, unlike ND (pre-STZ, 361 ± 9 ; post-STZ, 358 ± 9 , g), diabetes in PUFA animals was associated with a profound loss of body weight (pre-STZ, 450 ± 11 ; post-STZ, 393 ± 11 , g). This loss in body weight could not be attributed to any change in food or fluid intake but could be a result of excessive lipolysis and loss of adipose tissue mass with subsequent increases in both serum FFA and TG in PD animals. Additionally, dietary ω -6 PUFA for 4 weeks increased serum leptin levels compared to the NC group, whereas diabetes reduced leptin in both ND and PD groups. ω -6 PUFA feeding induced a 6-7 fold increase in total cardiac FFA and a 40-80 fold increase in total cardiac TG in both PC and PD groups, and indicated a cardiac lipid overload in these animals..

6.3.2 Free PUFA Content In The Heart

Although several fatty acid classes (e.g., saturated and monounsaturated FA) were measured, only the cardiac PUFA content in the free form are described in Fig. 6.1 (as per the focus of this study). Feeding 20% w/w sunflower oil successfully magnified both ω -6 (mainly linoleic and arachidonic acid) and ω -3 (mainly eicosapentaenoic acid and docosahexaenoic acid) PUFA content in the heart. However, the increase in ω -6 PUFA was substantially greater than the

increase in ω -3 PUFA, and was reflected in an approximately 2 fold higher ω -6/ ω -3 PUFA ratio in the PC group. Diabetes had no further effect on either the ω -6 or ω -3 PUFA content.

6.3.3 Cardiac Cell Death

Previous studies have associated cardiac lipid overload to cell death via apoptosis. To determine cardiac apoptosis following ω -6 PUFA feeding, caspase-3 activity, crucial for myocardial apoptosis, was estimated and determined to be unchanged in both PC and PD groups compared to NC (Fig. 6.2). In ND, the caspase-3 like activity was almost 2.5 fold higher than control and indicates acceleration of apoptotic cell death, as has been previously described [4]. As apoptosis can proceed in a caspase-3-independent manner, TdT mediated dUTP nick end labeling (TUNEL) on myocytes was also performed and found to be increased in ND but insignificant in PD hearts (data not shown).

Absence of apoptosis may not signify lack of cell death as cell demise may also progress via a necrotic pathway. Incidence of necrosis by measurement of serum LDH revealed that only PD had higher circulating levels of this enzyme marker (Fig. 6.3, bottom right). However, as serum LDH alone does not substantiate the incidence of cardiac necrosis, Masson's Trichrome staining was performed on cardiac ventricles [25]. In hearts treated with isoproterenol, a β -agonist, severe disruption of the contractile apparatus (solid arrow; Fig. 6.3E) together with focal necrosis (indicated by grayish discoloration instead of the more usual deep red coloration demonstrated by intact muscles) (hollow arrow; Fig. 6.3E) was observed. No such morphology was evident either in NC, ND or PC hearts (Fig. 6.3, A, B or C; magnification, 400X). Following diabetes, PD hearts showed necrotic changes, which on higher magnification (600X) demonstrated extensive grayish patches and focal necrosis (hollow arrow; Fig. 6.3D) with hypercontracted muscle fibers (solid arrow; Fig. 6.3D) characteristic of contraction band necrosis [29].

6.3.4 Cardiac GSH

Factors like GSH levels, which dictate the redox environment of the cell and also the mode of cell death [30], were also measured. PUFA feeding was associated with a significant decrease in GSH. Interestingly, diabetes for 4 days in normal chow fed rat hearts could not decrease cardiac GSH, whereas superimposition of diabetes in PUFA fed animals led to a further decrease in cardiac GSH levels (Fig. 6.4, top panel). Although cardiac GSSG was marginally increased in ND compared to NC hearts, this difference did not reach significance. PUFA feeding had no effect on GSSG. However, induction of diabetes in PD animals increased cardiac GSSG almost 3-fold (Fig. 6.4, middle panel). Evaluation of the GSH/GSSG ratio revealed that PD hearts had the lowest values, indicating substantial oxidative stress in this group (Fig. 6.4, lower panel) [31].

6.3.5 Cardiolipin And Mitochondrial Morphology

Cardiolipin is a mitochondrial membrane phospholipid that is essential for optimal functioning of oxidative phosphorylation enzyme complexes, and ATP generation [32]. Diabetes in both normal chow and high fat groups did not alter cardiolipin levels compared to the respective controls (Fig. 6.5, top panel). ω -6 PUFA feeding induced a profound loss of cardiac cardiolipin levels (Fig. 6.5, top panel) together with a drop in total ATP (NC 8.9 ± 0.6 , ND 8.1 ± 0.8 and PC 6.9 ± 0.6 vs. PD 5.1 ± 0.2 ; nmol ATP/mg protein; $P < 0.05$). As changes in cardiolipin have also been associated with mitochondrial structural alterations, we scrutinized mitochondrial morphology in all of the above groups. Figure 6.5 (lower panel, A) depicts a mitochondrion with double membrane and lamellar cristae that are typical in NC, ND or PC hearts. A novel observation in this study was that abnormal condensed mitochondria were observed, but only in the PD group (Fig. 6.5, lower panel, B).

6.3.6 Glucose And Palmitate Oxidation

As condensed mitochondria suggests lack of energy production, glucose and palmitate oxidation (Fig. 6.6) in the isolated heart were studied in parallel with heart function. At low afterload (80 mm Hg), rates of oxidation of exogenous glucose decreased in ND and PC with a parallel increase in palmitate oxidation in these groups. Interestingly, in PD hearts, although glucose oxidation dropped further compared to both ND and PC hearts, palmitate oxidation remained unchanged. To meet the energy demand at high afterload (135 mm Hg), all groups except PD increased their glucose oxidation. Regarding FA oxidation, only NC was able to increase palmitate oxidation suggesting that in the ND, PC and PD groups, FA oxidation was already operating at its maximum at low afterloads.

6.3.7 Electron Microscopy And Ultrastructural Morphology

In the milieu of increased intracardiac fatty acid levels (Table 6.2) and in the absence of any further increase in FA oxidation, we hypothesized that intracardiac TG will build up in PD hearts. Figure 6.7 (a-d) demonstrates ultrastructural morphology of hearts isolated from the different groups. Expectedly, although lipid droplets were visible in both ω -6 PUFA fed groups, only PD hearts demonstrated abundant lipid-like vacuoles (L). Additionally, NC, ND and PC hearts showed highly packed myofibrils separated by rows of mitochondria. Myofibrillar sarcomeres in these two groups exhibited clear Z-lines (indicated by hollow arrows) whereas PD hearts demonstrated more diffuse Z-lines indicating myofibrillar damage (indicated by hollow arrows). These findings are consistent with necrosis present in the PD hearts (Fig. 6.3), and could precipitate an early contractile defect.

6.3.8 Estimation Of Heart Function

Cardiac mechanical function measured as rate pressure product (RPP) of the different groups is summarized in Fig. 6.8. There were no differences in RPP between the groups at normal workload (80 mmHg). Subsequent to an increase in workload to 135 mmHg, RPP increased in

all groups. Compared to NC, neither fat feeding nor STZ-diabetes affected heart function in response to this high workload. Interestingly, superimposition of diabetes for 4 days in PD rats induced the least increase in cardiac function.

6.4 Discussion

Numerous clinical and epidemiological reports have confirmed that human diabetics are particularly susceptible to cardiomyopathy, independent of vascular disease and recent evidence points towards cell death as a potential contributing factor [1]. Indeed, in a recent study, we detected a 5-fold increase in apoptosis in the diabetic heart using TUNEL assay, and further validated this observation by identifying an increase in caspase-3 activity, the proteolytic enzyme involved in the execution of apoptosis [10]. In this study, an increase in cardiac caspase-3 activity was also observed in ND rats. Despite this augmented apoptosis in the diabetic heart, the number of cells affected in this and other studies [3, 4] is relatively minor and may explain the lack of functional changes at this early time point. However, given that apoptotic cells are cleared rapidly by phagocytes [23], and in the absence of any significant regeneration of the adult myocardium, low cell death rates over chronic periods of hyperglycemia may still result in progressive loss of myocytes and contractile failure [23].

Interestingly, compared to ND, superimposition of diabetes in 20% w/w PUFA fed rats did not change caspase-3 activity or TUNEL positive myocytes, and questions the prevalence of apoptosis in this group. At least in cell lines, ω -6 PUFA is also known to block apoptosis [16, 17]. Overall, our data suggest that rather than the amount; it is the type of FA that controls lipotoxic apoptosis [33]. Indeed, feeding a 20% w/w palm oil diet [rich in palmitic acid that is converted to pro-apoptotic ceramide [33]] increases apoptosis in control hearts, a process that was further aggravated following diabetes [10].

Although apoptosis is the predominant form of cellular demise, the diabetic heart is also characterized by necrosis, an 'accidental' form of cell death [3]. Assessment of serum markers or histochemical detection of necrosis did not reveal any necrotic change in NC, ND or PC hearts. However, PD animals demonstrated a rise in serum LDH, and severe cardiac necrosis as evidenced by light microscopy and appropriate staining. Furthermore, ultrastructural

evidence demonstrated diffuse Z-lines suggesting myofibrillar damage in PD hearts, indicative of necrotic changes. In an effort to determine the elements that induce necrosis, we measured the cardiac FFA species and determined that sunflower oil feeding increased cardiac ω -6 PUFA, predominantly linoleic acid. In clinical studies, accumulation of linoleic acid has been strongly associated with an increased inflammatory response in endothelial cells, in addition to an augmented risk of myocardial infarction and necrosis [34]. Additionally in vitro, linoleic acid precipitates necrosis via pro-inflammatory pathways in various cell types [35, 36].

Oxidative stress has also been implicated in the etiology of several lipotoxic and diabetic complications [2, 8], and can arise as a result of an imbalance between the production of free radicals and its neutralization by antioxidants like GSH [2, 31]. In ventricular myocytes, under conditions of oxidative stress, GSH is converted to GSH disulfide (GSSG) and the GSH/GSSG ratio decreases (i.e., a high GSH/GSSG ratio maintains a normal reducing intracellular environment). Interestingly, ω -6 PUFA feeding induced a loss of reduced GSH that decreased further with diabetes, an effect that could be directly linked to an increased conversion of reduced GSH to GSSG under conditions of augmented oxidative stress [31]. A major functional impact of a decreased GSH/GSSG ratio is oxidation of regulatory proteins at cysteine residues [30, 31]. For caspase activation and propagation of the apoptotic pathway, an intact cysteine moiety is necessary, that can easily be oxidized under increased oxidative stress and GSH depleted conditions [37]. Overall, our data suggests that increased cardiac ω -6 PUFA content and depleted cardiac GSH operate in concert to allow a 'switch' to necrosis in PD hearts.

Compromised myocardial energy status may also dictate necrotic death. Cardiolipin, a mitochondrial membrane phospholipid essential for optimal functioning of oxidative phosphorylation enzyme complexes and ATP generation [32], was estimated along with total

myocardial ATP levels. In myocardial ischemia, disappearance of total cardiolipin resulted in loss of OXPHOS activity [38] and could explain the drop in mitochondrial ATP synthesis. Total cardiolipin decreased almost 6-fold following ω -6 PUFA feeding with a significant drop of ATP only in PD hearts, that could have contributed towards cardiac necrosis [39]. Changes in cardiolipin have been associated with mitochondrial morphological alterations. In Barth Syndrome, a genetic disorder characterized by dilated cardiomyopathy, a lack of cardiolipin in mitochondria has been described together with abnormal mitochondria with concentric cristae [40]. A novel observation in this study was that analogous to Barth Syndrome, ω -6 PUFA feeding for 4 weeks together with 4 days of hyperglycemia led to similar changes in some mitochondria. Interestingly, abnormal condensed mitochondria have been recently recognized in skeletal muscle and cardiac atrial neurons from diabetic patients [41]. Given that both control and diabetic PUFA groups showed similar changes in cardiolipin, it could be assumed that the altered mitochondrial morphology in PD animals is a consequence of an additional effect of hyperglycemia i.e., when hyperglycemia sets in, there is augmented oxidative stress and extensive damage to the mitochondria, already compromised by depleted cardiolipin levels [42].

Structural damage to the mitochondria is associated with derangements in fuel utilization, and could lead to cardiac contractile impairment. Compared to glucose, FA's are the preferred substrate consumed by the heart, providing approximately 70% of its energy requirements. With hypoinsulinemia or insulin resistance that characterizes ND and PC respectively, glucose utilization is compromised and the heart is forced to switch to using higher levels of FA [7]. Interestingly, PD animals, characterized by a combination of insulin resistance initially followed by eventual hypoinsulinemia after STZ, demonstrated a precipitous drop in cardiac glucose utilization with no further increase in FA oxidation. Despite these changes in substrate preference, heart function at low afterloads remained unchanged. At higher afterloads, both

glucose and FA oxidation increased in the NC group. In ND and PC hearts, the only increase observed was that of glucose oxidation as presumably, FA oxidation was already operating at its maximum. PD was the only group that failed to increase its glucose oxidation in response to a higher energy demand, an aspect that could have contributed to the drop in cardiac function. Interestingly, when diabetic hearts are perfused with both glucose and FA, addition of dichloroacetate, a stimulator of glucose oxidation to the perfusion buffer improved heart function [43]. More recently, shifting substrate utilization from FA to glucose using trimetazidine improved left ventricular function in patients with diabetes and ischemic cardiomyopathy [44], and in transgenic db/db mice that overexpress GLUT-4 glucose transporters, glucose metabolism and contractile function were normalized [45]. Our study provides novel evidence that unlike other models of diabetic cardiomyopathy that exhibit cardiac dysfunction only after chronic hyperglycemia, ω -6 PUFA feeding coupled with only four days of diabetes can precipitate metabolic and contractile abnormalities in the heart.

