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PARP-MEDIATED STRUCTURAL ALTERATIONS IN DIABETIC CARDIOMYOPATHY

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PARP-MEDIATED STRUCTURAL ALTERATIONS IN DIABETIC CARDIOMYOPATHY

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Abstract

Diabetic cardiomyopathy is characterized structurally by fibrosis and cardiomyocyte hypertrophy. Chronic hyperglycemia leads to increased oxidative stress and results in DNA strand breaks. This activates poly (ADP-ribose) polymerase (PARP) in an attempt to repair the damage and may also regulate transcription through transcriptional co-activator p300. We hypothesized that increased activity of PARP from oxidative stress and DNA damage may lead to transcriptional alterations and structural changes in the heart in diabetes. Two *in vivo* models of diabetic complications were examined utilizing two methods of PARP inhibition; genetic ablation and pharmacological inhibition with 3-aminobenzamide. The findings were confirmed in an *in vitro* model system. Hyperhexosemia was found to increase oxidative stress and induce cardiac hypertrophy and fibrosis. These changes were prevented with PARP inhibition. These findings elucidate, for the first time, a specific pathway involving PARP in the development of structural alterations in diabetic cardiomyopathy.

Keywords: PARP, diabetic cardiomyopathy, oxidative stress

Dedication

I would like to dedicate this thesis to my family, especially my mother for her unwavering support in my academic pursuits.

To Aimee, Ava, Javy, John, Kris and Remi, thank you for your loving support, for putting up with my scientific shenanigans, for picking me up when I was down, for making me feel that much more special and for believing in me without which I would not be where I am today.

Although I would never fully be able to express my gratitude and appreciation for having you with me through this experience, at least in spirit, I hope that with these 100 or so pages you will see that it was not all in vain. I am a better person because of you.

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List of Abbreviations

8-OHdG	8-hydroxy-2'-deoxyguanosine
ABA	3-aminobenzamide
AGE	advanced glycation endproducts
ANP	atrial naturetic protein
AP-1	activator protein 1
AR	aldose reductase
BM	basement membrane
CAD	coronary artery disease
CHF	congestive heart failure
CO	control; mice = normal rodent feed, rat = citrate buffer
DAG	diacylglycerol
DCCT	diabetes control and complications trial
DM	diabetes mellitus
ECM	extracellular matrix protein
eNOS	endothelial nitric oxide synthase
ET-1	endothelin 1
FN	fibronectin
G	30% galactose enriched rodent feed
HAT	histone acetyltransferase
HDAC	histone deacetylase
HG	high glucose, 25 mmol/L
HO-1	heme oxygenase 1

LG	low glucose, 5 mmol/L
PARG	poly (ADP-ribose) glycohydrolase
PARP	poly (ADP-ribose) polymerase
PKC	protein kinase C
MHC	myosin heavy chain
NAD+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NFκB	nuclear factor κB
NO	nitric oxide
NT	nitrotyrosine
RAGE	AGE receptor
ROS	reactive oxygen species
STZ	streptozotocin
UKPDS	United Kingdom prospective diabetes study
WT	wild type
XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide

Chapter 1: Introduction

1.1 Diabetes Mellitus

Diabetes is a debilitating disease characterized by the body's inability to properly utilize glucose. There are two main types of diabetes, type 1 and type 2. Typically, type1 is associated with an autoimmune response leading to the destruction of β islet cells in the pancreas. The patient cannot produce their own insulin therefore they require exogenous insulin. Thus it is also known as insulin-dependent diabetes mellitus. Type 2 diabetes has multiple etiologies and is largely attributed to lifestyle. The patients have functioning β islet cells and thus usually may not require exogenous insulin initially. This type of diabetes is also known as insulin-independent diabetes mellitus. However, exogenous insulin may be needed in the later stages. Two mechanisms have been proposed for type 2 diabetes. Type 2 diabetes may result from low insulin receptor expression preventing sufficient insulin stimulation or may be due to defective signal transduction[1]. Both of these mechanisms impede the insulin-induced effect of increased glucose uptake, via the glucose transporter 4 (GLUT4), into the adipose and striated muscle tissues thus preventing adequate glycogen formation and is termed insulin resistance. As a result of the inability of insulin to perform its action, chronic hyperglycemia results in the patients.

1.2 Epidemiology

Currently, over 2 million Canadians have been diagnosed with diabetes with the number projected to reach 3 million by the end of the decade[2]. Worldwide, the World Health Organization estimates that there are more than 177 million people with this disease in 2000 and that this number will increase to over 300 million by 2025[3]. Furthermore, a Canadian with this disease is twice as likely to die a premature death when compared to an individual without diabetes[3]. Those with Type 1 diabetes can expect their lives to be shortened by as much as 15 years compared to their non-diabetic counterparts, while those with Type 2 diabetes can expect their lives to be shortened by 5 to 10 years[3].

1.3 Diabetic Complications and their pathogenesis

Approximately 40% of all diabetic patients will develop chronic complications[4]. These complications target the heart, retina, kidneys and peripheral nerves. Diabetic retinopathy is the most common cause of blindness in those under 65 years of age and is the most common cause of new blindness in North America[5]. Diabetic nephropathy is the leading cause of end stage renal failure in the western world[6]. Diabetic neuropathy is a major contributor to lower extremity amputations with the rates of lower extremity amputation 10 times higher for those with diabetes than individuals without[7]. These diseases manifest as a result of abnormalities in the micro- and macrovasculature.

Diabetic complications contribute to significant morbidity and mortality, of which the most devastating are microvascular in nature[8,9]. Chronically elevated blood glucose levels have been pinpointed by several large scale clinical trials, such as DCCT and UKPDS, to be the key initiating factor in the development of diabetic complications[8,10]. Evidence suggests that hyperglycemia induces its adverse effects through the activation of several interrelated pathways such as: protein kinase C (PKC) activation, hexosamine pathway, aldose reductase (AR) activation and nonenzymatic formation of advanced glycation end (AGE) products[11].

1.3.1 PKC Pathway

Hyperglycemia causes *de novo* synthesis of diacylglycerol (DAG), an endogenous activator of PKC[12,13]. Activation of PKC mediates a variety of different effects such as increased endothelial cell permeability through the increased production of vascular endothelial growth factor which is also an angiogenic factor[12-15]. PKC directly alters the production of vasoactive factors such as endothelial nitric oxide synthase (eNOS)[14] and endothelin 1 (ET-1)[16] which leads to abnormalities in blood flow. PKC activation may also induce the expression of extracellular matrix (ECM) proteins e.g. collagen and fibronectin (FN) through the increased expression of transforming growth factor β, activation of nuclear factor κB (NFκB) and increase the production of reactive oxygen

species (ROS)[11] [Figure 1.1]. Evidence also suggests that PKC activation, via hyperglycemia, can be achieved indirectly through the ligation of AGE receptors[17] and increased AR activity[18]. Furthermore, increased ROS production may further mediate these effects.

1.3.2 Hexosamine Pathway

Glucose is usually metabolized by glycolysis. However, excess amounts of intracellular glucose may be shunted to other pathways such as the hexosamine pathway[19]. In this pathway, fructose-6-phosphate is diverted from glycolysis to form glucosamine-6-phosphate. This molecule inhibits glucose-6-phosphate which is required for the regeneration of nicotinamide adenine dinucleotide phosphate (NADPH). A decrease in the NADPH:NADP+ ratio contributes to increased oxidative stress in two ways. Firstly, NADPH is required for the conversion of cellular antioxidant oxidized glutathione to reduced glutathione[20]. Secondly, the decreased availability of NADPH decreases the activity of catalase, a scavenger of oxidants thereby preventing the conversion of ROS and H_2O_2 to H_2O contributing to an overall increase in oxidative stress[21] [Figure 1.2].

Figure 1.1

Hyperglycemia-induced activation of the protein kinase C (PKC) pathway is through *de novo* synthesis of diacylglycerides (DAGs). This leads to increased expression of vasoactive factor endothelin 1 (ET-1) and fibronectin (FN) and the production of reactive oxygen species (ROS).

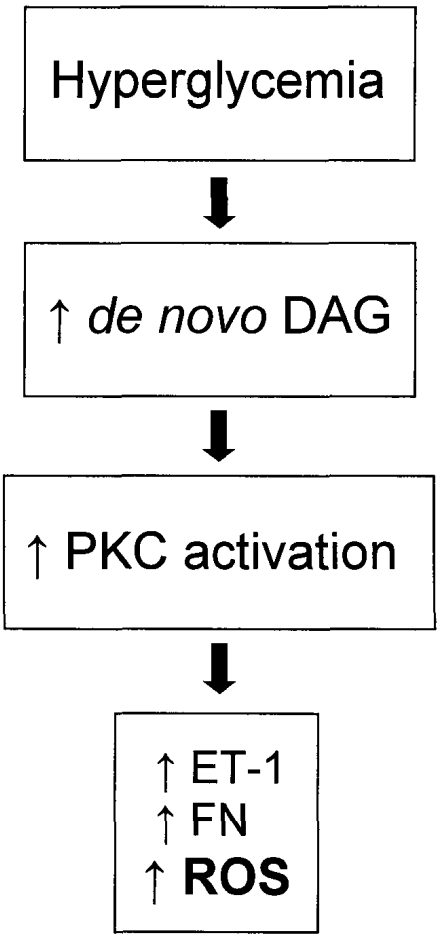
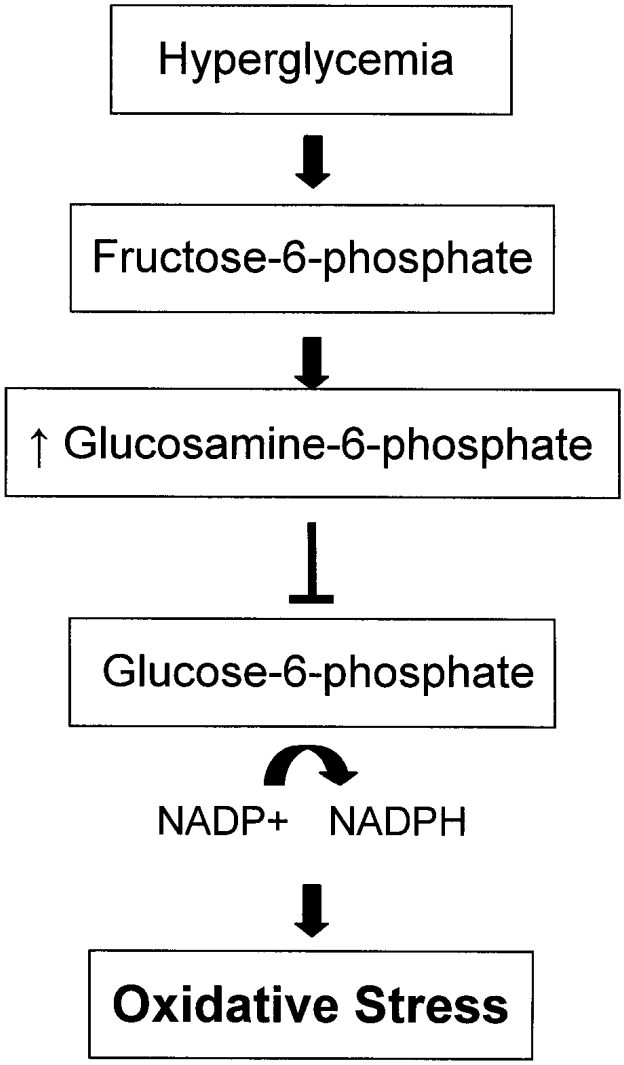

Hyperglycemia
↑ *de novo* DAG
↑ PKC activation
↑ ET-1
↑ FN
↑ **ROS**

Figure 1.2

Hyperglycemia-induced increased influx of glucose into the hexosamine pathway leads to the increased production of glucosamine-6-phosphate, an inhibitor of glucose-6-phosphate which regenerates NADPH. This leads to redox imbalance in the cell contributing to oxidative stress.