The heart and other non-adipose tissues have inadequate ability to handle excess lipids and accumulation of TG has been proposed to impair cardiac function. [5, 46]. In our study, ω -6 PUFA feeding substantially increased cardiac FA in both PC and PD groups, but only PD hearts demonstrated a considerable increase in lipid droplets. Given that fatty acid oxidation is likely operating at maximum in PC hearts, the excess circulating fatty acids in PD is likely channeled towards TG synthesis and could explain the increase in lipid droplets in these hearts. Interestingly, in Zucker fatty rats, when glucose utilization is compromised (as during fasting), increased lipid availability is associated with cardiac lipid accumulation [46]. At present it is unclear why PD hearts were unable to increase its fatty acid oxidation even in the presence of excess fatty acids. In this regard, leptin, a liporegulatory hormone increases FA entry and TG storage in adipose tissue, but activates fatty acid oxidation and decreases TG accumulation in

the heart [47]. Although leptin was high in PC animals, PD animals demonstrated low insulin and leptin levels, factors that could have contributed towards FA being channeled from oxidation to increased cardiac TG assembly.

It should be noted that although ω -6 PUFA rich diet was utilized as a beneficial regimen in this study, sunflower oil per se induced obesity and insulin resistance like other saturated fat diets. However, insulin resistance, hyperinsulinemia and impaired glucose tolerance that characterize obese humans, is usually associated with transition to frank diabetes occurring when secretion of insulin is no longer able to overcome insulin resistance and impaired glucose disposal. In rodents fed high fat diets, insulin resistance does not progress to hyperglycemia in the absence of genetic defects [20]. Thus, our current model in which a low dose of STZ was used to precipitate overt hyperglycemia following insulin resistance is a close approximation of the human condition where there is a gradual evolution of insulin resistance to hyperglycemia.

In summary, chronic caloric excess of ω -6 PUFA when coupled with acute diabetes of only 4 days precipitated mitochondrial abnormalities, a steep drop in GSH, altered substrate utilization and myocardial TG deposition. Given that these hearts also demonstrated necrosis and extensive myocardial cell loss, a feature that is predominant only in chronic diabetes [3, 4, 6, 48], our data suggests that this mode of cell death in PUFA fed diabetic hearts is an important factor in accelerating diabetic cardiomyopathy. Although these effects of ω -6 PUFA in the diabetic animal would seem contrary to accepted belief as being beneficial, in countries like Israel, with high dietary ω -6 PUFA consumption, there is an excessive incidence of obesity, insulin resistance, hypertension, and Type 2 diabetes [49]. Thus, the dilemma with recommending vegetable oils like sunflower oil as a source of PUFA is that it contains high levels of ω -6 PUFA but negligible amounts of ω -3 PUFA, the primary candidate for conferring cardiovascular benefit. Given the concurrent scarcity of fish oil in the Western diet, the main source of ω -3 PUFA, the ω -6 / ω -3 PUFA ratio has amplified from a traditional balance of 1:1

to an astounding 15:1 to 20:1 in the modern diet [50] and thus advocating diets rich in ω -6 PUFA to diabetic patients could accelerate the impairment of myocardial contractility.

6.5 Tables and Figures

Table 6.1 Composition of experimental diets

<i>Caloric Composition</i>	PUFA diet	Normal diet
	g%	g%
Protein	24.1	23.4
Carbohydrate	44.9	49.9
Fat	20.7	4.5
Fibre	6.0	8.3
kcal/g	4.6	4.0
<i>Fatty Acids (g%)</i>		
Saturated	1.9	1.5
Monounsaturated	3.8	1.6
ω-6 PUFA	10.6	1.2
ω-3 PUFA	0.1	0.3

Ingredients

PUFA Diet: Casein, 80 mesh, DL-Methionine, LoDex 10, Corn Starch, Sucrose, Cellulose, BW200, Corn Oil, Sunflower Oil, Mineral Mix,[†] Vitamin Mix,[‡] Choline Bitartrate

Normal Diet: Ground corn, soybean meal, beet pulp, fish meal, wheat, cane molasses, ground oats, Mineral Mix,[†] Vitamin Mix[‡]

[†]**Mineral Mix-** Di-Calcium Phosphate, Magnesium Oxide, Potassium citrate, Potassium Sulfate, Sodium Chloride, Chromium KSO₄.12H₂O, Cupric Carbonate, Potassium Iodate, Ferric Citrate, Manganous Carbonate, Sodium Selenite, Zinc Carbonate

[‡]**Vitamin Mix-** Vitamin A, Vitamin D₃, Vitamin E, Menadione Sodium bisulfite, Biotin1%, Cyancocobalamin 0.1%, Folic Acid, Nicotinic Acid, Calcium Pantothenate, Pyridoxine-HCl, Riboflavin, Thiamin HCl

Table 6.2 General characteristics of experimental animals

	<i>NC</i>	<i>ND</i>	<i>PC</i>	<i>PD</i>
Body weight (g)	381 ± 9	358 ± 8.7	485 ± 14 [†]	393 ± 11*
Cardiac FFA (µg/mg protein)	1.9±0.3	1.7±0.2	13.9±1 [†]	15.5±1 [†]
Cardiac TG (µg/mg protein)	0.01 ± 0.01	0.02 ± 0.0	0.5 ± 0.2 [†]	0.8 ± 0.2 [†]
Serum parameters				
Glucose (mmol/l)	8.6 ± 0.2	24.2 ± 0.8 [†]	11.1 ± 0.5	23.8 ± 0.5* [†]
FFA (mmol/l)	0.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	1.8 ± 0.8 [†]
Triglyceride (mmol/l)	0.8 ± 0.1	1.2 ± 0.2	4.5 ± 1.2 [†]	11.8 ± 2.9 [†]
Insulin (ng/ml)	2.8 ± 0.4	0.8 ± 0.3 [†]	5.5 ± 1.1 [†]	1.5 ± 0.6*
Leptin (ng/ml)	8.0± 0.8	1.9 ± 0.4 [†]	12.7± 1.6 [†]	1.2± 0.2* [†]

Values are means ± SE for 6 animals in each group. Animals were maintained on normal chow (NC) or a ω-6 PUFA (PC) rich diet for 4 weeks prior to diabetes induction (55 mg/kg STZ). Diabetic animals (ND, PD) were kept for 4 days before being killed. Values are those obtained before death and were from fed animals. *Significantly different from PC, $P < 0.05$. [†]Significantly different from NC, $P < 0.05$. ‡Significantly different from all groups, $P < 0.05$; FFA, free fatty acids; TG, triglycerides.

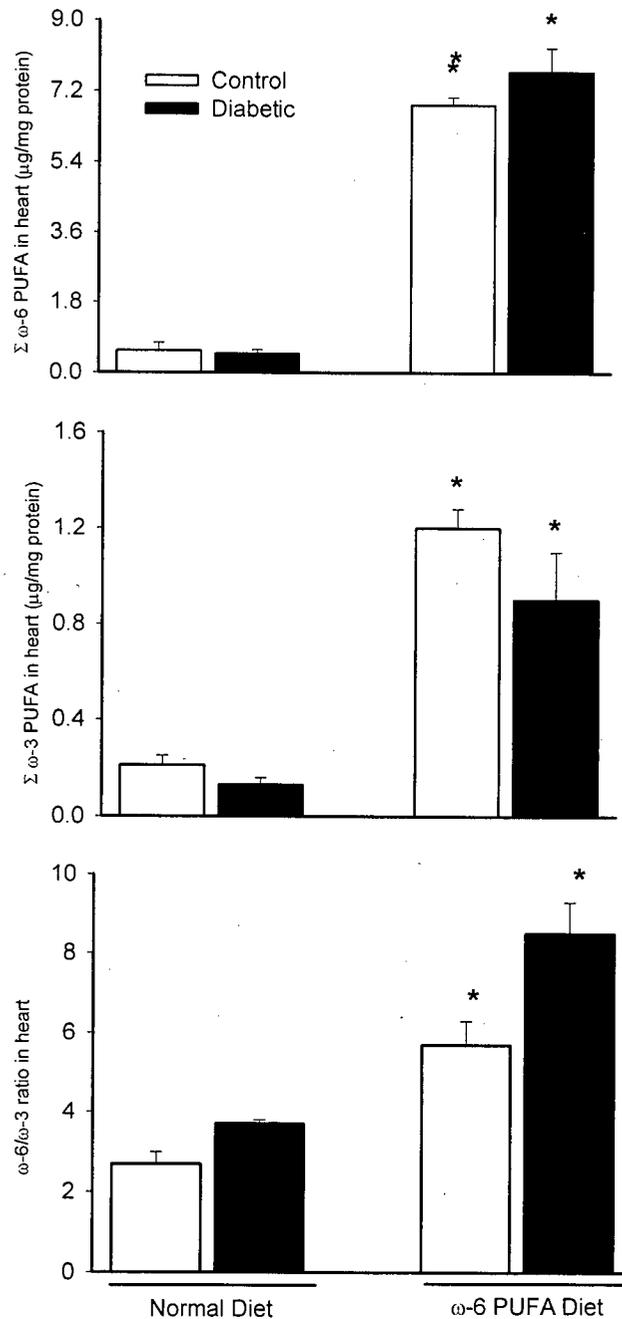


Fig. 6.1 *Effects of sunflower oil feeding and diabetes on cardiac PUFA levels* Cardiac FFA's were extracted with chloroform:methanol:acetone:hexane solvent and were converted to their respective methyl esters, and separated by gas chromatography. Results are the means \pm SE of 4 rats in each group. Top panel: Total cardiac ω -6 PUFA levels (mainly linoleic and arachidonic acid). Middle Panel: Total cardiac ω -3 PUFA levels (mainly eicosapentaenoic acid and docosahexaenoic acid). Lower panel: Ratio of ω -6 and ω -3 PUFA in the heart. *Significantly different from normal chow fed groups; $P < 0.05$.

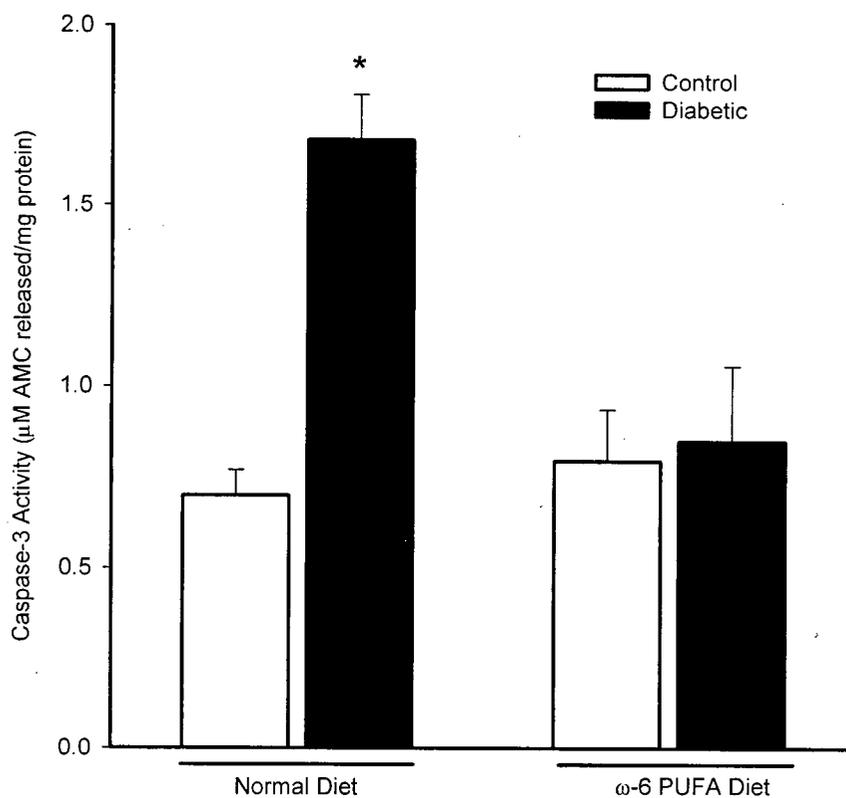


Fig.6.2 *Quantitative estimation of cardiac caspase-3 activity* Heart tissue was placed in cell-lysis buffer and homogenized. Subsequently, the suspension was centrifuged, supernatant separated and reacted with Z-DEVD-AMC substrate. Results are the means \pm SE of 4 rats in each group. *Significantly different from all other groups; $P < 0.05$.

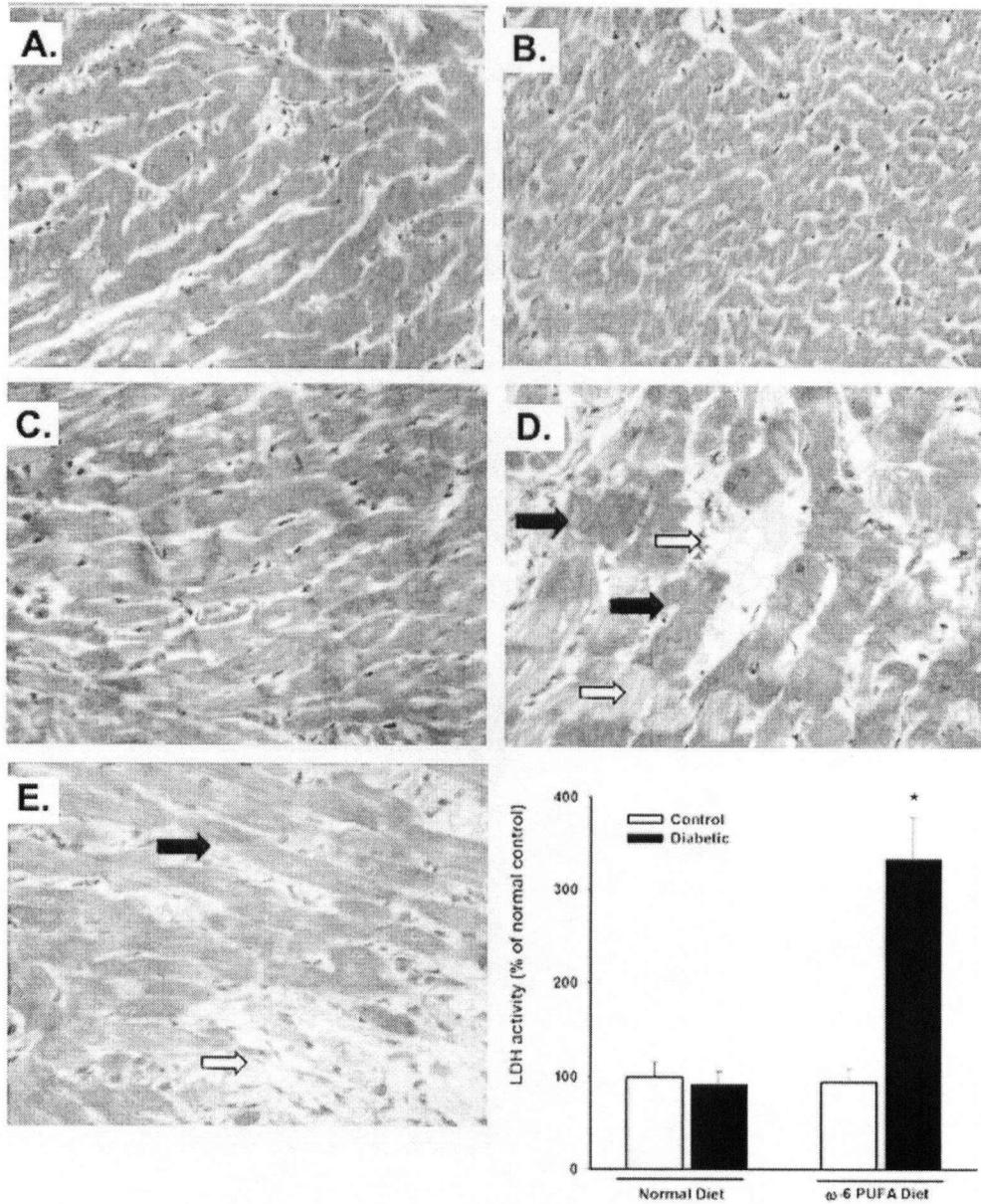


Fig. 6.3 Effect of 4 days of diabetes on cardiac cell death in animals fed a moderately high PUFA diet for 4 weeks. Heart sections were treated with Masson's Trichrome stain. Solid arrows depict severe disruption of the contractile apparatus (hypercontracted muscle and thickened fibres) whereas the hollow arrows indicate focal necrosis (indicated by grayish discoloration) in positive control (+Isoproterenol; E, 400x) and PD hearts (D, 600x). Cardiac necrosis was absent in NC (A, 400x), ND (B, 400x) and PC (C, 400x) hearts. Bottom right panel: Serum was collected at death and LDH activity measured with an appropriate kit. Results were expressed as a percentage of normal chow fed controls and are the means \pm SE of 4 rats in each group. *Significantly different from all other groups; $P < 0.05$.

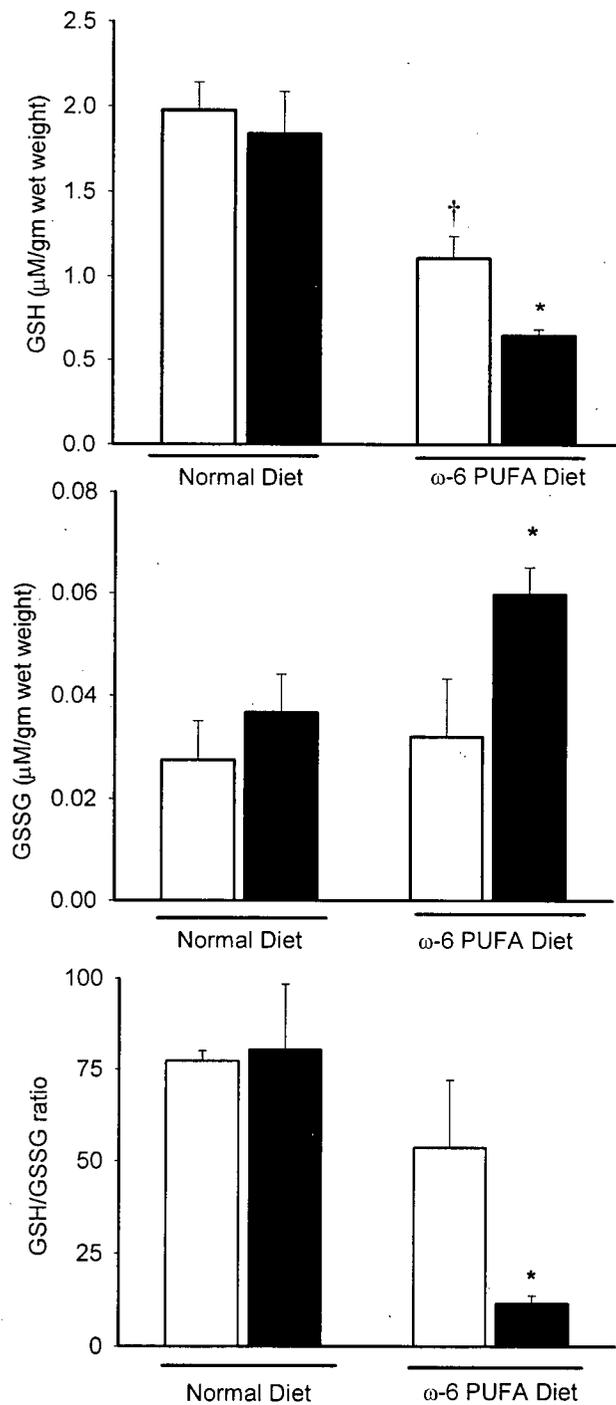


Fig. 6.4 Status of myocardial glutathione system as estimated by enzyme-recycling assay. Upper panel: Reduced GSH. Middle panel: Oxidized GSSG. Lower panel: Cardiac GSH/GSSG ratio. Results are the means \pm SE of 4 rats in each group. †Significantly different from normal chow fed control, *Significantly different from all groups. $P < 0.05$.

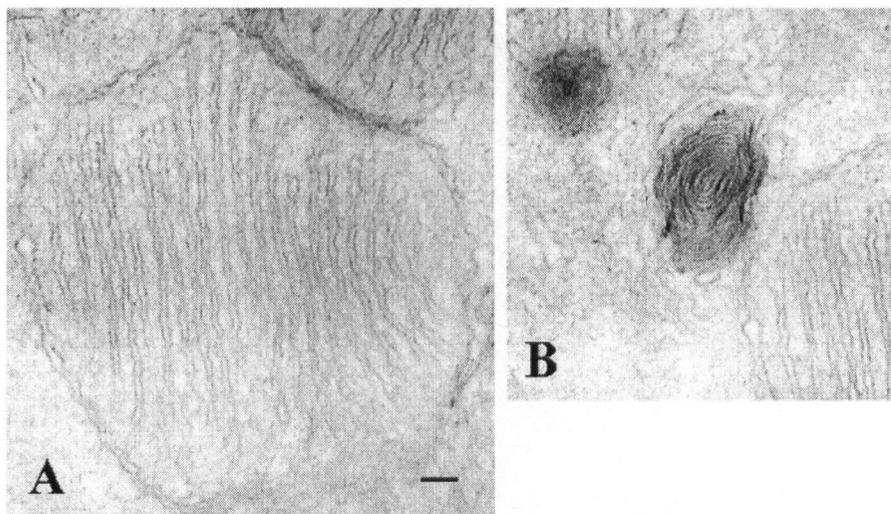
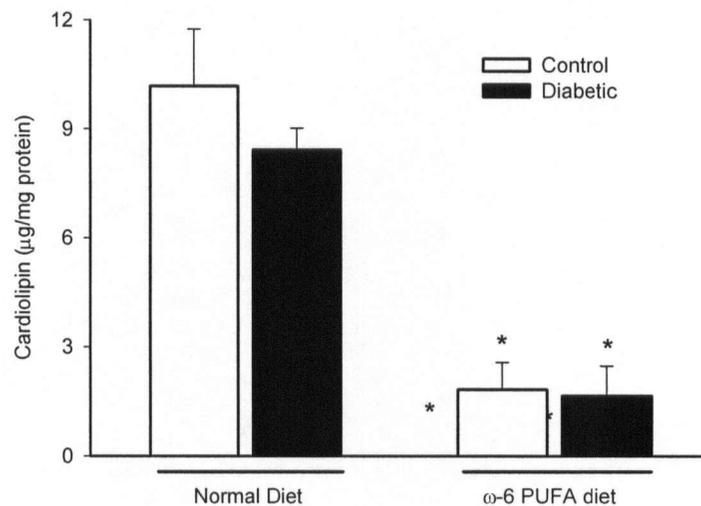


Fig. 6.5. *Biochemical and ultrastructural changes in cardiac mitochondria following PUFA feeding and diabetes* Top Panel: Separation of total cardiolipin was achieved by HPLC. Results are the means \pm SE of 4 rats in each group. *Significantly different from normal chow fed groups, $P < 0.05$. Lower Panel: Representative electron micrographs of myocytes showing normal (A) and condensed (B) mitochondrion. Some condensed mitochondria were only observed in PD hearts. The scale bar for the micrograph in A (100 nm) also apply to B.

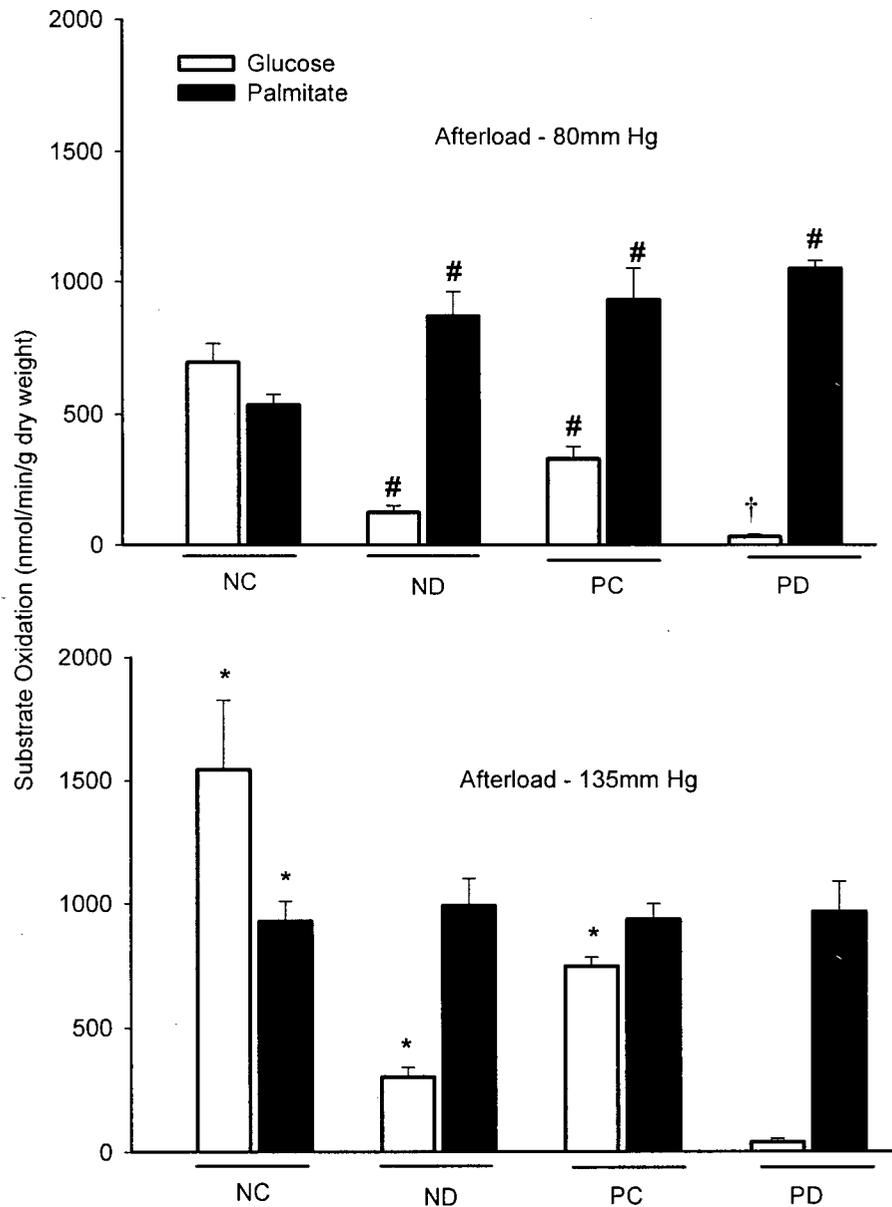


Fig. 6.6. *Glucose and palmitate oxidation at low (top panel, 80 mm Hg) and high (lower panel, 135 mm Hg) afterloads* During measurement of heart function, samples of perfusate and hyamine hydroxide were taken every 10 min for measurement of substrate oxidation. Results are the means \pm SE of 4 rats in each group. Top panel: [#]Significantly different from NC; [†]Significantly different from all groups. Lower panel: Comparison was only made between results obtained at high and low afterloads, ^{*}Significantly different from low afterload; $P < 0.05$.