Hyperglycemia
Fructose-6-phosphate
↑ Glucosamine-6-phosphate
Glucose-6-phosphate
NADP+ NADPH
Oxidative Stress

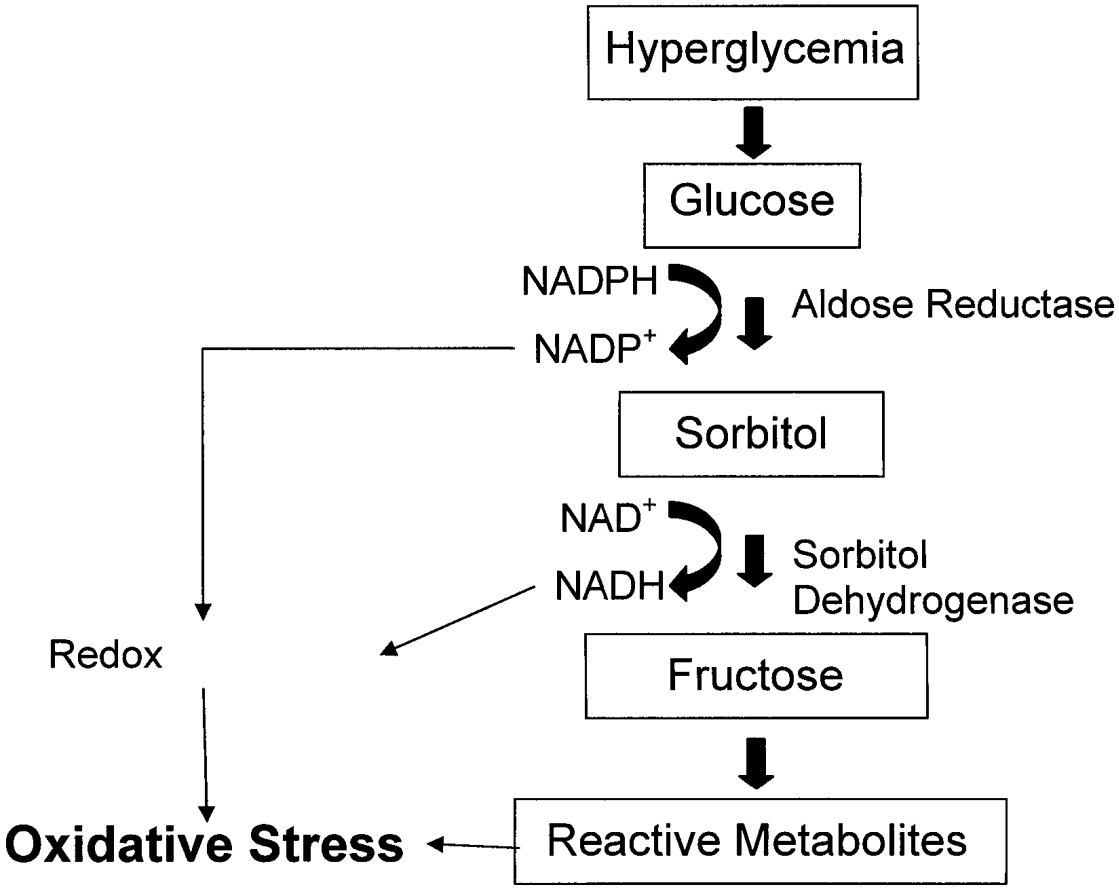
1.3.3 AR Pathway

Another pathway involved in diabetic complications is the AR pathway. Under normoglycemic conditions, very little glucose is metabolized through this pathway. However, hyperglycemia increases the activity of AR which leads to sorbitol accumulation[22] and the depletion of intracellular NADPH[22,23]. Since NADPH is required by glutathione (see above), this antioxidant system is compromised. Sorbitol is then metabolized to fructose by sorbitol dehydrogenase, utilizing nicotinamide adenine dinucleotide (NAD+) as a co-factor. This further exacerbates the oxidative state of the cell by decreasing the intracellular NAD+:NADH levels[24,25]. In addition, increased NADH facilitates the formation of AGE products as it acts as a co-factor for glyceraldehyde-3-phosphate dehydrogenase[26]. Therefore extraneous glucose metabolized through this pathway acts doubly in its pathogenetic effects [Figure 1.3].

In addition, aldose reductase metabolizes galactose to galactitol through the consumption of NADPH. Hypergalactosemia has been shown to produce similar lesions in the retina[27,27], kidneys[28,29], peripheral nerves[30,31] and heart[32] as in diabetes. Since insulin does not respond to galactose, these lesions are produced in the absence of hormonal input thus providing a hyperhexosemic, normoinsulinemic model of diabetic complications which permits the study of the effect of hexose sugars only on these organs. This model was utilized to understand the mechanism(s) regulating structural lesions in the heart.

Figure 1.3

Hyperglycemia-induced increased glucose metabolism via the aldose reductase pathway. The metabolism of glucose to sorbitol by aldose reductase and sorbitol to fructose by sorbitol reductase depletes intracellular NADPH and NAD+ levels, respectively. Fructose metabolism yields reactive metabolites which, in conjunction with the decreased NADPH:NADP+ and NAD+:NADH ratios, leads to oxidative stress.


Hyperglycemia
Glucose
NADPH
NADP+
Aldose Reductase
Sorbitol
NAD+
NADH
Sorbitol Dehydrogenase
Redox
Fructose
Oxidative Stress
Reactive Metabolites

1.3.4 Non-Enzymatic Glycation and AGE Products

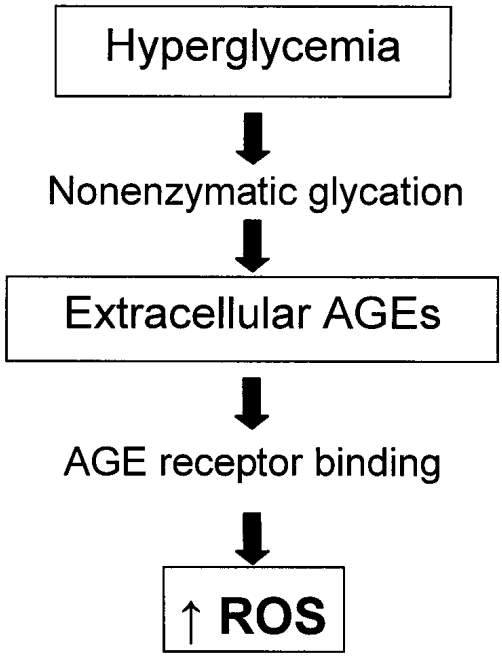
Non-enzymatic glycation and the accumulation of glycated products is a normal part of aging but this process is accelerated in diabetes[33-40]. Reducing sugars, including glucose, can react with proteins, DNA and lipids through the Maillard reaction creating reactive dicarbonyls: 3-deoxyglucosone, methylglyoxal and glyoxal[33]. These intermediates react with intracellular and extracellular amino groups to form AGEs. AGEs have the ability to interfere with protein and ECM function[41]. As well, AGEs can interact with its receptor (RAGE) located on endothelial cells, smooth muscle cells and macrophages[36-40,42]. Although the activities and functions of RAGE are unclear, AGE-RAGE binding in diabetic complications has been shown to upregulate cytokines and growth factors and in the process, activates redox sensitive transcription factor NFκB and produces ROS[33,36,39,43] [Figure 1.4].

1.3.5 Oxidative stress

The four aforementioned pathways contribute to the pathogenecity of chronic hyperglycemia through increased cellular oxidant damage in the target organs of diabetic complications. It is suggested that each of the pathways discussed above maybe activated as a result of hyperglycemia-induced overproduction of superoxide by the mitochondrial electron transport chain[11].

Figure 1.4

Hyperglycemia leads to accelerated non-enzymatic glycation of proteins, DNA and lipids to form advanced glycation end-products (AGEs). Extracellular AGEs bind plasma proteins and interacts with the AGE receptor to generate reactive oxygen species (ROS).


Hyperglycemia
Nonenzymatic glycation
Extracellular AGEs
AGE receptor binding
↑ ROS

Excess oxidative stress, as a result of mitochondrial superoxide overproduction, can have dramatic downstream effects including activation of important nuclear enzymes and alterations in gene expression which will be addressed below.

1.4 Diabetes and the Heart

In 1999, the American Heart Association stated, "Diabetes is a cardiovascular disease"[44]. Diabetes increases the risk of an individual from suffering a vascular disease and at an earlier age. As a matter of fact, approximately 80% of all diabetic patients will die of a heart attack or stroke[45]. Cardiovascular complications are most common cause of morbidity and mortality in diabetic patients[3]. This disease detrimentally affects the heart in 3 ways: autonomic neuropathy, coronary artery disease (CAD) and diabetic cardiomyopathy.

1.4.1 Autonomic Neuropathy

Autonomic neuropathy is a progressive disease with an estimated mortality rate of 44% within 2.5 years of symptomatic diagnosis[46]. The development of this disease occurs with increased age, longer durations of disease and poor glycemic control[47,48]. Diabetic patients with autonomic neuropathy are at an increased risk of developing cardiovascular disease. In

addition, CAD in the presence of autonomic neuropathy yields poorer prognosis for the patients[48].

Damage to the autonomic nerve fibers that innervate the heart and blood vessels leads to abnormal heart rate and altered vascular dynamics[49]. The parasympathetic nerve fibers are the first to be affected. This leads to a relative increase in sympathetic tone resulting in tachycardia along with defective exercise-induced increases in heart rate and blood pressure[48]. Autonomic dysfunction is associated with an increased incidence of arrhythmias. This disease can lead to resting tachycardia, orthostatic hypotension, silent myocardial infarctions, congestive heart failure (CHF) and sudden death[48].

1.4.2 Coronary Artery Disease

The Honolulu Heart Program found a dose-response relationship between glucose tolerance at baseline and the incidence of CAD and all causes of death including those cardiovascular in nature[50]. A patient with diabetes has a 2-4 fold increase in risk of mortality from CAD and increased likelihood of developing severe carotid atherosclerosis[48]. CAD is the most common disease affecting the heart in diabetes and is often the cause of symptomatic left ventricular systolic or diastolic dysfunction[51]. In diabetes, there is accelerated deposition of atherosclerotic plaques in the macrovasculature[52]. Typically a large number of vessels are involved and the distribution of atherosclerotic lesions is more diffuse[48]. In North America, 65-80% of all deaths among diabetic patients are

due to atherosclerosis as compared to 1/3 of all deaths in the general population[52].

Acute myocardial ischemia is a major cause of mortality in patients with diabetes. Furthermore, diabetic patients who suffer from a myocardial infarction have increased risk of mortality compared to their non-diabetic counterparts[48]. In fact, studies have found that in-hospital mortality rate for patients with diabetes is 1.5-2.0 folds higher compared to those without[53-57]. This is primarily due to an increased incidence of CHF with diastolic dysfunction as an important cause. According to the Framingham Study, 15-25% of patients with CHF have diabetes. In men, there is a 2-4 fold increased risk of symptomatic CHF and in women, this risk is increased 5 fold[58]. CHF is frequently a clinical manifestation of end stage cardiovascular complications in patients with diabetes.

1.4.3 Diabetic Cardiomyopathy

Diabetic cardiomyopathy is defined as CHF in the absence of an identifiable etiology. This term was first introduced by Rubler in 1972 when he identified 4 diabetic patients with nephropathy that suffered from CHF with normal coronary arteries and no other etiologies of CHF[59]. However, there was myofibrillar hypertrophy and diffuse interstitial fibrosis. Although there was initial controversy with the existence of this disease, the Washington DC Dilated Cardiomyopathy Study determined that there was an association between diabetes and idiopathic cardiomyopathy[60].

Chronic diabetes affects the cardiac structure and function with patients exhibiting increased left ventricular mass and increased heart rate compared to those without diabetes[48]. Ventricular diastolic dysfunction and decreased compliance of the heart ensue ultimately leading to CHF. In Type I diabetic patients without any known CAD, echocardiographs revealed these patients had diastolic dysfunction with reduced early diastolic filling, increased atrial filling, extension of isovolumetric relaxation and increased numbers of supraventricular premature beats[48]. There is also evidence of impaired coronary blood flow as a result of structural alterations in the microvasculature such as perivascular fibrosis and myocardial capillary basement membrane (BM) thickening[51]. In addition, poor glycemic control is related to abnormal coronary blood flow reserve.

1.4.3.1 Pathogenesis of Diabetic Cardiomyopathy

Chronic hyperglycemia increases oxidative stress through the activation of PKC, hexosamine, AR and nonenzymatic formation of AGEs. In the heart, this leads to endothelial dysfunction and decreased blood flow to the heart through the increase of vasoconstrictor ET-1 and decreased nitric oxide (NO) bioavailability[61] which results in an ischemic environment in the heart. In response, there is an upregulation of ECM proteins, BM thickening and fibrosis. The alteration in blood flow also causes myocyte dysfunction. Both of these factors contribute to myocyte necrosis and as a result there is compensatory

cardiomyocyte hypertrophy. Ultimately, this develops into diabetic cardiomyopathy[61]. Furthermore, the Framingham Heart Study showed that diabetic patients had increased incidence of heart failure, as mentioned above[58].

Diabetic cardiomyopathy involves both cardiomyocytes and endothelial cells and is characterized by cardiomyocyte hypertrophy and focal fibrosis due to the increased production and accumulation of ECM proteins such as collagen and FN[61]. FN is a 250 kDa protein involved in a variety of cellular events such as survival, migration and proliferation[62]. We have previously shown hyperglycemia-induced upregulation of FN in the heart and other target organs of diabetic complications[63]. We have further shown that this upregulation is ET-1-dependent, involves transcription factors NFκB and activator protein 1 (AP-1)[63,64] and is mediated through transcriptional co-activator and histone acetyltransferase (HAT) p300[65].