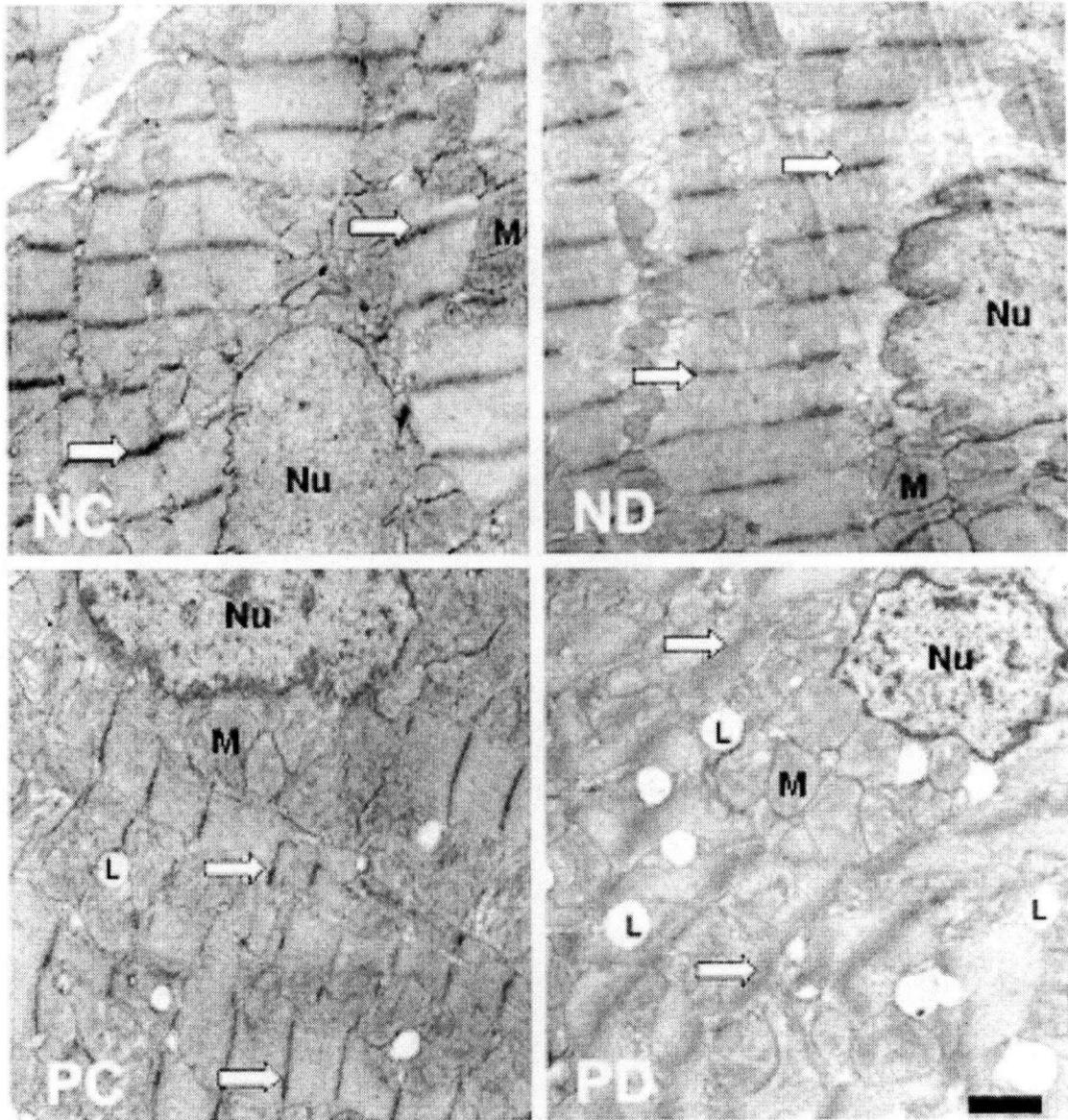


Fig. 6.7 *Evaluation of ultrastructural cardiac morphology* A representative electron micrograph of hearts from NC , ND, PC and PD animals are depicted. The scale bar on PD represents 1 μ m. M, mitochondria; Nu, nucleus; L, lipid like vacuoles. Hollow arrows indicate Z-lines.

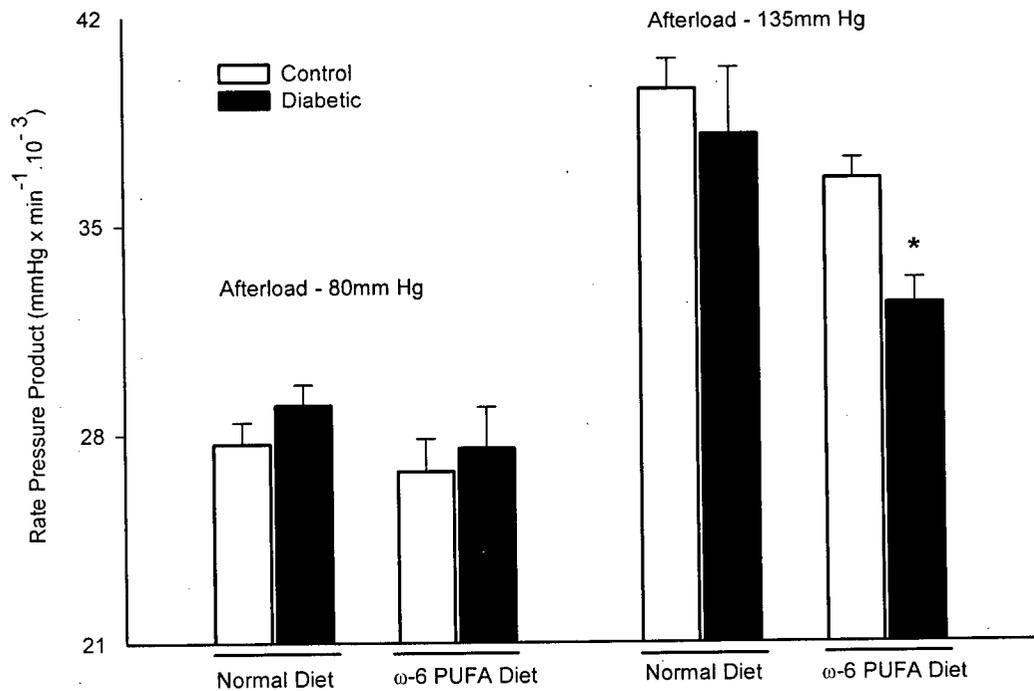


Fig.6.8 *Cardiac function at low (80 mm Hg) and high (135 mm Hg) afterloads.* Isolated hearts were perfused in the working mode at an afterload of 80 mmHg for the first 30 min. Subsequently, the afterload is clamped off causing the peak systolic pressure to increase to ~135 mmHg, and the perfusion continued for another 20 minutes. Results are the means \pm SE of 4 rats in each group. RPP, rate pressure product. *Significantly different from all other groups at afterload ~135 mmHg, $P < 0.05$

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7. Chronic Provision Of Dietary ω -6 PUFA Induces Mitochondrial Nitrosative Damage And Cardiac Dysfunction

7.1 Introduction

Obesity, a common disorder in the Western world, increases the incidence of diseases like hypertension and diabetes. In the last few decades, heightened awareness of obesity and its complications has led to an indiscriminate substitution of atherogenic saturated cooking fats with vegetable oils containing ω -6 polyunsaturated fatty acids (PUFA) [1]. In addition to this intentional modification of human dietary intake, farmed animals are fed grains and seeds containing high amount of ω -6 PUFA like linoleic and arachidonic acid [2]. Consumption of these animals (and their products) has led to an even greater increase in dietary ω -6 PUFA. Thus, intake of linoleic acid in the US and Western Europe has risen from <3% of energy in the 1950s to the current 6–7%, with a parallel decrease in saturated fats [3]. . A few populations (Northern Belgium and Israel) consume even higher proportions of linoleic acid (8–12% of their total energy) [3]. Additionally, following increased ω -6 PUFA consumption, competition for desaturase enzymes reduces the biosynthesis of the beneficial ω -3 PUFA like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [1]. Finally, a reduction in dietary fish oils, a rich source of ω -3 PUFA, has also been documented. Taken together, the present human dietary ω -6: ω -3 PUFA ratio has increased from a balanced 1:1 to an unexpected 15:1 to 20:1 in the current Western diet [1]. Given that the anti-arrhythmic and hypocholesterolemic effects of PUFA have mainly been attributed to ω -3, excess ω -6 PUFA may not provide the desired cardiovascular benefits [1, 4]. In fact, a number of recent studies have identified ω -6 PUFA to be pro-inflammatory, with effects ranging from smooth muscle

proliferation to oxidative endothelial injury, mitochondrial damage and activation of transcription factors in the vasculature [5-8]

The majority of studies that have evaluated the effects of ω -6 PUFA on cell systems used high levels of individual fatty acids like linoleic or arachidonic acid [5-7]. Little attempt was made to evaluate these effects in the presence of low ω -3 PUFA, which would mimic the current Western diet. Additionally, although widely studied with respect to coronary vascular disease, the consequences of increased ω -6 PUFA on the heart muscle itself, are largely unknown. In a recent study, we reported that in diabetic rats fed 20% w/w sunflower oil (which increases the cardiac ω -6: ω -3 PUFA ratio), abnormal mitochondria and decreased ATP levels were evident [9]. In these hearts, cardiac damage was characterized by necrosis, a process of cell death that requires extensive mitochondrial damage and loss of ATP [10, 11]. Interestingly, in experimental models of diabetes fed normal chow (with a balanced ω -6: ω -3 PUFA ratio), or saturated fats, cardiac damage is usually characterized by an increase in apoptosis, a process of programmed cell death characterized by transient mitochondrial permeability changes [10, 11]. Thus, one of the key 'switches' that converts apoptotic to necrotic cell death is persistent mitochondrial damage [10, 11]. Interestingly, simply feeding 20% w/w sunflower oil to control rats led to an extensive loss of cardiolipin, a mitochondrial phospholipid [9]. Depletion of cardiolipin is not only associated with loss of oxidative phosphorylation, but also extensive generation of free radicals [12]. We hypothesized that chronic dietary ω -6 PUFA can induce free radical generation, predisposing the cardiac mitochondria to oxidative damage. In this study, we demonstrate that ω -6 PUFA feeding for 4 weeks, in addition to inducing loss of cardiolipin, can precipitate mitochondrial nitrosative stress, DNA damage and lead to cardiac dysfunction at high workloads.

7.2 Materials And Methods

7.2.1 Experimental Animals

The investigation conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the University of British Columbia. Previous studies have established that feeding 20% w/w sunflower oil for 4 weeks increases the ω -6 PUFA content of the heart [9, 13]. To investigate the cardiac effects of this increased ω -6 PUFA, male Wistar rats (220-240 g) were fed a diet [(AIN)-76A; Research Diets Inc.] supplemented with 20% w/w sunflower oil (High PUFA; HP) for 4 weeks. The composition of the HP diet in g% was as follows: protein, 24.1; carbohydrate, 44.9; fats, 20.7; fiber, 6.0; kcal/g, 4.6; total ω -6 PUFA, 10.6; total ω -3 PUFA, 0.3. In this diet, the energy contribution from ω -6 PUFA was 10.6 g%, and is reflective of the human diets in North America and Europe (around 5-10% of the total energy intake) [14, 15]. A control group fed normal laboratory chow [5% corn oil; Low PUFA, LP] (PMI Feeds) was utilized to compare the effects of ω -6 PUFA feeding. The composition of the LP diet in g% was as follows: protein, 23.4; carbohydrate, 49.9; fats, 4.5; fiber, 8.3; kcal/g, 4.0; total ω -6 PUFA, 1.2; total ω -3 PUFA, 0.1. Water was provided ad libitum. Following 4 weeks of feeding the two diets, glucose was measured using a glucometer from blood obtained from the tail vein (Advantage Comfort, Roche Diagnostics). Subsequently, the rats were anesthetized with sodium pentobarbital (65 mg/kg, i.p.), the thoracic cavity opened, and hearts removed.

7.2.2 Separation And Characterization Of Cardiac Lipids

Total cardiac lipids were extracted and solubilized in chloroform:methanol:acetone:hexane (4:6:1:1v/v/v/v). Separation of cardiolipin, lysophosphatidylcholine and free fatty acids was achieved using HPLC (Waters 2690 Alliance HPLC, Milford, MA) equipped with an auto-sampler and column heater, as previously described [9, 13]. Individual fatty acids were

quantified as their respective methyl esters using heptadecaenoic acid (17:0) as the internal standard with a Varian 3400 GLC equipped with a flame ionization detector, a Varian Star data system, and a SP-2330 capillary column (30 m x 0.25 mm internal diameter) (Supelco, Bellefonte, PA). Value of cardiac FA, cardiolipin and lysophosphatidylcholine were expressed as $\mu\text{g}/\text{mg}$ protein.