1.5 Poly (ADP-Ribose) Polymerase (PARP)

PARP is a highly conserved, family of nuclear enzymes that plays a crucial role in eukaryotic cell survival and genomic stability. Poly (ADP-ribosyl)ation is a post-translational modification of glutamate, aspartate and lysine residues catalyzed by PARP activation[66,67]. PARP-1 is the major isoform in intact cells synthesizing approximately 90% of the poly (ADP-ribose)[68] and is one of the most abundant proteins in the nucleus. This 116 kDa protein has 3 main domains: an N-terminal DNA-binding domain with 2 zinc fingers, an

automodification domain and a C-terminal catalytic domain[69,70] [Figure 1.5]. PARP functions as a sensor for both single stranded and double stranded DNA strand breaks as well as a signaling molecule. PARP is activated by DNA strand breaks and bind to the damaged DNA mainly through its second zinc finger domain forming homodimers[70]. This catalyzes the cleavage of NAD+ into nicotinamide and ADP-ribose[70]. The ADP-ribose is used to synthesize nucleic acid-like branched polymers which are covalently attached to nuclear acceptor proteins with the size of these poly (ADP-ribose) polymers varying from a few to up to 200 units[71]. Due to its high negative charge, these polymers can affect target protein functions. Histones are considered to be major acceptors of poly (ADP-ribose) as the negative charge creates electrostatic repulsion between the DNA and the histones[70]. As a result, PARP has been implicated in chromatin remodeling, DNA repair as well as transcriptional regulation (discussed below) [Figure 1.6].

A major regulatory mechanism for this enzyme is auto-poly (ADP-ribosylation) which serves to downregulate enzyme activity[72,73]. PARP activity is also counteracted by two enzymes, poly (ADP-ribose) glycohydrolase (PARG)[74] and ADP-ribosyl protein lyase; both of which break down poly (ADP-ribose). PARG is the only protein known to catalyze the hydrolysis of ADP-ribose polymers to free ADP-ribose. There are 3 splice variants of which the largest is targeted to the nucleus while the smaller two are targeted to the cytoplasm[75]. This enzyme contains a caspase-3 cleavage site which is used during apoptosis

Figure 1.5

A schematic representation of the human PARP-1 protein. The DNA binding domain, located on the N-terminal, includes 2 zinc fingers which bind DNA and is involved in some protein to protein interactions and the nuclear localization signal (NLS) which acts as a DNA-nick sensor and contains a caspase cleavage site. The automodification domain contains the breast cancer-susceptibility protein-carboxy terminus (BRCT) motif common to DNA-repair proteins to serve protein to protein interactions. The C-terminal contains the catalytic domain which shows a high degree of similarities in different species. The NAD+ binding site can be found between residues 785 and 1010.

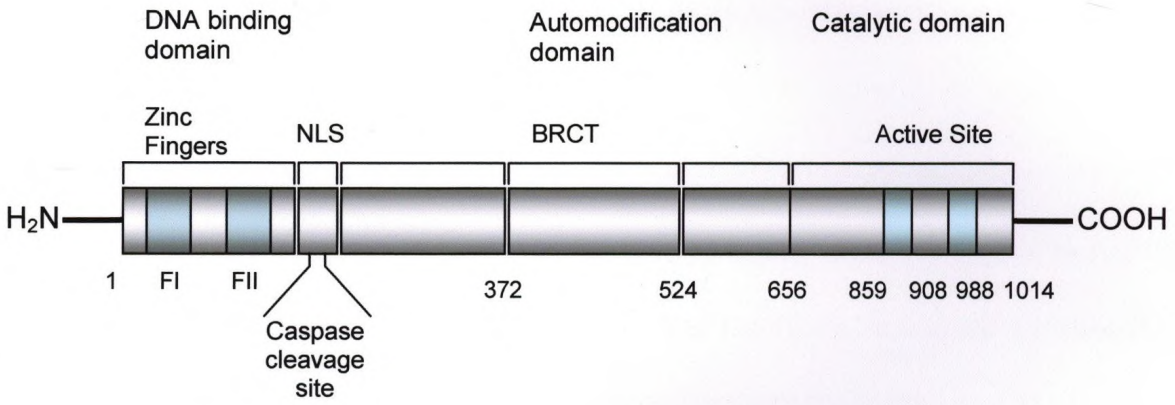
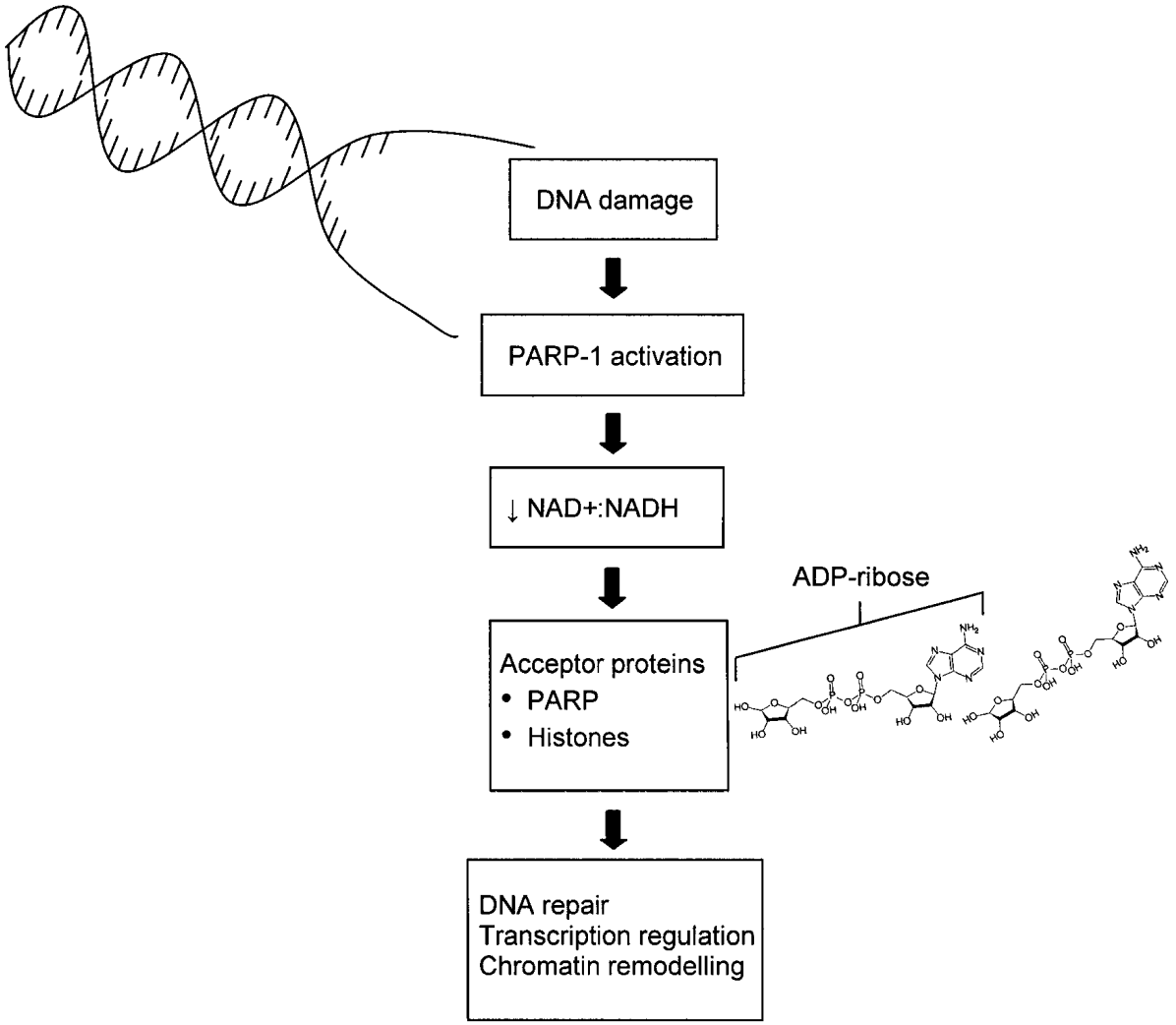

DNA binding domain
Automodification domain
Catalytic domain
Zinc Fingers
NLS
BRCT
Active Site
H2N
COOH
1
FI
FII
372
524
656
859
908
988
1014
Caspase cleavage site

Figure 1.6

A schematic representation of PARP-1 activation. DNA damage leads to the activation of PARP-1 which depletes intracellular NAD+ during poly (ADP-ribosyl)ation of acceptors proteins such as PARP and histones. Poly (ADP-ribosyl)ation of acceptor proteins can result in DNA repair, transcriptional regulation and chromatin remodelling.


DNA damage
PARP-1 activation
↓ NAD+:NADH
ADP-ribose
Acceptor proteins
• PARP
• Histones
DNA repair
Transcription regulation
Chromatin remodelling

and facilitates PARP-induced apoptosis[76]. On the other hand, the role of ADP-ribosyl protein lyase in ADP-ribose metabolism is to remove the protein proximal ADP-ribose monomer[77].

1.5.1 PARP and Oxidative Stress

PARP overactivation depletes NAD+, its substrate, creating a redox imbalance by decreasing the intracellular NAD+:NADH levels[78]. This detrimental process inhibits glycolysis, mitochondrial respiration and ultimately depletes intracellular high energy phosphate levels[70,79,80]. Furthermore, NAD+ is the precursor for NADP+ which is a critical co-factor in the maintenance of reduced glutathione, the primary intracellular antioxidant in the eukaryotic cell (discussed above). This additionally contributes to the susceptibility of the cell to oxidative stress leading to further increases in DNA strand breaks. Low levels of NAD+ also prevent the cell from generating an adequate amount of energy which can lead to cell death. This process acts as a positive feedback mechanism which continues to intensify the oxidative stress experienced by the cells[80].

The role of PARP and increased oxidative stress as a pathogenetic mechanism is best illustrated by rodent models of various diseases whose pathogenesis can be attenuated or prevented with pharmacological inhibition PARP or gene ablation of PARP-1. These animals are protected against a variety of insults including lipopolysaccharide-induced septic shock[81], angiotensin II-induced cardiac hypertrophy[82] and streptozotocin (STZ)-induced diabetes[83,84].

However, in circumstances where PARP-1 is functional this overactivation further exacerbates the oxidative state within the cells[85,86].

1.5.2 PARP and Transcriptional Regulation

PARP-1 has been implicated in the regulation of transcriptional activity in two ways. First, the removal of histones through either poly (ADP-ribosyl)ation or non-covalent association with poly (ADP-ribosyl)ated PARP-1 in response to DNA damage leads to an open chromatin conformation, thereby destabilizing the nucleosome[87,88]. This allows the transcriptional machinery access to the DNA and mobilizes the DNA repair apparatus[89,90]. Secondly, PARP-1 can interact directly with transcription factors and transcriptional co-activators by acting as a component of enhancer/promoter binding complexes[90,91]. Depending on the protein with which PARP is interacting with, this can either have a stimulatory or inhibitory effect on transcription such as NFκB[92] and PARP-1[93], respectively.

It appears that not only is the catalytic activity of PARP is of importance in transcription but also the expression of the protein itself. PARP-1 has been shown to activate NFκB-mediated gene expression through its direct interaction with the transcription factor. Neither the enzymatic nor DNA-binding activity of PARP-1 is required for its co-activator function[94]. Furthermore, the regulation of PARP-1 on gene expression involves transcription co-activator p300. Acetylation of PARP-1 by p300 not only favours its association with NFκB but results in the synergistic co-activation of NFκB by p300[92]. Histone acetylation is believed to

trigger (ADP-ribosyl)ation[95]. Whether the catalytic activity of PARP-1 or the protein itself is in effect may depend on the availability of its substrate, NAD+ [90,91]. On the other hand, although the DNA binding domain allows for the binding of PARP-1 to the nucleosome, the catalytic domain, which does not bind to the nucleosome on its own, cooperates with the DNA binding domain to promote chromatin compaction thus regulating transcription through repression[96]. There also appears to be a reciprocating interplay between PARP-1 and histone H1. Genomic localization of PARP-1 is positively correlated with gene expression while increased H1 binding showed a negative correlation with gene expression[97]. Thus PARP-1 plays a dynamic role in regulating gene transcription.

1.5.3 PARP in Diabetes and its Complications

1.5.3.1 Islet Destruction

Research has shown that mice deficient in the PARP gene are resistant to STZ-induced diabetes[83,84,98,99]. STZ is a widely used endotoxin used to induce experimental diabetes through the destruction of the β cells in the pancreatic islets. STZ is taken up by the GLUT2 transporters[100] where the drug is then metabolized and initiates its damaging effects to DNA by either alkylation[101] or through NO generation[102,103]. PARP deficiency leads to the prevention of NAD+ depletion, the principle event in STZ-induced islet cell death. Wild type mice

injected with STZ showed significantly reduced NAD+ levels compared to their controls however, heterozygous and homozyogous disruption of the PARP gene prevents this STZ-induced effect[83]. Burkart *et al* found that the reduction of NAD+ levels in the $PARP^{+/-}$ and $PARP^{-/-}$ animals was less than 5% of the controls after a 160 mg/kg dose of STZ[83]. These results can be explained by the lack of detectable PARP protein in the $PARP^{+/-}$ and $PARP^{-/-}$ animals[83].

The protective effect of PARP inhibition in the development of diabetes is further corroborated through studies utilizing a pharmacological PARP inhibitor. Studies have found that chemical PARP inhibition can protect against two different models of Type I diabetes, STZ-induced as well as in nonobese diabetic mice[104-107]. The various inhibitors with variable efficacy along with the different models used confirms an important role for PARP in the development of Type I diabetes.

1.5.3.2 Endothelial Dysfunction

In response to neurohormonal mediators and mechanical stress, endothelial cells regulate vascular tone and reactivity. The main endothelium-derived vasodilator is NO. In diabetes, we have shown that eNOS is upregulated[108-110] which would suggest increased NO formation and thus causing vascular relaxation. However, in endothelial cells exposed to high glucose, the production of superoxide anion ($\bullet O_2^-$) may sequester NO thereby reducing its bioavailability[111]. The superoxide anion reacts with NO to form peroxynitrite[112,113]

which readily crosses membranes and thus can travel significant distances[114]. This molecule is considered to be a main trigger of DNA strand breakage. The increase of free radicals is associated with many hyperglycemia-activated biochemical pathways such as glucose auto-oxidation, the aldose reductase pathway, increased DAG formation and subsequent activation of PKC, prostanoid synthesis and accelerated AGE formation. Oxygen- and nitrogen-derived oxidants and free radicals are considered to play a significant role in diabetes-induced endothelial dysfunction[80]. The mechanism by which increased oxidative stress activates the aforementioned pathways associated with diabetic complications appears to be through the inhibition of GAPDH, which becomes poly (ADP-ribosyl)ated by PARP-1[115].

Previous findings showed that diabetes-associated endothelial dysfunction can be prevented through PARP inhibition[116]. In addition, PARP inhibition rapidly reverses the diabetes-induced loss of endothelium function[117]. *In vitro* studies found that PARP inhibition can improve endothelium-dependent relaxant response in diabetic blood vessels[117]. Furthermore, endothelial cells exposed to high glucose had severely depressed levels of cellular high energy phosphates and suppressed NAD+ and NADPH levels[117]. These effects were found to last 1 to 2 days but were prevented with PARP inhibition, both chemically and genetically[80]. *In vivo* studies found that PARP-1 activation in the blood vessels of STZ-induced diabetic mice was apparent 2 weeks after disease onset[116]. This slightly preceded endothelial dysfunction which occurred between the 2nd and 4th

week of diabetes[116]. PARP inhibition 1 week after STZ injection prevented PARP-1 activation and restored normal function of the vasculature[116].

Two mechanisms have been proposed with regards to the protective effects of PARP inhibition on the vasculature. First, PARP inhibition conserves the cellular energetic pools as the activation of PARP-1 depletes NAD+. The second mechanism by which PARP inhibition may be protective is by the prevention of the upregulation of proinflammatory mediators through the activation of NFκB since PARP-1 is a co-activator of this transcription factor.

1.5.3.3 Diabetic Retinopathy

Diabetic retinopathy is associated with many structural changes in the microvasculature of the retina. These include capillary basement membrane thickening, increased permeability of the vessels, retinal pericyte loss and the formation of capillary microaneurysms[118]. With these structural changes, hemodynamic alterations occur such as decreased retinal blood flow, leukostasis and capillary occlusion. Advanced retinopathy manifests as angiogenesis, hemorrhages, formation of fibrotic tissue and tractional retinal detachment and ultimately leads to blindness[118].

The activation of PARP-1 has been shown in microvessels and the ganglionic layer of the retina in diabetes[119]. The increased activation of PARP-1 due to high glucose has also been demonstrated in bovine retinal endothelial cells and pericytes, *in vitro*[120]. This diabetes-induced effect was prevented

through the administration of PJ34, a PARP inhibitor, for 2 months to STZ-treated rats[114]. PARP inhibition also prevented early lesions in diabetic retinopathy such as endothelial and pericyte death. This beneficial effect appears to be mediated through transcription factor NFκB[120]. Furthermore, PARP inhibition has been shown to prevent the diabetes-induced upregulation of vascular endothelial growth factor, an angiogenic factor, in the retinas of rats[119].

As mentioned previously, peroxynitrite plays an important role in the activation of PARP-1 as this molecule readily crosses membranes and reacts with DNA to induced damage. Leukocyte entrapment, an early event in diabetic retinopathy, has been shown to be prevented with the treatment of FP15, a peroxynitrite decomposition catalyst, or PJ34, a PARP inhibitor[121]. We have also shown ET-1-dependent expression of ECM protein FN is mediated by PARP-1 in both diabetic and hyperhexosemic rodents in the retina[122]. Taken together, these studies demonstrate an important role for PARP-1 in mediating the development of diabetic retinopathy.

1.5.3.4 Diabetic Nephropathy

Diabetic nephropathy is characterized by glomerular basement membrane thickening and mesangial expansion as a result of ECM protein production and accumulation[123,124]. Alterations in the permeability of the capillaries results in increased protein excretion such as albumin and can be attributed to increased glomerular filtration pressure. Changes in the basement membranes lead to

glomerular occlusion, fibrosis, decreased filtering capacity and eventual kidney failure[118].

Although few studies have been conducted to determine the role of PARP-1 in the development of diabetic nephropathy, the ones which have been done indicates the importance of this enzyme in the pathogenesis of this disease. Inhibition of PARP-1 with nicotinamide in diabetic rodents significantly reduced glomerular deposition of ECM proteins and thus mesangial expansion[125]. Furthermore, other pharmacological inhibitors of PARP, ABA and 1,5-isoquinolinediol, attenuated the diabetes-induced activation of PARP-1 in the tubuli early in the disease[126]. The inhibition of PARP-1 in the diabetic animals also led to decreased mRNA expression of ET-1[126]. In keeping which such studies, we have shown diabetes- and galactosemia-induced upregulation of ET-1 in the kidneys of rats and mice, respectively. PARP inhibition prevented this hyperhexosemia-induced effect which coincided with reduced oxidative stress[122]. These findings illustrate the importance of PARP-1 activation in the early stages of this disease as well as in the progression of this disease.

1.5.3.5 Diabetic Peripheral Neuropathy

There are several types of diabetic peripheral neuropathies, of which the most common are distal sensory neuropathy, a mild distal sensory impairment with minimal motor deficits and distal small fiber neuropathy, which is paradoxically characterized by painfulness and impaired perception of pain and

temperature[127]. Due to the long axons of the peripheral nerves, they are highly dependent on their endoneurial microenvironment for oxygen, nutrients and metabolic waste removal[127]. Diabetes-induced damage can result from both hyperglycemia and ischemia as both decrease neural blood flow[128,129]. Ultimately, diabetic peripheral neuropathy can lead to ulceration of the lower limb and eventual amputation.

The progressive slowing of sensory and motor neuron conductance is observed in diabetic rats and mice. This was prevented with PARP inhibition which maintained neuronal phosphocreatine levels as well, improved endoneural blood flow[130]. In addition, established diabetic peripheral neuropathy in mice can be reversed with PARP inhibition by restoring sensory and motor neuronal conduction[131]. Of interest to note, nicotinamide, a weak PARP inhibitor with antioxidant properties, has been shown to reverse endoneural blood flow in a dose-dependent manner as well, prevent diabetes-induced decreased sensory nerve conduction velocity[132]. In advanced diabetic neuropathy, PARP inhibition has been shown to prevent intraepidermal nerve fiber loss and neuropathic pain in both rats and mice[133].These findings suggest an important role for PARP overactivation in the diabetes-induced peripheral neuronal dysfunction.

1.5.3.6 Diabetic Cardiomyopathy

As mentioned previously, diabetic cardiomyopathy manifests as a result of biochemical, structural and mechanical changes brought on by chronically

elevated blood glucose levels. Increased oxidative stress has been implicated as a major mediator of these alterations as treatment with antioxidants such as curcumin has been shown to attenuate these effects[110]. Endothelial dysfunction, an early manifestation of hyperglycemia, precedes changes in the structure of the vasculature. Endothelial dysfunction and diabetic cardiomyopathy maybe be interrelated as an impaired endothelium can result in myocardial ischemia and secondarily affect cardiac function[114].

Increased PARP-1 activation has been shown in the heart of both STZ-induced diabetic rats and nonobese diabetic mice. These animals exhibited altered vascular function as determined by decreased left ventricular systolic pressure and increased left ventricular end-diastolic pressure[134]. Pharmacological PARP inhibition with PJ34 significantly improved both parameters in the two models of diabetes[134]. Previously, our lab has shown that the diabetes-induced expression of ECM protein FN is dependent on vasoactive factor ET-1[63]. Furthermore, we demonstrated that these changes were mediated through transcription factors NFκB and AP-1[64]. We also demonstrated a role for p300 in this pathogenetic pathway possibly acting in conjunction with PARP-1 in the retina and hearts of diabetic rodents[65]. Although some of the functional alterations of PARP-1 have been demonstrated in the heart in diabetes[117], it is not clear whether PARP-1 activation is important in mediating these structural changes.

1.6 Hypothesis

Hyperhexosemia-induced structural alterations in the heart can be prevented by PARP inhibition.

1.7 Specific Aims

1. To determine the effects of PARP-1 gene ablation in the development of galactosemia-induced structural alterations in the heart of mice.

2. To determine the effects of pharmacological PARP inhibition in the heart of STZ-induced diabetic rats.

3. To determine whether PARP inhibition can prevent glucose-induced hypertrophy and oxidative DNA damage in cardiomyocytes.

Chapter 2: Materials and Methods

2.1 *In Vivo* Studies

All animals were cared for according to the Guiding Principle in the Care and Use of Animals. All experiments were approved by the University of Western Ontario Council on Animal Care Committee.

2.1.1 Mice

$PARP^{-/-}$ mice and their corresponding wild type (WT) strain of a similar background (129S/SvlmJ) were purchased from Jackson Laboratories (ME, USA). At 6 weeks of age, males from both $PARP^{-/-}$ and WT groups were randomly divided into two groups. They were given either a standard rodent diet (CO) containing 19% protein, 5% fat and 5% crude fiber or a similar feed enriched with 30% galactose (G, Test Diet, IN, USA). These animals were maintained for a period of 2 months.

2.1.2 Rats

Male Sprague-Dawley rats weighing 200-250 g were purchased from Charles River (QC, Canada) and randomly divided into 3 groups: control (CO), diabetic (DM) or diabetic treated with 3-aminobenzamide (DM-ABA). Diabetes was induced by a single dose of STZ (65 mg/kg in citrate buffer, pH = 5.6), intravenously, while the control animals were injected with the same volume of citrate buffer. STZ-induced diabetes in the rat is a well-established model for

type 1 diabetes. ABA, reconstituted in saline, was administered (30 mg/kg/day, Sigma Aldrich, ON, Canada) intraperitoneally for a period of 4 months. This dose is based on previous studies[130]. The animals were monitored daily and the diabetic animals were implanted with insulin implants that released small doses of insulin to prevent ketonuria (release rate of 2 U/day, Linshin Canada Inc, ON, Canada). Clinical monitoring of the mice and the rats was performed through the regular assessment of body weight and blood glucose concentrations (see Results).

The animals were anesthetized and sacrificed. Their heart tissues were weighed and were snap frozen for gene expression analysis as well placed in 10% formalin to embed in paraffin for immunohistochemical analysis.

2.2 *In Vitro* Studies

2.2.1 Human Umbilical Vein Endothelial Cells

Endothelial cells (American Type Culture Collection, MD, USA) were cultured in endothelial cell growth medium (Clonetics, MD, USA) and exposed to varying concentrations (0.5 mmol/L, 1 mmol/L, 2.5 mmol/L and 5 mmol/L) of PARP inhibitor ABA dissolved in ddH_20. The cells were serum-starved overnight and treated with ABA for 1 hr. Next, 5 mmol/L of glucose (low glucose, LG) or 25 mmol/L of glucose (high glucose, HG) was added and the cells were maintained

for 24 hrs. The endothelial cells were subsequently used for RNA extraction and cDNA synthesis (see below) to determine the dose-response.

2.2.2 Cardiomyocyte Isolation and Culture

Myocytes were prepared from neonatal Sprague-Dawley rat heart ventricles as previously described[135,136]. Six day old rat hearts were removed under aseptic conditions. The ventricles were minced and digested through a series of 0.1% collagenase treatments. Non-myocytes were removed through differential attachment. The isolated cardiomyocytes were then plated onto 6 well culture plates (Primaria™ Falcon, NJ, USA) at a density of 3.0×10^4 cells/cm^2. The cells were maintained for 48 hrs in Dulbecco's Modified Eagle's Medium/Ham's F-12 supplemented with 10% fetal bovine serum, 10 µg/mL transferrin selenium, 10 µg/mL insulin, 10 ng/mL selenium, 50 units/mL penicillin, 50 µg/mL streptomycin, 2 mg/mL bovine serum albumin, 5 µg/mL linoleic acid, 3 mN pyruvic acid, 0.1 mmol/L minimum essential medium non-essential amino acids, 10% vitamin, 0.1 mmol/L bromodeoxyuridin, 100 µm L-ascorbic acid, and 30 mmol/L HEPES (pH 7.1). The cells were serum-starved overnight and treated with 5 mmol/L of PARP inhibitor ABA for 1 hr. Either 5 mmol/L of glucose or 25mmol/L of glucose was subsequently added to the cardiomyocytes and they were maintained for 24 hrs. LG was used as controls. L-glucose was used as a control to account for differences in optical density. The cardiomyocytes were used for hypertrophy and DNA damage analyses.