7.2.3 Total Phospholipase A₂ (PLA₂) Activity

After removal of the heart, the apex was cut, washed in cold PBS buffer, and homogenized in cold buffer containing 50mM HEPES and 1mM EDTA (pH 7.4). The sample was centrifuged at 10,000g for 15 mins, and supernatant separated. Supernatant corresponding to 50 μg of protein from each heart was then subjected to PLA₂ assay. Briefly, PLA₂ cleaves off fatty acids at the sn-2 position of glycerophospholipids to yield free fatty acid and lysophospholipid as by-products. Arachidonyl thio-phosphatidylcholine is a synthetic substrate that contains an arachidonyl thioester bond at the sn-2 position. Hydrolysis of this bond releases free thiol that is detected by DTNB [5,5'-dithiobis(2-dinitrobenzoic acid)] to give yellow-colored 5-thio-2-nitrobenzoic acid (λ_{max} 412nm) [16]. The sample was reacted with assay buffer (160 mM HEPES, 300mM NaCl, 20mM CaCl₂, 8mM Triton X, 60%glycerol and 2mg/ml BSA) and 1.5mM Arachidonyl thio-PC for 60 mins at room temperature. Bee venom PLA₂ was used a positive control. Finally, 10ul of 25mM DTNB/EDTA was added to develop color, which was measured spectrophotometrically at 405nm.

7.2.4 Separation Of Cytosolic And Particulate Fractions

Heart sections were immediately snap frozen in liquid nitrogen and stored at - 80°C. 50 mg of tissue was ground under liquid nitrogen, and homogenized in cytosolic buffer (20 mM Tris HCl, 250mM EGTA, 200mM EDTA, 100mM PMSF, 100mM NaF, 2-mercaptoethanol, leupeptin and aprotinin). After centrifugation at 35,000g for 1 hour at 4°C, the 'cytosolic

fraction' (supernatant) was isolated. The pellet containing all membrane bound fractions including nucleus and mitochondria were washed, recentrifuged and supernatant discarded. The pellet was resuspended in particulate buffer containing detergents (cytosolic buffer + NP-40, 10%SDS and 5%DCA) to rupture and dissolve all membranes. The mixture was centrifuged at 35,000g for 1 hour at 4°C, and the supernatant collected as the 'particulate fraction'. The purity of the fractions was estimated by performing immunoblotting for manganese superoxide dismutase (MnSOD; a mitochondrial protein) and copper/zinc SOD (Cu/Zn SOD; a cytosolic protein). No detectable signals for MnSOD or Cu/Zn SOD were present in the cytosolic or particulate fractions respectively. Protein content of the fractions was quantified using a Bradford protein assay.

7.2.5 Western Blotting Procedures

Either heart fractions or whole heart homogenates were diluted, boiled with sample loading dye, and samples corresponding to 50 µg protein were used in SDS-polyacrylamide gel electrophoresis. After transfer, nitrocellulose membranes were blocked in 5% skim milk overnight in Tris-buffered saline containing 0.1% Tween-20 (TBS-T). Membranes were incubated with antibodies raised in rabbit [inducible nitric oxide synthase (iNOS), peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1)], sheep [Cu/Zn superoxide dismutase (Cu/ZnSOD), Mn superoxide dismutase (MnSOD)], mouse (nitrotyrosine) or goat (p22^{phox}, p47^{phox}) for 2 hours at room temperature. Following 3x wash in TBS-T, the membranes were incubated for the next 2 hours at room temperature with secondary goat anti-rabbit, goat anti-mouse, donkey anti-sheep or donkey anti-goat HRP-conjugated antibodies, and visualized using ECL detection kit.

7.2.6 Immunofluorescence

Part of the left ventricle was fixed in 10% neutral buffered formalin, embedded in paraffin, and 5 μm sections prepared. Double immunostaining was performed for both nitrotyrosine and 8-hydroxyguanosine (8-OHG) [17]. For the first labeling, sections were deparaffinized in xylene, rehydrated using various grades of ethanol, and pretreated with 10 $\mu\text{g}/\text{ml}$ proteinase K (to permeabilize the nucleus) for 30 min at 37°C. Non-specific binding of immunoglobulins was blocked by incubating the sections in 10% BSA for 20 minutes. Nitrotyrosine immunolabeling was performed using a rabbit polyclonal anti-nitrotyrosine antibody (1:50, 2 hours at 37°C) (Molecular Probes, Oregon, USA) with Alexa Fluor 594[®] conjugated goat anti-rabbit IgG (Molecular Probes; 90 min at 37°C). For the second labeling, the same sections were incubated with primary goat anti-8-OHG antibody (Alpha Diagnostics) (1:200, overnight at room temperature), and secondary donkey anti-goat antibody conjugated with Alexa Fluor 488[®]. Sections were finally counterstained with DAPI (to visualize nuclei), and examined using confocal fluorescent microscopy. Slides were visualized using FITC, DAPI and Texas Red filters under a multiphoton confocal microscope.

7.2.7 Gene Expression

Gene expressions of mitochondrial respiratory subunits encoded by mitochondrial DNA (mtDNA) and 8-oxoG-DNA glycosylase (OGG-1), a mitochondrial oxidative DNA repair enzyme were measured using reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, total RNA from snap frozen hearts (100mg) was extracted using Tri-Zol (Invitrogen). After spectrophotometric quantification and resolving of RNA integrity using a formaldehyde agarose gel, reverse transcription was carried out using an oligo(dT) primer and superscript II RT (Invitrogen). cDNA was amplified using specific primers for NADH dehydrogenase subunit 1 (ND-1), cytochrome b (CYTb), cytochrome-c-oxidase subunit 1 (COX 1), ATPase 6

and OGG-1 (primers are listed in Table 1). 18S rRNA or β -actin was amplified as an internal control. The linear range was found to be between 15 and 30 cycles. The amplification parameters were set at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, for a total of 30 cycles. The PCR products were electrophoresed on a 1.7% agarose gel containing ethidium bromide and identified according to bands corresponding to specific base pairs (as per Table-1). Expression levels were represented as the ratio of signal intensity for target mRNA relative to either 18S or β -actin mRNA.

7.2.8 Cardiac Function

Hearts from halothane (2-3%)-anesthetized rats were perfused for 30 min with Krebs-Henseleit (KH) solution in the working heart mode at a preload of 11.5 mmHg, as previously described [18]. The KH solution contained 1.2mM palmitate prebound to 3% fatty acid-free albumin, 5.5mM glucose, 0.5mM lactate, 2.5mM calcium, and 100mU/l of insulin, and was continuously circulated through the closed perfusion system. A high concentration of palmitate was used to minimize differences in fatty acid oxidation, between HP and LP hearts. To ensure that glucose uptake was not limiting, a high physiological concentration of insulin was utilized. The solution was oxygenated with 95% O₂-5% CO₂ and maintained at 37°C throughout the perfusion. A pressure transducer (Viggo-Spectramed, Oxnard, CA) inserted in the afterload line was used to measure heart rate and peak systolic pressure. Cardiac output and aortic flow was measured via external flow probes (Transonic Systems, Ithaca, NY) on the preload and afterload lines, respectively. An afterload of 80mmHg was kept for the first 30 min. Subsequently, the afterload was clamped off causing the peak systolic pressure to increase to ~135 mmHg, and the perfusion continued for another 20 minutes [9].

7.2.9 Statistical Analysis.

Values are means \pm SE. Paired student's *t*-test was used to determine differences between group mean values. The level of statistical significance was set at $P < 0.05$.

7.3 Results

7.3.1 Cardiac Content Of ω -3 And ω -6 PUFA

Compared to LP fed animals, HP feeding for 4 weeks did not affect food or water intake (data not shown), body weight (LP, 431 ± 7 ; HP, 454 ± 23 ; g) or blood glucose (LP, 5.8 ± 0.4 ; HP, 6.4 ± 0.1 ; mmol). The cardiac PUFA content in the free form is described in Table 7.2. Feeding 20% w/w sunflower oil successfully magnified both the ω -6 (mainly linoleic and arachidonic acid) and ω -3 (mainly linolenic, EPA and DHA) PUFA content in HP fed hearts. However, the increase in ω -6 was substantially greater than the increase in ω -3 PUFA, and resulted in an approximate 3-fold increase in the ω -6: ω -3 ratio in HP fed rats.

7.3.2 Phospholipid Metabolism

Cardiolipin is a mitochondrial membrane phospholipid that is essential for oxidative phosphorylation (OXPHOS). HP feeding brought about a severe drop in total cardiolipin levels. Evaluation of individual fatty acids within this phospholipid revealed a substantial reduction in the linoleic acid fraction of cardiolipin, with no significant change in the saturated and monounsaturated fatty acid fraction (Fig 7.1). Hearts from HP rats also demonstrated a substantial increase in PLA₂ activity (Fig 7.2A). Given the non-selective nature of PLA₂, we also measured total phospholipids. A 50% decrease in total cardiac phospholipids with a corresponding increase in lysophosphatidylcholine (LPC), a lysophospholipid by-product of increased PLA₂ activity, was noted (Fig 7.2B, C).

7.3.3 Nitrosative Stress

Under pathological conditions, excess nitric oxide (NO) is predominantly generated by nitric oxide synthases (NOS) like inducible nitric oxide synthase (iNOS). HP hearts demonstrated increased iNOS protein expression (Fig 7.3A). NO when combined with superoxide, can form the most damaging free radical, peroxynitrite (ONOO⁻). ONOO⁻ nitrates tyrosine residues of

proteins to produce nitrotyrosine. HP hearts were characterized by a significant rise in the particulate, but not cytosolic nitrotyrosine levels (Fig 7.3B).

7.3.4 Mitochondrial Damage

In addition to proteins, peroxynitrite can also oxidize macromolecules like DNA, present in both mitochondria and nucleus, to produce 8-hydroxyguanosine (8-OHG) [19]. As evidence of peroxynitrite activity in the particulate fraction could indicate mitochondrial and/or nuclear damage, immunofluorescence for nitrotyrosine and 8-OHG was carried out on rat heart sections (Fig 7.4). LP fed hearts demonstrated negligible 8-OHG and nitrotyrosine staining (Fig 7.4A, B, C). In contrast, the HP group demonstrated widespread 8-OHG staining (Fig 7.4D, G) that co localized with increased nitrotyrosine (Fig 4E, H) staining in the extra nuclear area (Fig 4 I), suggesting a predominantly mitochondrial location for this DNA damage. The mRNA expression of OGG-1, the DNA repair enzyme specific for 8-OHG, also increased in HP hearts (Fig. 7.4J). Nuclear staining for nitrotyrosine and 8-OHG was absent in all groups. The mitochondrial origin of peroxynitrite was also supported by decreased levels of Mn superoxide dismutase (SOD), the antioxidant responsible for neutralizing superoxide in the mitochondria (Fig 7.5A). Interestingly, Cu/Zn SOD, the cytosolic antioxidant for superoxide demonstrated no change in protein levels (Fig 7.5B) between LP and HP fed hearts. To evaluate the role of cytosol in the generation of free radicals, we measured p22^{phox} and p47^{phox} subunit expression of the NADPH oxidase system. Both p22^{phox} (LP, 2.5±0.3; HP, 3.0±0.4, arbitrary units) and p47^{phox} (LP, 2.8±0.2; HP, 3.0±0.4, arbitrary units) demonstrated no change in their protein expression.

7.3.5 Loss Of Gene Expression Of Respiratory Enzyme Subunits

A mammalian mitochondrion encodes 13 essential components of oxidative phosphorylation enzyme complexes. In lieu of investigating the gene expression of all 13 subunits, we

identified 4 genes, each representative of a different enzyme complex, (viz. NADH dehydrogenase subunit 1 (ND-1) from Complex I, cytochrome b (CYTb) from Complex III, cytochrome-c-oxidase subunit 1 (COX 1) from Complex IV and ATPase 6 from Complex V) which is affected by oxidative stress in the heart. It should be noted that subunits from Complex II are not encoded by mitochondrial DNA but by nuclear DNA, and were thus excluded from gene expression studies. Interestingly, RT-PCR studies revealed a drop in gene expression of only ND-1 (Fig 7.6B) and ATPase 6 (Fig 6C), but not Cytb (LP, 2.0 ± 0.57 ; HP, 1.7 ± 0.1 , arbitrary units; Fig 6A) or COX-1 (LP, 1.01 ± 0.07 ; HP, 1.02 ± 0.07 , arbitrary units; Fig 6A) in the HP group. Protein expression of PGC-1 (a nuclear co activator for biogenesis of mitochondrial OXPHOS enzymes) was negligible in LP hearts but increased almost 5-fold in the HP hearts (Fig 7.6D).

7.3.6 Cardiac Functional Parameters

Cardiac output (CO) is the total volume of blood pumped by the ventricle per minute. There were no differences in CO or heart rate (HR) between the LP and HP groups at normal workload (80 mmHg). However, subsequent to an increase in workload to 135 mmHg, CO and HR decreased in all groups. Interestingly, compared to LP, HP hearts demonstrated a more robust decline in both CO and HR (Table 7.3).

7.4 Discussion

Changes that disrupt mitochondrial structure or function can compromise cardiac contractility. Cardiolipin, a mitochondrial membrane phospholipid, is absolutely essential for the activity of key OxPhos enzymes [12, 20]. Recently, we demonstrated that cardiac cardiolipin levels were reduced in rats fed 20% w/w sunflower oil (rich in linoleic acid, an ω -6 PUFA) [9]. In the current study, we confirmed this observation and extended our findings to a specific loss of the linoleic acid fraction in cardiac cardiolipin, following HP feeding. As linoleic acid represents around 80% of the total cardiolipin mass [12], and given that the HP diet is rich in linoleic acid, this observation appeared paradoxical. It should be noted that in the rat heart, other studies have also demonstrated a loss of cardiolipin following chronic ω -6 PUFA feeding [8, 21]. A mechanism behind such an occurrence is yet to be determined. However, high ω -6 PUFA feeding has been shown to increase cardiac phospholipase A₂ (PLA₂) activity [22]. PLA₂ specifically cleaves the acyl group at the *sn*-2 position of most phospholipids, releasing constituent arachidonic acid and lysophosphatidylcholine (LPC). Similar effects on linoleic acid rich cardiolipin can lead to a reduction in this fatty acid fraction, and a loss in total cardiolipin mass [12]. Indeed, increased PLA₂ activity has been reported to decrease cardiolipin levels in rat brain and liver [23, 24]. In the current study, the increase in total PLA₂ activity matched the drop in total phospholipids, and the increase in LPC in HP fed hearts. Given the ability of LPC and arachidonic acid to generate superoxide (O₂⁻), this free radical can also lead to peroxidation of the highly unsaturated linoleic acid fraction of cardiolipin, leading to a further loss in this phospholipid [25-27].