2.2.3 Cell Viability

Cell viability of the endothelial cells was measured by trypan blue dye exclusion, and cell proliferation was evaluated by the microculture of tetrazolium assay using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT)[137]. Cells were seeded in 96-well plates (3,200 cells/well, 10,000 cells/cm^2). After 24 hrs, experimental agents were applied (100 µL) and the cultures were incubated for 1, 3 and 5 days at 37°C. XTT (50 µg) and 0.38 mg of phenazine methosulfate were added to each well (50 µL) after cell inoculation. The cells were incubated at 37°C for 4 hrs, and the plates were mixed on a mechanical plate shaker. Absorbance at 450 nm was measured with the Bio-Rad model 3550 microplate reader (Bio-Rad Laboratories, CA, USA). All experiments were performed in triplicate.

2.3 RNA Extraction and Real Time RT-PCR Analysis

RNA was isolated from rat and mice heart tissues as previously described[63]. Tissue homogenized in TRIzol reagent (Invitrogen Canada Inc, ON, Canada) was used to isolate the RNA from 50-100 mg of tissue and chloroform was added. Following centrifugation, the aqueous phase was collected and the RNA was precipitated out with isopropyl alcohol. cDNA was subsequently synthesized from the total RNA by using the Superscript II system (Invitrogen Canada Inc, ON, Canada). The mRNA levels of ET-1, FN, p300, heme

oxygenase 1 (HO-1), atrial naturetic peptide (ANP) and α- and β-myosin heavy chain (MHC) were quantified using LightCycler™ (Roche Diagnostics Canada, QC, Canada). The reaction mixture (total volume 20µl) consisted of the following reagents: 10 µl of SYBR® Green Taq Ready Mix (Sigma-Aldrich, ON, Canada), 1.6µl of 25 mM $MgCl_2$, 1 µl of each forward and reverse 10 µM primers, 5.4 µl of H_2O and 1 µl of cDNA template. The data was normalized to housekeeping gene 18S rRNA to account for reverse transcription efficiencies. The primer sequences are outlined in Table 2.1.

2.4 Histological Analysis

Formalin fixed tissues were embedded in paraffin, sectioned to 5 µM thickness and placed on positively charged slides. Trichrome and hematoxylin and eosin staining was performed to assess ECM protein deposition and fibrosis.

2.5 Immunohistochemistry

The heart tissues were also analyzed for 8-hydroxy-2'-deoxyguanosine (8-OHdG, Chemicon International, Inc, CA, USA), a sensitive marker for oxidative DNA damage[138] and nitrotyrosine (NT, Cayman Chemical Company, MI, USA), a marker for oxidative protein damage. The slides were stained using Vectastain

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Table 2.1 Real Time RT-PCR Primer Sequences

Gene	Primer Sequence (5' →3')
18S rRNA (human/rat/mouse)	GTAACCCGTTGAACCCCATT CCATCCAACGGTAGTAGCG
ET-1 (rat)	GCTCCTGCTCCTCCTTGATG CTCGCTCTATGTAAGTCATGG
ET-1 (mouse)	TTAGCAAGACCATCTGTGTG GAGTTTCTCCCTGAAATGTG
FN (human)	GATAAATCAACAGTGGGAGC CCCAGATCATGGAGTCTTTA
FN (rat)	CCAGGCACTGACTACAAGA CATGATACCAGCAAGGAGTT
FN (mouse)	CGGTAGGACCTTCTATTCCT GATACATGACCCCTTCATTG
p300 (rat)	GGGACTAACCAATGGTGGTG ATTGGGAGAAGTCAAGCCTG
p300 (mouse)	AGGCAGAGTAGGACAGTGAA CTCAGTCTGGGTCACTCAAT
HO-1 (mouse)	TGAACACTCTGGAGATGACA AACAGGAAGCTGAGAGTGAG
ANP (mouse)	CTGCTAGACCACCTGGAGGA AAGCTGTTGCAGCCTAGTCC
α-MHC (rat)	TCTACCAGATCCTGTCCAAC ACTTCATGTTCCCATAGTGC
β-MHC (rat)	CCTACGATTATGCGTTCTTC CTGACTTGTCAGCTTCTTCC

Elite (Vector Laboratories Canada, Inc, ON, Canada) for 8-OHdG and EnVision (Dako Canada, Inc, ON, Canada) for NT. The chromagen 3,3' diaminobenzine (Sigma-Aldrich, ON, Canada) was used for detection. Ten random fields were examined by two investigators unaware of the experimental treatment. 8-OHdG immunoreactivity was assessed by the presence of positively stained nuclei while NT was evaluated by comparing the relative staining intensity in the cytoplasm.

2.6 Immunofluorescence

Paraffin-embedded rat heart tissues were analyzed for PARP-1 activation. A monoclonal mouse PARP-1 antibody was used to assess PARP-1 activity (1:200, Biomol International L.P., PA, USA). The cardiomyocytes were stained for phospho-H2A.X which indicates repaired DNA, to assess for double stranded DNA breaks[139]. The cells were fixed in methanol and incubated with a mouse monoclonal phospho-H2A.X antibody (1:200, Abcam, Inc, MA, USA). An Alexa Fluor® 488-labelled anti-mouse secondary antibody (Invitrogen Canada, Inc, ON, Canada) was used for detection using a fluorescent microscope (Olympus BX51, Olympus Canada Inc, ON, Canada) and Northern Eclipse software (Empix Inc, ON, Canada). Hoechst 33342 dye (1μg/mL, Invitrogen Canada Inc, ON, Canada) was used to visualize the nuclei. Ten random fields were examined by two investigators unaware of the experimental treatment.

2.7 Histone Deacetylase (HDAC) Activity Assay

Nuclear protein extracts were prepared from the heart tissues (50-100 mg) of both rats and mice as previously described[140]. HDAC activity from 100 µg of nuclear extract was determined using the HDAC activity assay kit (ab1432, AbCam Inc, MA, USA) as per manufacturer's instructions. HDAC activity is expressed as the absolute amount of deacetylated lysine generated in each sample (µmol/L).

2.8 Measurement of Cardiomyocyte Hypertrophy

Cell surface area of cardiomyocytes was measured to assess cellular hypertrophy. The cells were visualized using a Leica DMIL inverted microscrope (Leica Wetzlar, Germany) equipped with a Polaroid digital camera. The images were captured at 10X magnification. Cell surface area was determined using Mocha™ Software (SPSS, IL, USA) from 50 randomly selected cells per experiment, then averaged and expressed as μm^2.

2.9 Statistical Analysis

The data are expressed as mean ± SEM and were analyzed by Bonferroni/Dunn test for multiple comparisons. Differences were considered to be statistically significant at values of $p < 0.05$.

Chapter 3: Results

3.1 Clinical Monitoring

Both rats and mice were monitored for diabetic dysmetabolism by evaluating body weight gain and blood glucose levels. The diabetic rats showed significantly reduced body weight gain and increased blood glucose levels compared to their non-diabetic controls [Table 3.1]. $PARP^{-/-}$ had no effect on body weight gain. Furthermore, no significant difference in body weight gain between the mice fed a normal diet and the mice fed a high galactose diet were seen in the $PARP^{-/-}$ and WT animals. However, both strains of mice fed a high galactose diet exhibited increased blood hexose sugar levels [Table 3.1].

3.2 Hyperhexosemia-Induced PARP Activation and Increased Oxidative Stress is Prevented with PARP Inhibition

We first determined whether diabetes does in fact increase the activation of PARP-1 in the heart. Rat heart sections were immunofluorescently assessed for PARP-1 activation using a monoclonal PARP-1 antibody. In the diabetic animals, there was increased nuclear positivity for PARP-1 compared to the control counterparts [Figure 3.1]. PARP inhibition with ABA treatment prevented this diabetes-induced effect.

Next, we assessed hyperhexosemia-induced oxidative damage in the heart. The WT mice fed a galactose rich diet showed increased nuclear staining

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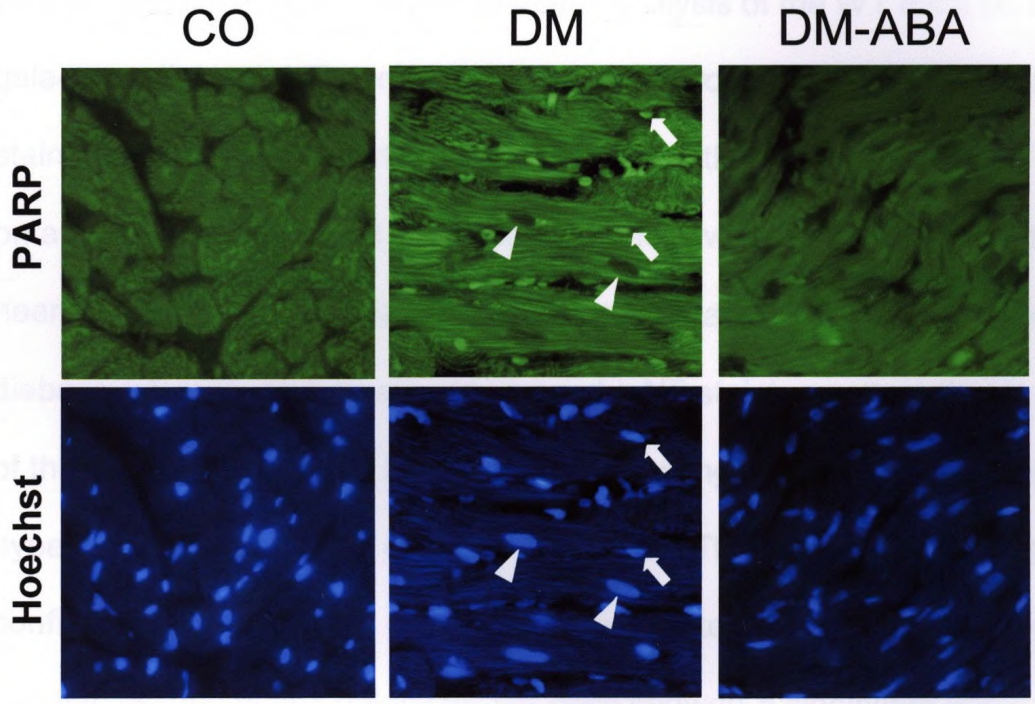
Table 3.1 Clinical Monitoring of Animals

Animal Groups	Body Weight (g)	Blood Reducing Sugar (mmol/L)
Mice		
WT CO	25.6±0.92	6.7±0.51
WT G	24.27±1.54	19.6±1.48*
$PARP^{-/-}$ CO	25.76±0.81	8.2±1.66
$PARP^{-/-}$ G	25.92±0.78	21.7±4.13*‡
Rats		
CO	613.5±13.8	7.22±1.6
DM	484.0±40.3*	18.4±6.8*
DM-ABA	526.1±17.9*	22.6±4.7*

* $p < 0.05$ vs. CO or WT CO, ‡ $p < 0.05$ $PARP^{-/-}$ CO

Figure 3.1

Micrographs demonstrating the prevention of PARP activation in the hearts of diabetic rats with PARP inhibitor ABA. The DM animals showed increased number of PARP positive nuclei (arrows) [CO = control, DM = diabetes mellitus, ABA = 3-aminobenzamide; arrowheads = negative nuclei, arrows = positive nuclei; bar represents 20μm; Hoechst = Hoechst 33342, a nuclear marker].

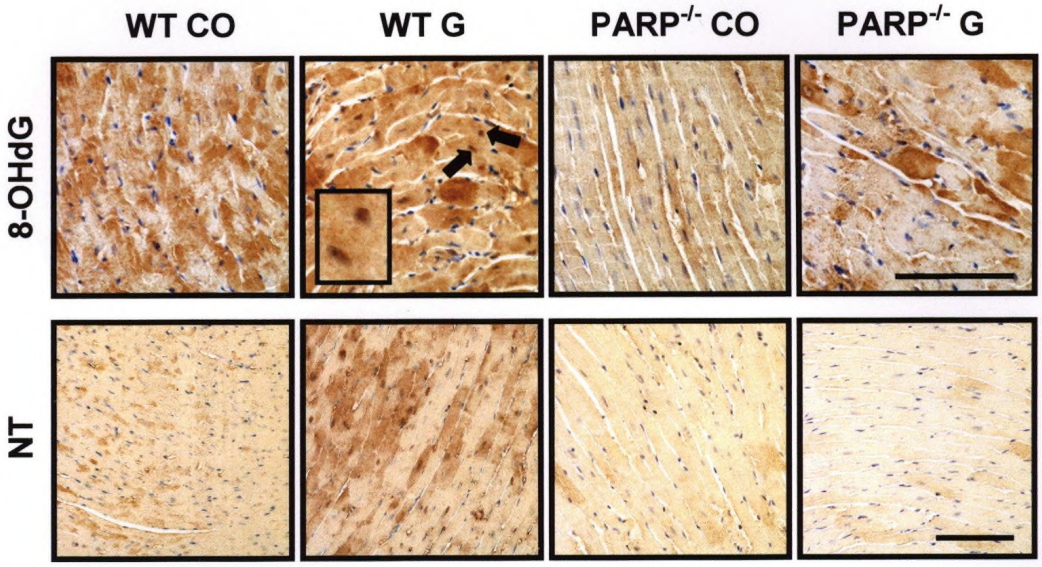

CO
DM
DM-ABA
PARP
Hoechst

of 8-OHdG, a marker of oxidative DNA damage, compared to the WT control mice [Figure 3.2]. This was paralleled in the rats where the STZ-induced diabetic rat hearts exhibited increased nuclear staining of 8-OHdG compared to their age-matched controls [Figure 3.2]. Interestingly, the galactose-fed $PARP^{-/-}$ mice and the diabetic rats treated with ABA showed complete normalization of the hyperhexosemia-induced effects [Figure 3.2].