In addition to generation of superoxide, LPC and other PLA₂ metabolites are known to increase inducible nitric oxide synthase (iNOS) expression, as observed following HP feeding [28]. iNOS overexpression produces large amounts of nitric oxide (NO), for a sustained period. Excess NO, when combined with superoxide, forms peroxynitrite (ONOO⁻), a highly

reactive anion [29]. Although NO was not directly measured, its increased generation is critical for the formation of peroxynitrite [30, 31]. Once formed, peroxynitrite can oxidize lipids and nitrate proteins to form nitrotyrosine. Based on its intracellular location, peroxynitrite can differentially affect cellular functions. Mitochondrial peroxynitrite can oxidize sulfhydryl groups on aconitase and other enzymes in the mitochondrial respiratory chain, thereby impairing energy production [32]. Additionally, peroxynitrite can oxidize the C-8 position of guanine in the DNA of both mitochondria and nucleus to form 8-hydroxyguanosine (8-OHG) [19]. Oxidative damage to nuclear DNA (nDNA) triggers DNA repair, increased NAD consumption and cell death [29].

Measurement of cytosolic and 'particulate' (consisting both nucleus and mitochondria) nitrotyrosine following HP feeding revealed an increase in particulate, and not cytosolic nitrotyrosine. To determine the nuclear and/or mitochondrial location of peroxynitrite, we used immunofluorescent co-localization techniques to identify 8-OHG and nitrotyrosine on intact cardiac sections. Mechanical separation of nucleus from mitochondria often leads to high levels of background oxidation and artificial DNA damage, and was avoided to prevent false positivity for nitrotyrosine and oxidized DNA [33, 34]. Our double-labeling immunofluorescence analysis revealed that both nitrotyrosine and 8-OHG were localized predominantly in the extranuclear space in HP heart sections, thus negating the oxidative effects of peroxynitrite on nuclear DNA. In spite of the presence of extensive free radical induced mitochondrial damage, absence of nuclear DNA damage may explain the lack of cell death (both apoptosis and necrosis) in rat hearts following HP feeding [9]. Thus, in the current study, cardiac mitochondria (the only other organelle with DNA) were believed to be the source for 8-OHG in HP hearts. This preferential damage to mDNA likely occurs as a consequence of a lack of protective histones and its proximity to the electron transport

complexes that produce free radicals [35]. Accumulation of 8-OHG in the mitochondria can cause GC to AT mutations [36]. To prevent this event, nuclear DNA encoded OGG-1, an 8-oxoG-DNA glycosylase can be targeted towards mitochondria to remove 8-OHG. Our data suggests that similar to humans and rodents, augmented expression of OGG-1 in HP hearts could result from increased formation of 8-OHG [36-38].

HP hearts were characterized by a specific loss of MnSOD, the antioxidant responsible for neutralization of mitochondrial O_2^- , an essential precursor for peroxynitrite formation [39]. As the cytosolic antioxidant Cu/Zn SOD, and the NADPH oxidase subunits p22^{phox} and p47^{phox} protein expression (chief source for extramitochondrial ROS generation within the cytosol), demonstrated no change in their protein expression, our data supports a predominantly mitochondrial location of peroxynitrite. It should be noted that high fat feeding with lard has been shown to induce p22^{phox} and p47^{phox} protein expression in rat brains [40]. It is likely that this differential effects of high fat on the NADPH system is due to differences in organs studied, and the type of dietary fat used (lard is a source of saturated fatty acids).

The mammalian mitochondrial DNA contains 13 polypeptide genes, all of which encode essential subunits of OxPhos complexes including complex I (ND 1, 2, 3, 4, 4L, 5, and 6), III (cytochrome b), IV (COX I, II and III) and V (ATPase 6 and 8) [17, 41]. Although mutations in these genes are known to decrease the rate of energy production, the relative importance of individual genes remains controversial. Nevertheless, with 13 potential candidates, we selected 4 genes from the different complexes that are affected by cardiac oxidative stress. For example, oxidative stress induced by lipopolysaccharide or doxorubicin alters ND-1 and ATPase-6, and COX I expression, respectively [42, 43]. Additionally, in humans, oxidative damage following myocardial infarction decreases ND-1 and cytochrome b [44]. In the present study, HP feeding reduced the gene expression of ND-1 of complex I, and ATPase 6 of complex V. Although the reason behind such losses in gene expression of specific subunits is

unknown, complex I inhibition has been suggested to work as a protective response to limit electron flow to Complex III, in an attempt to decrease superoxide generation [45]. Loss of mitochondrial respiratory subunits may promote a compensatory response from the nuclear genome through activation of PGC-1, as seen in HP hearts. PGC-1 interacts with nuclear respiratory factors (NRF-1 and NRF-2) to induce genes for OXPHOS and other mitochondrial functions [43]. With regard to the heart, oxidative stress induced by lipopolysaccharide exposure damages mtDNA, decreases respiratory subunits and concurrently elevates gene expression for PGC-1 [43].

In spite of considerable mitochondrial damage, both LP and HP fed hearts had identical cardiac output and heart rate at basal afterloads of 80 mmHg. It is likely that during such conditions, the vast mitochondrial reserves in cardiomyocytes (around 30% of the cardiomyocytes volume is occupied by mitochondria) may be responsible for this absence of any functional alterations in HP hearts [46]. However with the existing mitochondrial nitrosative damage, we hypothesized that HP hearts may not function optimally following increased workloads. Consistent with our hypothesis, both LP and HP fed hearts had identical cardiac output and heart rates at basal afterloads. However, when these hearts were exposed to a high afterload, HP hearts demonstrated a greater decline in both these functional parameters than LP hearts.

A limitation of this study is the lack of an isocaloric diet (e.g., a diet enriched with saturated fatty acid). Thus, the conclusions of mitochondrial nitrosative damage cannot be attributed solely to ω -6 PUFA, but could be a result of a high fat diet per se. However, it should be noted that the pro-inflammatory properties demonstrated in this study (activation of PLA₂, breakdown of membrane phospholipids, and induction of nitrosative stress) is a hallmark of ω -6 PUFA and not saturated fatty acids [4-7].

In summary, we conclude that a high fat diet enriched with ω -6 PUFA leads to activation of pro-inflammatory pathways within the heart causing mitochondrial nitrosative damage (Fig. 7.7) and cardiac dysfunction at higher afterloads. Given the postmitotic nature of cardiomyocytes, the heart would be particularly vulnerable to this damage inflicted by ω -6 PUFA [47]. Interestingly, accumulation of ω -6 PUFA has been strongly associated with an augmented risk of myocardial damage in humans [48, 49]. With the current shift towards dietary ω -6 PUFA, our study raises other epidemiological concerns. During aging and diseases associated with aging, loss of cardiolipin [8, 21], oxidative stress, mitochondrial membrane and DNA damage [50] are observed, and are similar to changes seen in this study. Whether the current 'epidemic' of cardiovascular disease is a function of high fat diet enriched with ω -6 PUFA warrants further investigation.

7.5 Tables and Figures

Table 7.1 Primers used for PCR amplification of genes.

Name	GeneBank Acc. No.	Forward (5'-3')	Reverse (5'-3')	Base pair
ND1	X 14848 (2729..3685)	CACCCCCTTATCAACCTCAA	ATTTGTTTCTGCGAGGGTTG	305
Cyt B	X 14848 (14124..15266)	TCTCATCAGTCACCCACATC	CATTCTGGTTTGATGTGGGG	627
COX 1	X 14848 (5309..6853)	AGCAGGAATAGTAGGGACAGC	TGAGAGAAGTAGTAGGACGGC	519
ATPase6	X 14848 (7904..8584)	CGAACCTGAGCCCTAATA	GTAGCTCCTCCGATTAGA	337
18S rRNA	X0 3205	TCAATCTCGGGTGGCTGAACG	GGACCAGAGCGAAACCATTG	495
OGG1	NM0 30870	ATCTGTTCTTCCAACAACAAC	GCCAGCATAAGGTCCCCACAG	500
β -actin	NM 031144	TTGTCACCAACTGGGACGATATG	GATCTTGATCTTCATGGTGCTAG	763

Table 7.2 Major cardiac ω -6 and ω -3 PUFA

	Low PUFA	High PUFA
Linoleic Acid (18:2 ω -6)	0.42 \pm 0.01	2.1 \pm 0.09*
Arachidonic Acid (20:4 ω -6)	0.12 \pm 0.00	3.7 \pm 0.07*
Linolenic Acid (18:3 ω -3)	0.14 \pm 0.00	0.71 \pm 0.13*
Eicosapentaenoic Acid (20:5 ω -3)	0.10 \pm 0.00	0.20 \pm 0.05*
Docosahexaenoic Acid (22:6 ω -3)	0.07 \pm 0.02	0.17 \pm 0.08*

Animals were fed either low or high PUFA for 4 weeks before termination and removal of the hearts. Cardiac lipids including phospholipids were extracted with chloroform: methanol: acetone: hexane solvent. Separation of phospholipids and fatty acids was achieved using HPLC, followed by their conversion to their respective methyl esters, and quantification by GLC. Data are expressed in μ g/mg protein. The results are means \pm SEM for 4-6 animals in each group. *Significantly different from low PUFA fed group, $P < 0.05$.

Table 7.3 Heart Rate and Cardiac Output of isolated working hearts

PARAMETER	Low PUFA		High PUFA	
	BASAL AL	ELEVATED AL	BASAL AL	ELEVATED AL
Heart Rate (beats/min)	257±11	305±7	243±3	283±6*
Cardiac Output (ml/min)	71±2.2	41±4.6	68±1.3	28±1.4*

Animals were fed either low or high PUFA for 4 weeks before termination and removal of the hearts. Various parameters of cardiac function were measured at either basal (80 mm Hg) or elevated (135 mm Hg) afterloads (AL). The results are means ± SEM for 4-6 animals in each group. *Significantly different from corresponding group fed low PUFA, $P < 0.05$.

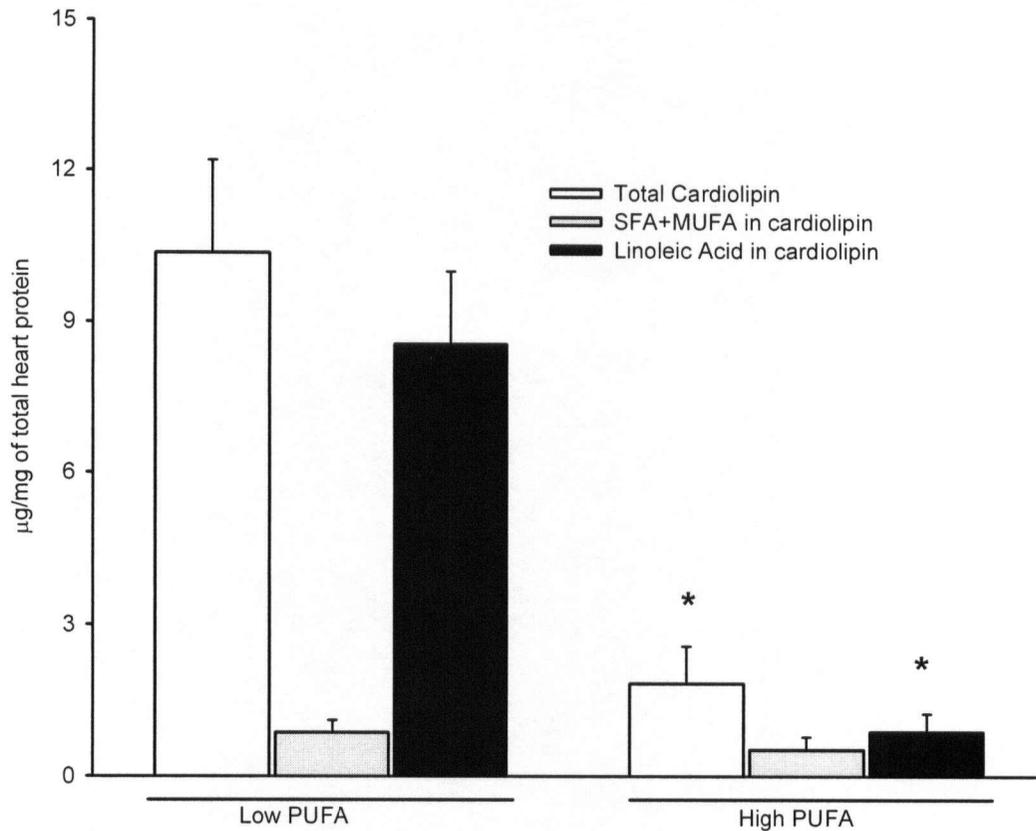


Fig. 7.1 *Effects of ω -6 PUFA feeding on cardiac cardiolipin* Separation of total cardiolipin was achieved by HPLC. Cardiolipin fatty acids were quantified as their respective methyl esters using heptadecaenoic acid (17:0) as the internal standard with a GLC equipped with a flame ionization detector. Results are the means \pm SE of 4-6 rats in each group. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid. *Significantly different from low PUFA fed group; $P < 0.05$.

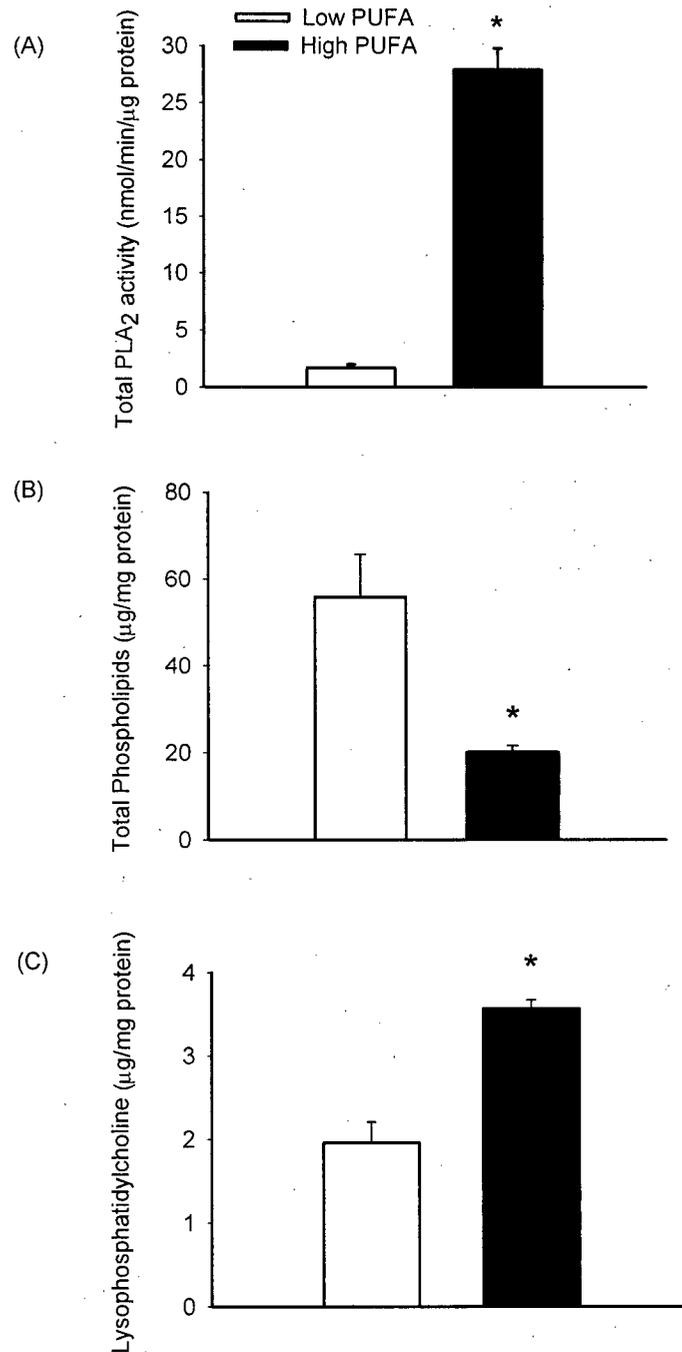


Fig. 7.2 *Cardiac phospholipid metabolism* Total cardiac phospholipase A₂ (PLA₂) activity as measured by a spectrophotometric assay using arachidonyl thio-phosphatidylcholine as a substrate (A). Total cardiac phospholipid (B) and lysophosphatidylcholine (C) levels were measured by HPLC. Results are the means ± SE of 4-6 rats in each group. *Significantly different from low PUFA fed group; $P < 0.05$.

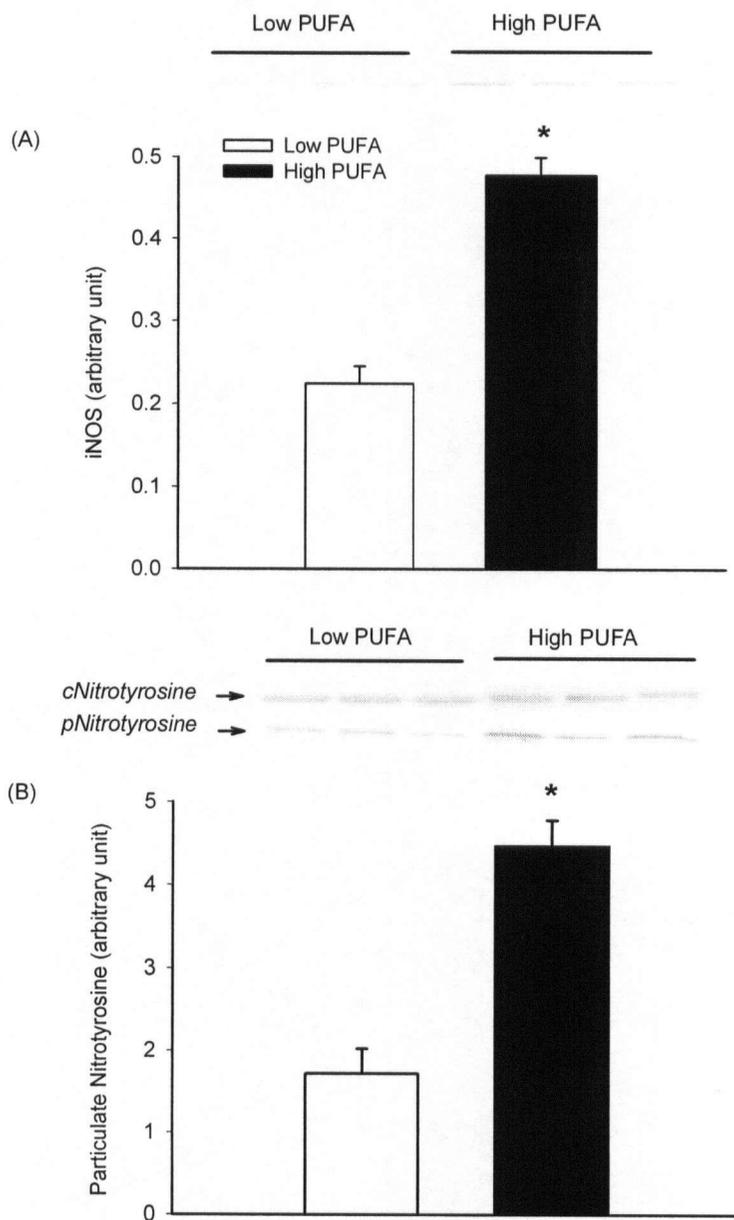


Fig. 7.3 *Cardiac nitrosative stress* Inducible nitric oxide synthase (iNOS) (A) and cytosolic and particulate nitrotyrosine (B) protein expression were measured by Western blot, followed by densitometry of the respective bands. Results are the means \pm SE of 4-6 rats in each group. *Significantly different from low PUFA fed group; $P < 0.05$.

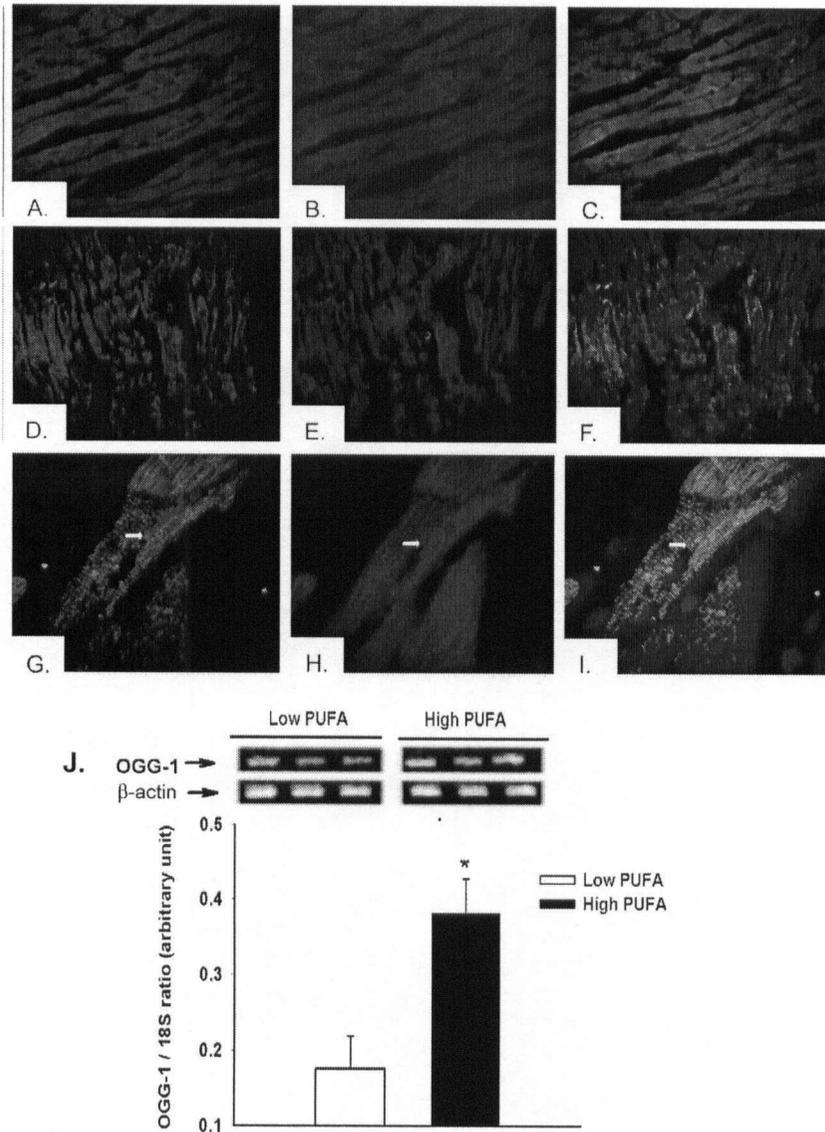


Fig. 7.4 *Intracellular localization of peroxynitrite and 8-hydroxyguanosine (8-OHG) by confocal microscopy* Formalin-fixed heart sections were analyzed by double immunostaining for 8-OHG (A, D, G) and nitrotyrosine (B, E, H). C, F and I represent composite images of 8-OHG, nitrotyrosine and nucleus. Images A-C represent low PUFA fed heart sections that were negative for both nitrotyrosine and 8-OHG (400x magnification). Images D-F represent high PUFA fed heart sections with both 8-OHG and nitrotyrosine at 400x magnification. Images G-I represent high PUFA fed hearts at a higher magnification (1200x) and demonstrate a predominantly extra nuclear location of 8-OHG and nitrotyrosine (arrows). OGG-1 gene expression is depicted in representative gels, followed by densitometric analysis (J). β -actin mRNA was utilized as an internal standard. Results are the means \pm SE of 4-6 rats in each group. *Significantly different from low PUFA fed groups; $P < 0.05$. OGG-1, 8-oxo-DNA glycosylase.

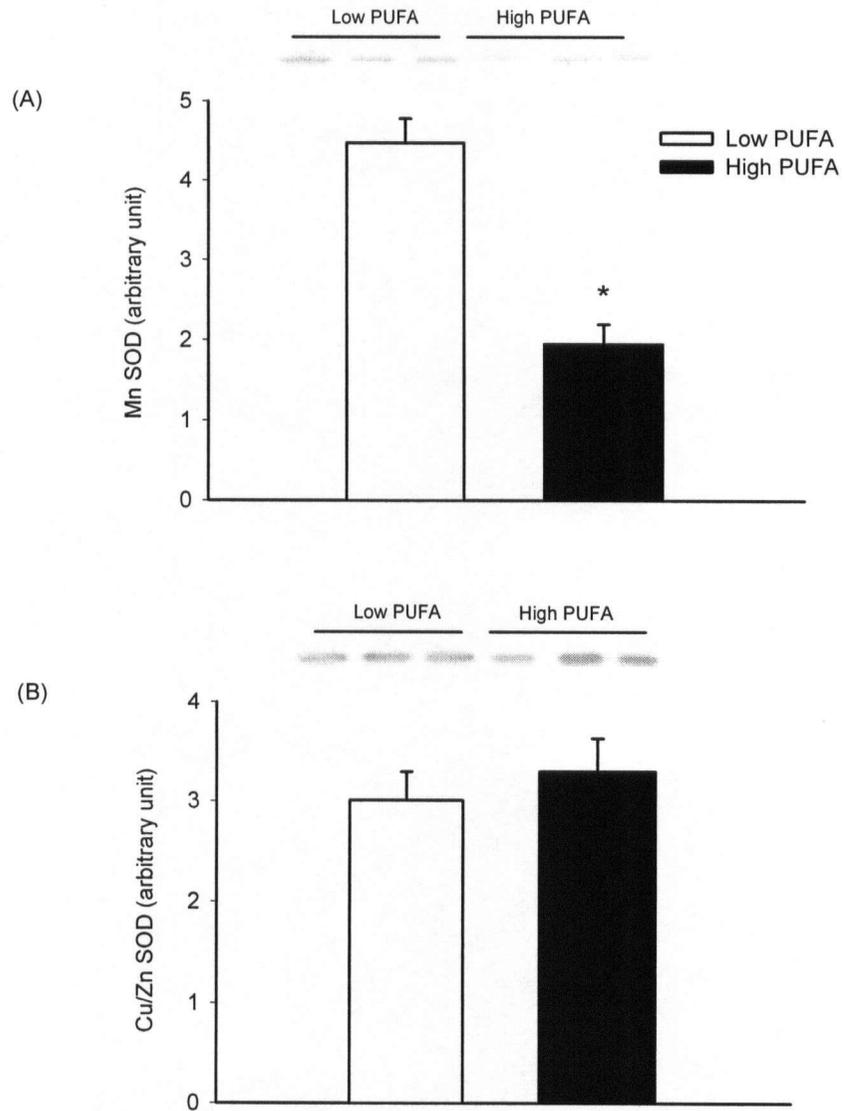


Fig. 7.5 *Intracellular superoxide dismutase (SOD) protein expression* Mitochondrial MnSOD (A) and cytosolic Cu/Zn SOD (B) protein expression were measured by Western blot followed by densitometry of the representative bands. Results are the means \pm SE of 4-6 rats in each group. *Significantly different from low PUFA fed group; $P < 0.05$.