We then tested the effect of PARP inhibition on oxidative stress by using another sensitive oxidant injury marker. Analysis of the WT mice on the high galactose diet with NT, a marker of oxidative protein damage, showed increased staining intensity in the cytoplasm compared with the hearts of the WT animals on a normal diet [Figure 3.2]. A similar pattern was also seen in the diabetic rat hearts [Figure 3.2]. Cardiac tissue from the galactose-fed $PARP^{-/-}$ mice and the diabetic rats with ABA treatment showed a NT staining pattern that is reminiscent of their respective control hearts; again indicating complete attenuation of the hyperhexosemia-induced effects [Figure 3.2]. These results were further confirmed by the analysis of a pro-oxidant marker HO-1 transcript levels. Cardiac tissues of WT galactose-fed mice showed a significant upregulation of HO-1 mRNA [Figure 3.3]. Inhibition of PARP also prevented this hyperhexosemia-associated increased in the mice [Figure 3.3]. These findings are in support of Garcia Soriano *et al* who found that PARP-1 activation increased NT positivity in the blood vessels of diabetic mice and that pharmacological PARP inhibition can prevent this diabetes-induced effect[116].

Figure 3.2

Representative micrographs showing immunohistochemical analyses of oxidative stress markers 8-OHdG and NT in the mice (upper panels) and rat (lower panels) heart tissues. Increased nuclear 8-OHdG and cytoplasmic NT staining were present in the WT G mice and DM rats indicating increased oxidative stress. Such changes were attenuated with PARP inhibition [8-OHdG = 8-hydroxy-2'-deoxyguanosine, NT = nitrotyrosine; WT = wild type, $PARP^{-/-}$ = PARP-1 knockout, CO = normal rodent diet, G = 30% galactose-enriched diet, DM = diabetes mellitus, ABA = 3-aminobenzamide; arrows = positive nuclei, inset shows higher magnification; bar represents 20μm, magnification is the same for all micrographs in each panel].


WT CO
WT G
PARP-/- CO
PARP-/- G
8-OHdG
NT

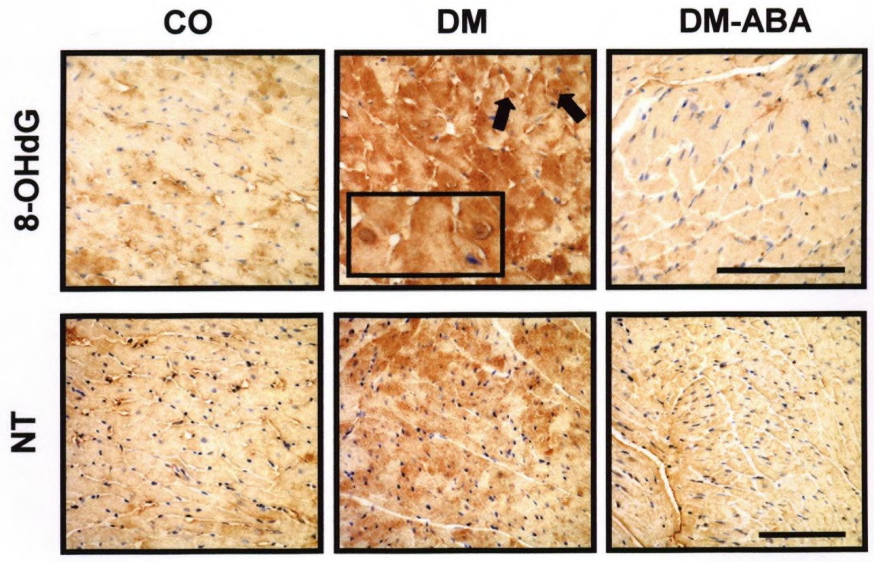
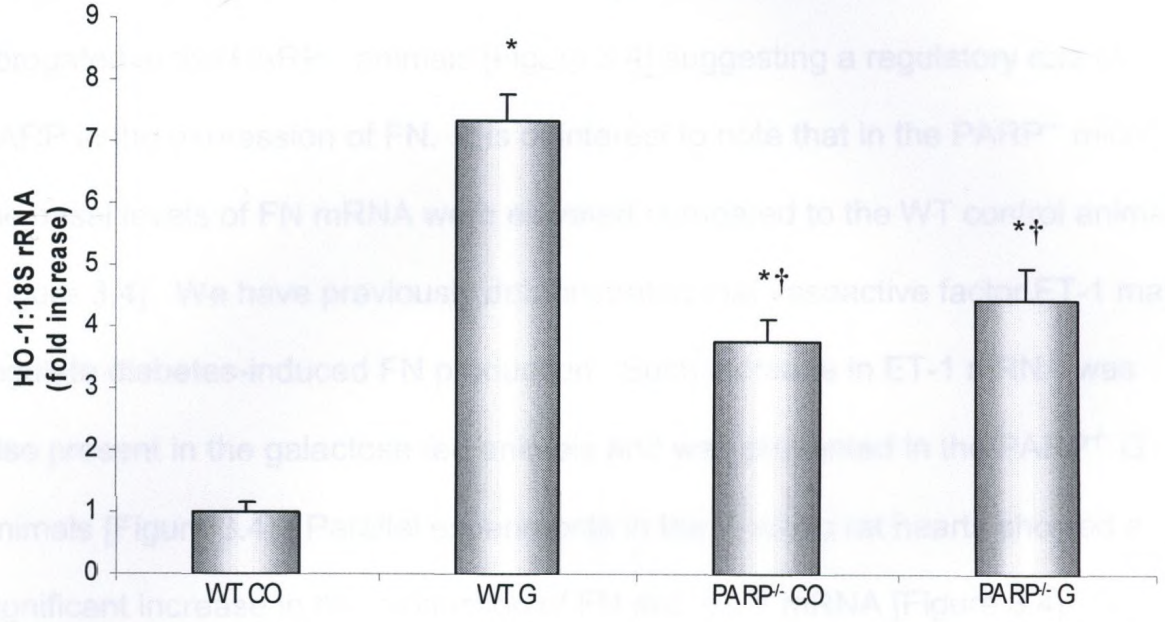

CO
DM
DM-ABA
8-OHdG
NT

Figure 3.3

Real time RT-PCR analysis of heme oxygenase 1 (HO-1), a marker of oxidative stress, in the hearts of mice. Galactose feeding resulted in significantly increased expression of the transcript in the WT animals. PARP gene ablation attenuated this galactose-induced effect [WT = wild type, $PARP^{-/-}$ = PARP-1 knockout, CO = normal rodent diet, G = 30% galactose-enriched diet; * $p < 0.05$ compared to WT CO, † $p < 0.05$ compared to WT G; n=5; data expressed as mean ± SEM].


HO-1:18S rRNA
(fold increase)
9
8
7
6
5
4
3
2
1
0
*
*†
*†
WT CO
WT G
PARP-/- CO
PARP-/- G

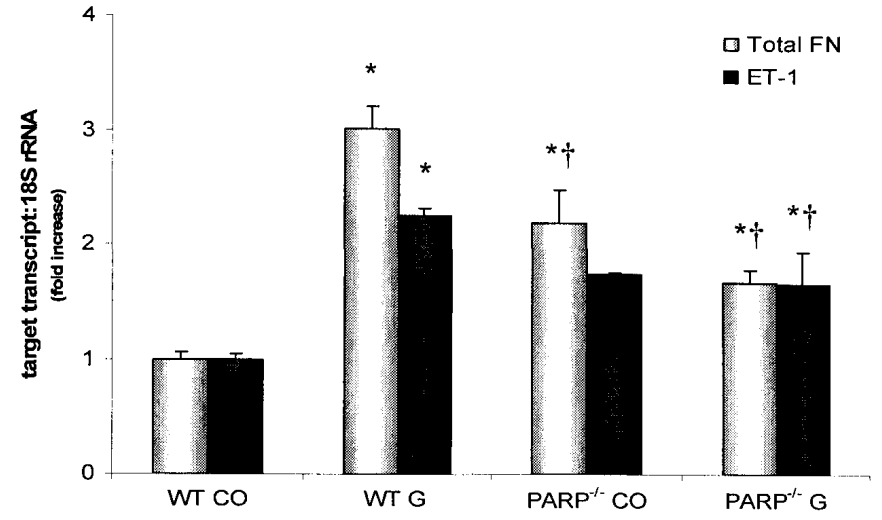
3.3 Hyperhexosemia-induced ECM Protein Production is mediated through a PARP-Dependent Pathway

First, we studied one of the two important parameters of diabetic cardiomyopathy, ECM protein production. WT mice fed with a galactose rich diet had significantly upregulated mRNA expression of FN when compared to their WT controls [Figure 3.4]. However, such galactose-induced effects were abrogated in the $PARP^{-/-}$ animals [Figure 3.4] suggesting a regulatory role of PARP in the expression of FN. It is of interest to note that in the $PARP^{-/-}$ mice, the basal levels of FN mRNA were elevated compared to the WT control animals [Figure 3.4]. We have previously demonstrated that vasoactive factor ET-1 may regulate diabetes-induced FN production. Such increase in ET-1 mRNA was also present in the galactose-fed animals and was prevented in the $PARP^{-/-}$ G animals [Figure 3.4]. Parallel experiments in the diabetic rat hearts showed a significant increase in the production of FN and ET-1 mRNA [Figure 3.4]. Inhibition of PARP through the daily administration of ABA in the diabetic animals completely prevented the diabetes-induced effects on these genes [Figure 3.4].

Histological analyses of the heart tissues were done on both the mice and the rats. As with the molecular studies, hyperhexosemia in the WT mice lead to areas of fibrosis as demonstrated by trichrome staining [Figure 3.5]. Inhibition of PARP prevented these hyperhexosemia-induced effects in the heart [Figure 3.5]. Furthermore, focal fibrosis was also present in the hearts of the untreated

Figure 3.4

Real time RT-PCR analyses of the vasoactive factor, endothelin 1 (ET-1) and the extracellular matrix protein, fibronectin (FN) in the hearts of mice and rats. Hyperhexosemia increased expression of both transcripts in the WT G and the DM animals which was prevented with PARP inhibition [WT = wild type, $PARP^{-/-}$ = PARP-1 knockout, CO = normal rodent diet, G = 30% galactose-enriched diet, DM = diabetes mellitus, ABA = 3-aminobenzamide; * $p < 0.05$ compared to WT CO and CO, † $p < 0.05$ compared to WT G and DM; n=5; data expressed as mean ± SEM].


target transcript:18S rRNA
(fold increase)
4
3
2
1
0
Total FN
ET-1
*
*
*†
*†
*†
WT CO
WT G
PARP-/- CO
PARP-/- G

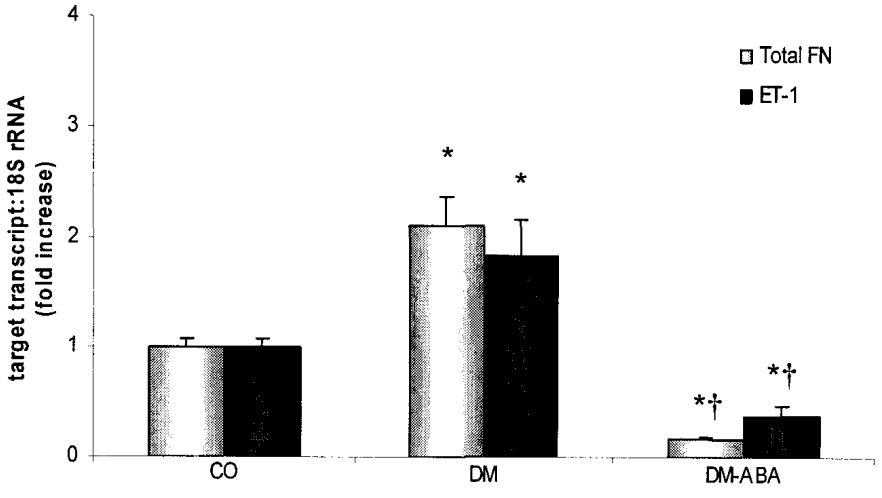
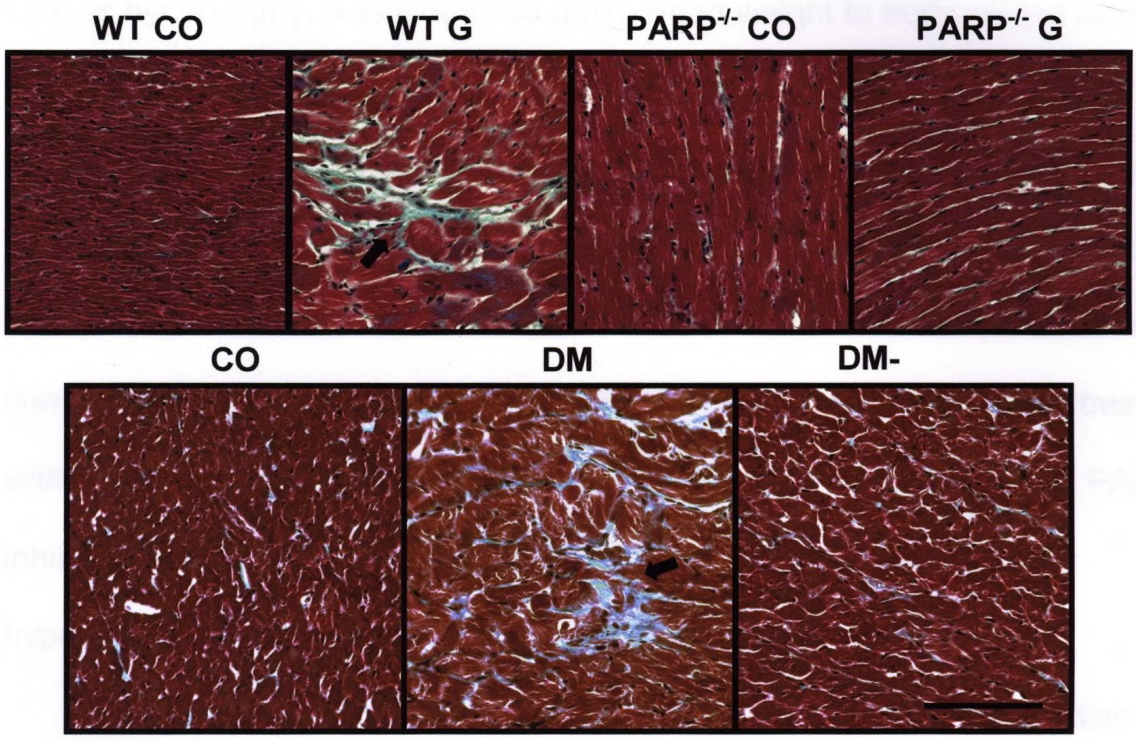

target transcript:18S rRNA
(fold increase)
4
3
2
1
0
Total FN
ET-1
*
*
*†
*†
CO
DM
DM-ABA

Figure 3.5

Micrographs depicting focal fibrosis (arrows) in the heart with trichrome stain in the WT G and DM mice (upper panel) and rats (lower panel), respectively. This hyperhexosemia-induced effect was prevented with PARP inhibition [WT = wild type, $PARP^{-/-}$ = PARP-1 knockout, CO = normal rodent diet, G = 30% galactose-enriched diet, DM = diabetes mellitus, ABA = 3-aminobenzamide; arrows = focal fibrosis; bar represents 20μm; n=3].


WT CO
WT G
PARP-/- CO
PARP-/- G
CO
DM
DM-

diabetic rats but was absent in the myocardium of ABA-treated diabetic rats [Figure 3.5].

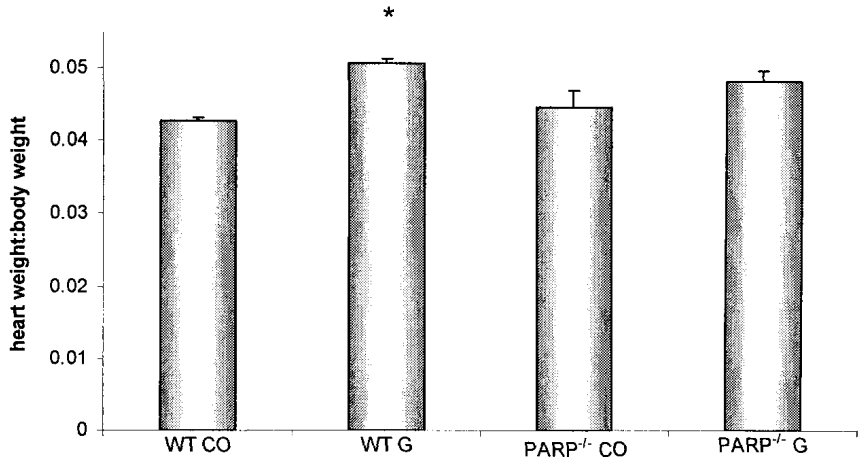
3.4 PARP Activation mediates Hyperhexosemia-Induced Cardiac Hypertrophy

The second structural characteristic important in diabetic cardiomyopathy, cardiac hypertrophy, was examined next. Heart weight to body weight ratios were determined to assess myocardial hypertrophy. These ratios were significantly increased in the hyperhexosemic mice and rats compared to their respective controls [Figure 3.6]. Interestingly, this hyperhexosemia-induced hypertrophy was not seen in the $PARP^{-/-}$ mice fed galactose compared to its corresponding control and was significantly lowered in the diabetic rats treated with ABA compared to the diabetic rats [Figure 3.6]. This indicates that PARP inhibition can, at least in part, prevent hyperhexosemia-induced cardiac hypertrophy.

We further used surrogate molecular markers to assess myocardial hypertrophy. Hence, the mice heart tissues were analyzed for cardiac hypertrophy marker ANP mRNA expression. The WT mice fed a galactose enriched diet showed a significant increase in ANP levels in comparison to the WT control animals, which were prevented in the galactose-fed $PARP^{-/-}$ mice [Figure 3.7]. Interestingly, similar to the pattern of expression of FN mRNA,

Figure 3.6

Heart weight to body weight ratios of mice and rats showing hyperhexosemia-induced cardiac hypertrophy which was prevented with PARP inhibition [WT = wild type, $PARP^{-/-}$ = PARP-1 knockout, CO = normal rodent diet, G = 30% galactose-enriched diet, DM = diabetes mellitus, ABA = 3-aminobenzamide; * $p < 0.05$ compared to WT CO and CO, † $p < 0.05$ compared to WT G and DM; n=5; data expressed as mean ± SEM].


heart weight:body weight
0.05
0.04
0.03
0.02
0.01
0
*
WT CO
WT G
PARP-/- CO
PARP-/- G

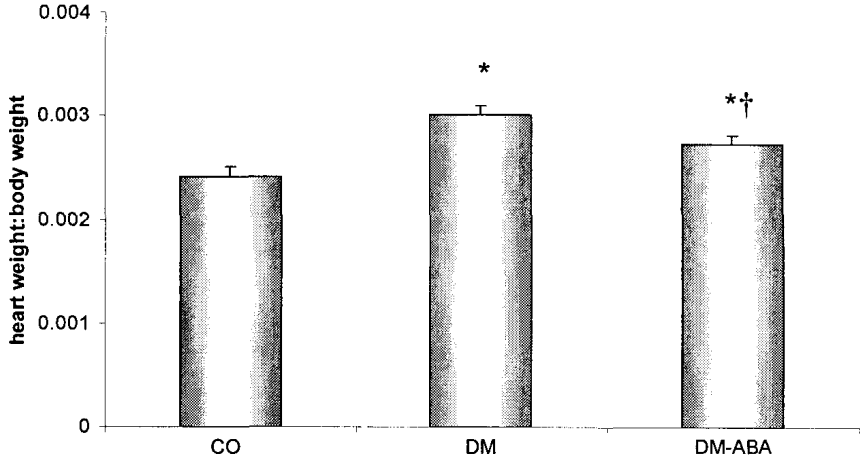
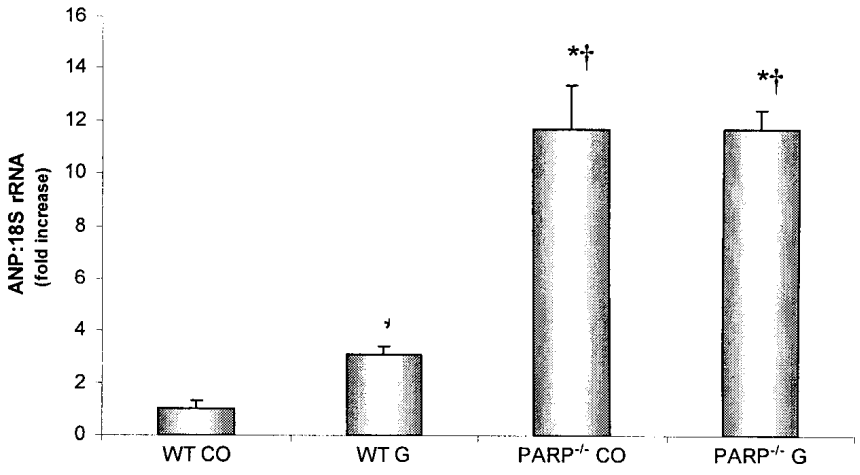
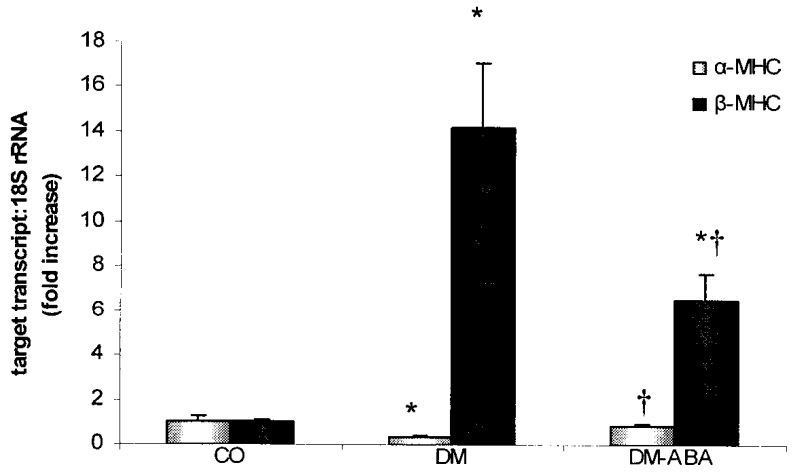

heart weight:body weight
0.004
0.003
0.002
0.001
0
*
*†
CO
DM
DM-ABA

Figure 3.7

Real time RT-PCR analyses of molecular markers of cardiac hypertrophy ANP and α- and β-MHC in the mice and rats, respectively. Hyperhexosemia-induced hypertrophy was prevented with PARP inhibition [WT = wild type, $PARP^{-/-}$ = PARP-1 knockout, CO = normal rodent diet, G = 30% galactose-enriched diet, DM = diabetes mellitus, ABA = 3-aminobenzamide; * $p < 0.05$ compared to WT CO and CO, † $p < 0.05$ compared to WT G and DM; n=5; data expressed as mean ± SEM].


ANP:18S rRNA
(fold increase)
16
14
12
10
8
6
4
2
0
*†
*†
†
WT CO
WT G
PARP-/- CO
PARP-/- G


target transcript:18S rRNA
(fold increase)
18
16
14
12
10
8
6
4
2
0
*
α-MHC
β-MHC
*†
*
†
CO
DM
DM-ABA

basal levels of ANP were also significantly elevated in the $PARP^{-/-}$ mice [Figure 3.7].

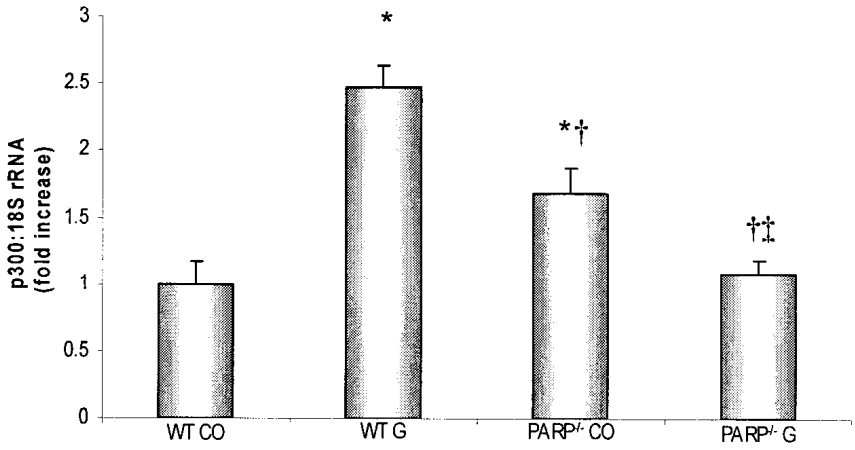
In rats, isotype switching from α-MHC to β-MHC is characteristic of cardiomyocyte hypertrophy[141]. We found that diabetes significantly downregulated α-MHC and that the treatment of the diabetic rats with ABA prevented the downregulation of α-MHC [Figure 3.7]. When β-MHC mRNA expression was determined, we found that the diabetic animals had significantly upregulated levels of the β-MHC transcript [Figure 3.7]. However, the diabetic animals treated with ABA had significantly lowered expression of β-MHC compared to the diabetic animals while α-MHC expression was the same as the CO animals [Figure 3.7]. These molecular findings are consistent with the heart weight to body weight ratios indicating myocardial hypertrophy.