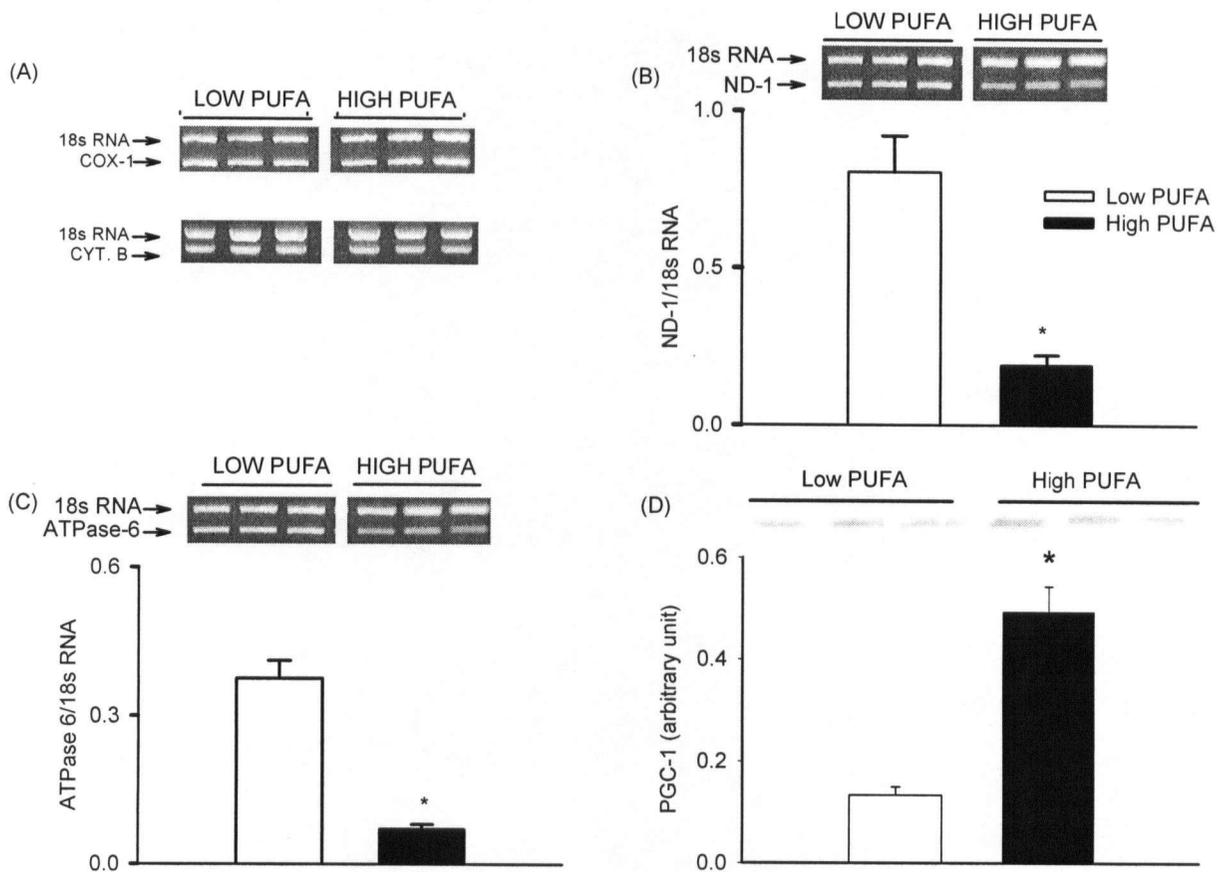


Fig. 7.6 *Respiratory subunit gene and PGC-1 protein expression* Gene expression of mitochondrial respiratory subunits encoded by mitochondrial DNA (mtDNA) was measured using reverse transcriptase-polymerase chain reaction. Four genes, each representative of a different enzyme complex were chosen. Representative gels of COX-1 and Cyt b gene expression are depicted in (A). No change was observed between the two groups. ND-1 (B) and ATPase 6 (C) gene expression are depicted in representative gels, followed by densitometric analysis. 18s RNA was utilized as an internal standard. PGC-1 protein expression was measured by Western blot followed by densitometry of the representative bands in (D). Results are the means \pm SE of 4-6 rats in each group. *Significantly different from low PUFA fed group; $P < 0.05$. ND1, NADH dehydrogenase subunit 1 from Complex I; CYTb, cytochrome b from Complex III; COX 1, cytochrome-c-oxidase subunit 1 from Complex IV; ATPase 6 from Complex V; PGC-1, peroxisome proliferator-activated receptor gamma co activator 1.

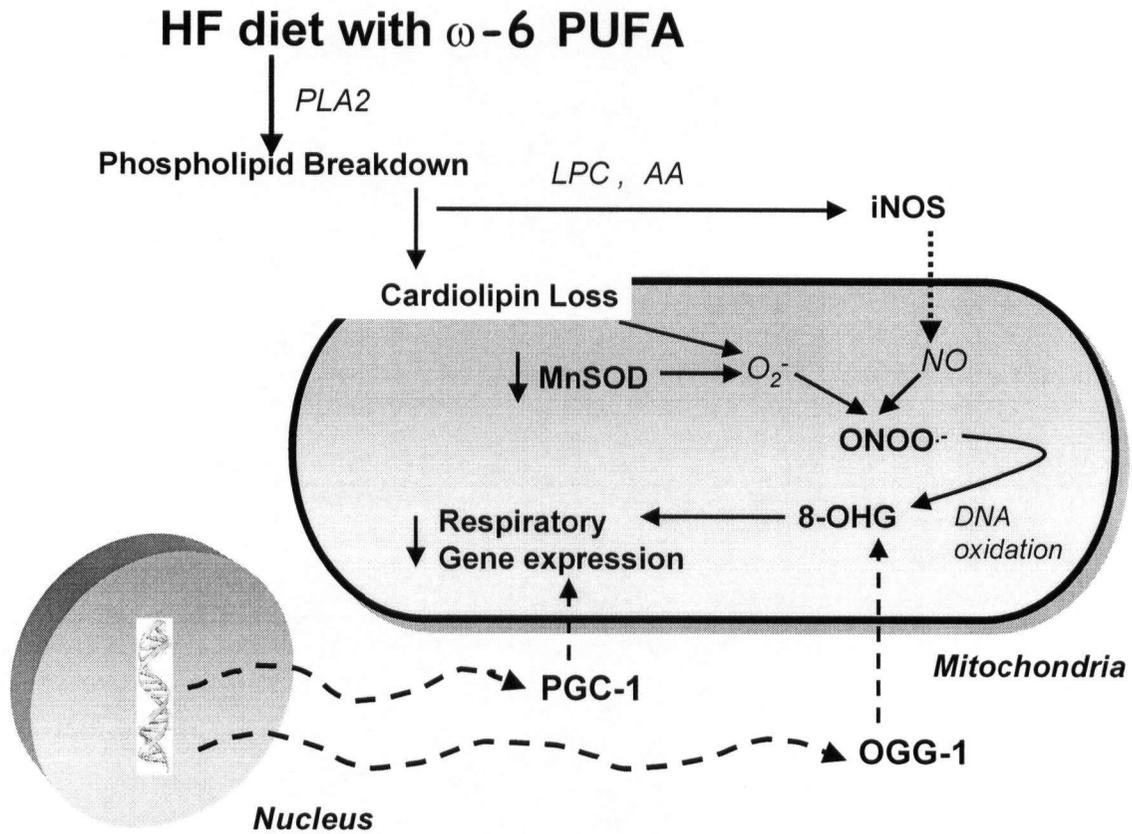


Fig. 7.7 Proposed pathways of mitochondrial damage and nuclear responses following consumption of a high fat (HF) diet rich in ω -6 PUFA. Excess ω -6 PUFA increases cardiac phospholipase A₂ (PLA₂) activity, breaking down cardiac phospholipids, including cardiophilin, and promoting free radical generation. Byproducts of PLA₂ activity like lysophosphatidylcholine (LPC), and AA (arachidonic acid) can stimulate inducible nitric oxide synthase (iNOS) expression, and production of nitric oxide (NO). Combination of NO and free radicals like superoxide O₂⁻ can produce peroxynitrite (ONOO⁻). Peroxynitrite in turn can damage mitochondrial DNA and produce 8-hydroxyguanine (8-OHG), thereby impairing gene expression of certain respiratory subunits. After oxidative damage to the mitochondria, the nuclear genome up regulates the production of OGG-1 (8-oxo-DNA glycosylase) and PGC-1 (peroxisome proliferator-activated receptor gamma co activator 1). OGG-1 removes 8-OHG from oxidized DNA, while PGC-1 helps to synthesize mitochondrial respiratory subunits (shown in dotted lines).

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8. Conclusions

There is strong experimental evidence supporting the involvement of apoptosis in the development of diabetic myocardial disease [1]. However, although the significance of cell death in the diabetic heart was highlighted more than 6 years ago, factors modulating such death are unclear [2]. Oxidative stress has been hypothesized to be the “unifying link” behind all diabetic complications including heart disease [3, 4]. However, large-scale trials have failed to demonstrate any cardiovascular benefits using antioxidants like vitamin E and β -carotene in diabetic patients [5, 6]. These regimens have failed probably because as ‘chain-breaking’ antioxidants, they could only prevent lipid peroxidation reactions, but not oxidative protein modifications or DNA damage, which can be only prevented by antioxidants that can directly inhibit free radical generation at its source. Further, it is possible that these patients with a long-standing history of diabetes were poor candidates for damage reversal. As this thesis and other laboratories have demonstrated mitochondria to be the main source of free radical generation in the diabetic heart, antioxidants that neutralize free radicals within the cardiac mitochondria are warranted [7]. Glutathione (GSH) is such an endogenous antioxidant that can directly prevent free radical formation in the cardiac mitochondria. With regards to GSH, this thesis provides the following evidence, which could shed new light on antioxidant therapy during diabetes:

- 1 The major source for free radical generation in the diabetic heart is the mitochondria and not the cytosol. Thus, therapies increasing cytosolic antioxidant levels only, may not prevent diabetic heart disease.
- 2 Decrease in mitochondrial glutathione and oxidative stress can be observed as early as 3 days following the induction of diabetes. Thus, therapy against oxidative stress should be initiated early.

3 Replenishment of mitochondrial GSH can reduce oxidative stress and cell death in the diabetic heart by reducing the generation of free radicals from the source (i.e. the mitochondria).

Although lipotoxicity is also considered a major player in the induction of diabetic heart muscle death, most of the work has been done in vitro with saturated fatty acids. Looking at the current western dietary trends, more emphasis on the role of PUFAs, specifically ω -6 PUFA are warranted. This thesis demonstrates that feeding a high fat diet enriched with ω -6 PUFA in rats leads to activation of pro-inflammatory pathways within the heart causing mitochondrial nitrosative damage. Given the postmitotic nature of cardiomyocytes, the heart would be particularly vulnerable to this damage inflicted by ω -6 PUFA [8]. Interestingly, accumulation of ω -6 PUFA has been strongly associated with an augmented risk of myocardial damage in humans [9, 10]. Upon superimposition of diabetes, with preexisting mitochondrial nitrosative damage, this thesis also demonstrates loss of ATP, increased cardiac necrosis and functional cardiac impairment at higher afterloads.

The findings of this thesis also raise some important epidemiological concerns. The loss of cardiolipin [11], oxidative stress [12], DNA damage and energy stress [13] are all hall marks of aging. Indeed in human studies, high ω -6 PUFA or low ω -3 PUFA feeding is positively correlated with metabolic and degenerative diseases like cancer [14] atherosclerosis [15, 16], Alzheimer's and Parkinson's disease [17], insulin resistance and diabetes [18, 19]. Even in the absence of any disease, high ω -6 PUFA feeding in young Wistar rats decreases cardiac inotropic responses to adrenergic agonists to that seen in senescent rats [20]. Whether the current epidemic of cancer, neurological or cardiovascular disease is a function of high ω -6 PUFA diet is a question that warrants further investigation.

8.1 Future Directions

This thesis demonstrates that cardiac mitochondrial GSH is lost following the induction of diabetes. It is possible that following generation of excessive ROS from the electron transport chain, the flux of cytosolic GSH to the mitochondria is impaired in the diabetic heart, which in turn leads to lower mitochondrial GSH levels and oxidative damage. Two major transporters, the dicarboxylate and 2-oxaloglutarate carriers, transport mitochondrial GSH in the liver [21]. However their regulation in the diabetic heart is still unknown. Apart from defective transporters, alteration of mitochondrial membrane phospholipids during diabetes [22] can also affect this transport and can precipitate a deficit in mitochondrial GSH [21]. My MSc. project also demonstrated a severe depletion of cardiac GSH following ω -6 PUFA feeding [23], a conclusion supported by other studies [24-26]. Interestingly, increasing dietary ω -3 PUFA like docosahexaenoic acid can reverse such a defect [27, 28]. In all of these cases, the exact sub cellular site of GSH depletion and the exact mechanisms leading to such a loss of cardiac GSH following ω -6 PUFA feeding remains unknown. Thus, future experiments should target the study of mitochondrial GSH transport and the effects of both ω -3 PUFA and ω -6 PUFA in modulating cardiac GSH levels in the diabetic heart.

8.2 References

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