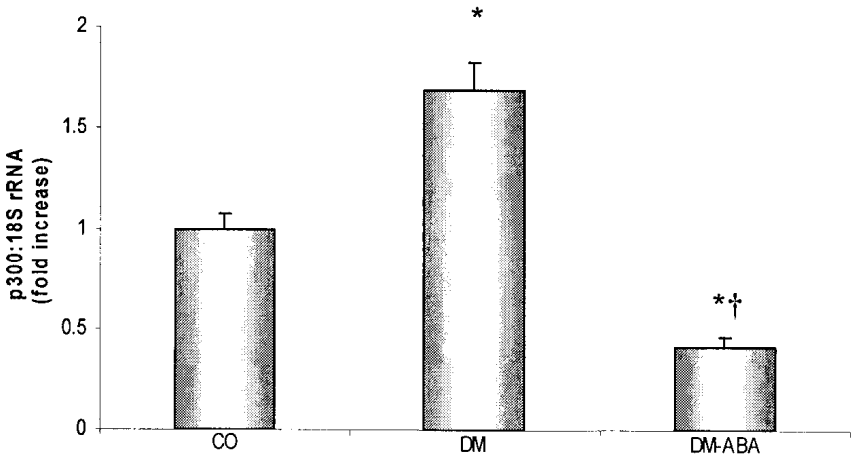
3.5 PARP may regulate Gene Expression at the Transcriptional Level

At the chromosomal level, alteration of transcriptional co-activators may influence PARP-1-dependent gene expression. Thus, we investigated transcriptional co-activator p300, a histone acetylator, and histone deacetylase activity. The WT mice showed increased cardiac p300 transcript levels in the animals given a high galactose diet compared to those fed normal rodent feed [Figure 3.8]. The $PARP^{-/-}$ mice fed a galactose rich diet expressed p300 levels similar to that of the WT control mice [Figure 3.8]. Similarly, in the rats, the hearts of the diabetic animals exhibited significantly increased levels of p300

Figure 3.8

Real time RT-PCR analyses of transcriptional co-activator p300 in the mice and rat hearts. Hyperhexosemia-induced transcript upregulation was prevented with PARP inhibition [WT = wild type, $PARP^{-/-}$ = PARP-1 knockout, CO = normal rodent diet, G = 30% galactose-enriched diet, DM = diabetes mellitus, ABA = 3-aminobenzamide; * $p < 0.05$ compared to WT CO and CO, † $p < 0.05$ compared to WT G and DM, ‡ $p < 0.05$ compared to $PARP^{-/-}$ CO; n=5; data expressed as mean ± SEM].


p300:18S rRNA
(fold increase)
3
2.5
2
1.5
1
0.5
0
*
*†
†‡
WT CO
WT G
PARP-/- CO
PARP-/- G


p300:18S rRNA
(fold increase)
2
1.5
1
0.5
0
*
*†
CO
DM
DM-ABA

mRNA when compared to the control animals [Figure 3.8]. ABA treatment prevented this hyperglycemia-induced increase of p300 mRNA [Figure 3.8].

As histone acetylation of p300 may be balanced by HDACs, we investigated HDAC activity. We found that in both the galactose-fed WT mice and the diabetic rat heart, level of deacetylated lysine were elevated compared to the WT mice and the control rats, respectively [Figure 3.9]. Surprisingly, PARP inhibition had no effect in either the $PARP^{-/-}$ galactose-fed mice or the diabetic rats [Figure 3.9].

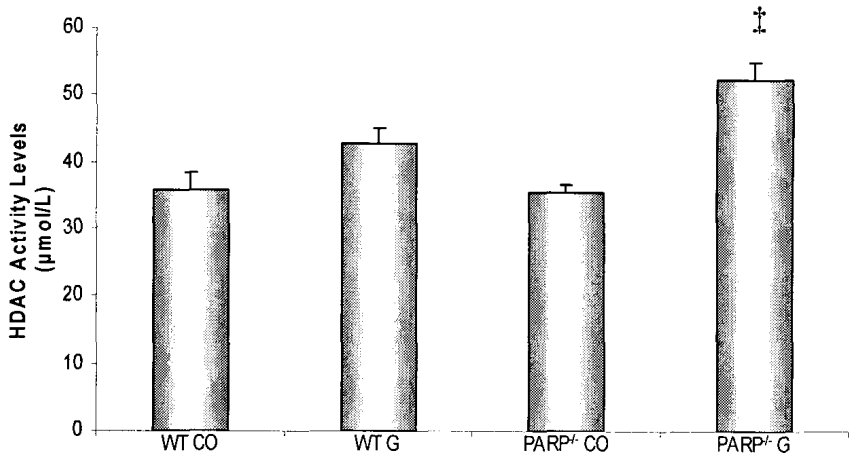
3.6 PARP Inhibition also Prevents Glucose-Induced Cardiomyocyte Hypertrophy and DNA Damage

We investigated whether the hyperhexosemia-induced effects, such as cardiomyocyte hypertrophy and oxidative stress, seen in our *in vivo* models cause similar effects in isolated heart cells. First, dose-response experiments in human endothelial cells were undertaken to determine the most effective concentration of ABA on glucose-induced FN gene expression. ABA treatment yielded a dose-dependent response with maximal inhibition of FN gene expression at 5 mmol/L concentration of ABA and thus all subsequent *in vitro* experiments were conducted with this concentration [Figure 3.10].

We isolated neonatal cardiomyocytes and exposed the cells to high levels of glucose. Twenty five mmol/L glucose caused cardiomyocyte hypertrophy after 24 hours as measured morphometrically [Figure 3.11]. ABA was effective in

Figure 3.9

HDAC activity analyses in the hearts of the mice and rats. Hyperhexosemia results in increased HDAC activity which was not prevented with PARP inhibition [WT = wild type, $PARP^{-/-}$ = PARP-1 knockout, CO = normal rodent diet, G = 30% galactose-enriched diet, DM = diabetes mellitus, ABA = 3-aminobenzamide; * $p < 0.05$ compared to CO, ‡ $p < 0.05$ compared to $PARP^{-/-}$ CO; n=3; data expressed as mean ± SEM].


HDAC Activity Levels (µmol/L)
60
50
40
30
20
10
0
‡
WT CO
WT G
PARP-/- CO
PARP-/- G

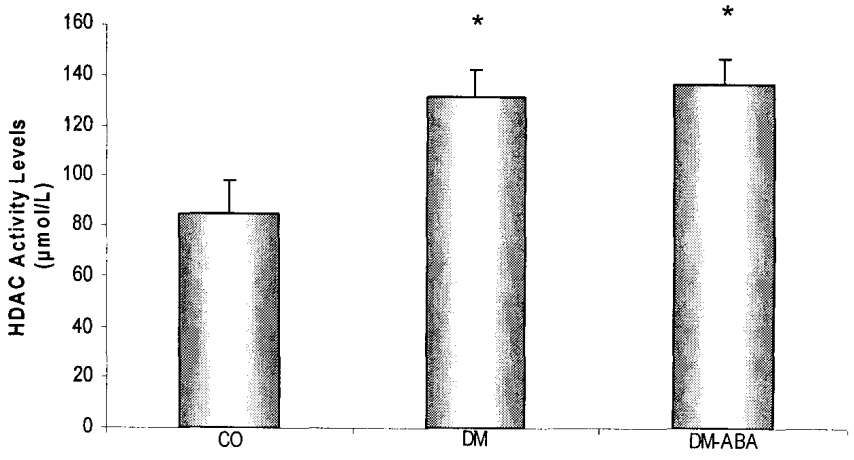

HDAC Activity Levels (µmol/L)
160
140
120
100
80
60
40
20
0
*
*
CO
DM
DM-ABA

Figure 3.10

Dose-response of FN expression when human umbilical vein endothelial cells, exposed to 25mmol/L glucose, were treated with 0.5 mmol/L, 1 mmol/L, 2.5 mmol/L and 5 mmol/L concentrations of ABA. As FN expression was not changed by 5mmol/L glucose such analyses were performed on 25mmol/L [FN = fibronectin, ABA = 3-aminobenzamide; n=3].

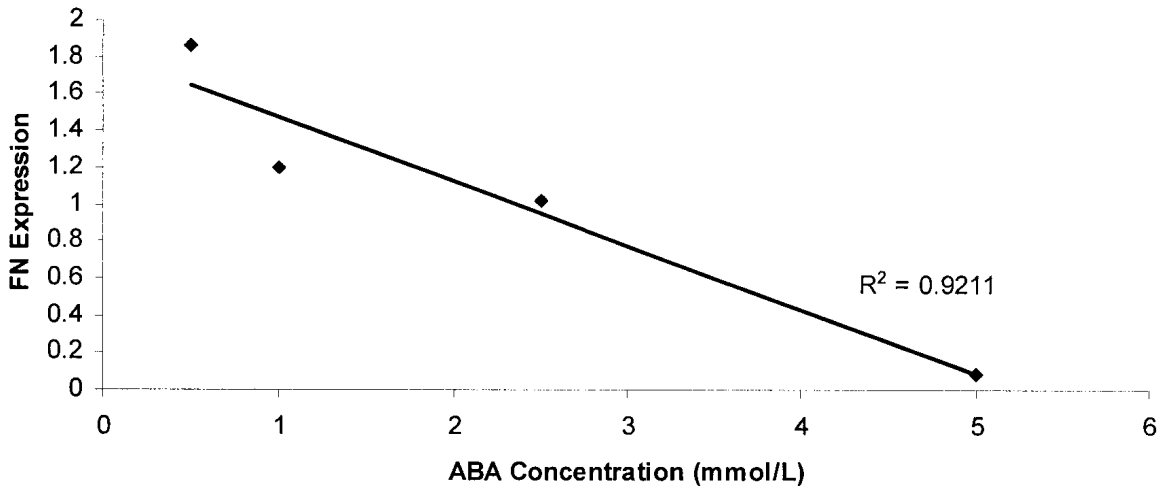
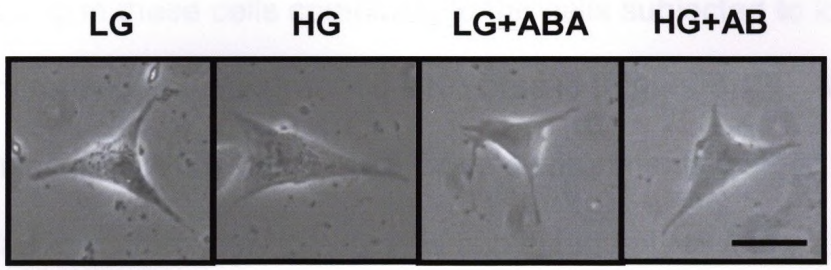
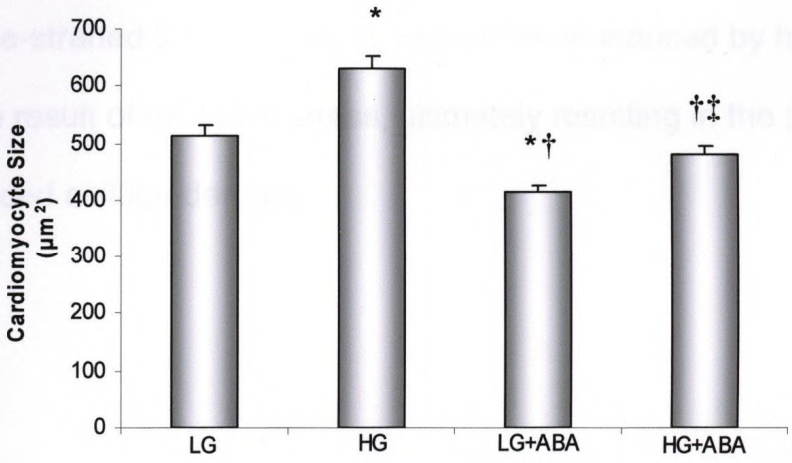

FN Expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
ABA Concentration (mmol/L)
R2 = 0.9211

Figure 3.11

Representative micrographs of neonatal rat cardiomyocytes and their morphometric analyses of glucose-induced hypertrophy which was prevented with 5 mmol/L ABA treatment [LG = 5 mmol/L glucose, HG = 25 mmol/L glucose, ABA = 3-aminobenzamide; bar represent 20μm; * $p < 0.05$ compared to LG, † $p < 0.05$ compared to HG, ‡ $p < 0.05$ compared to LG + ABA; n=25; data expressed as mean ± SEM].


LG
HG
LG+ABA
HG+AB

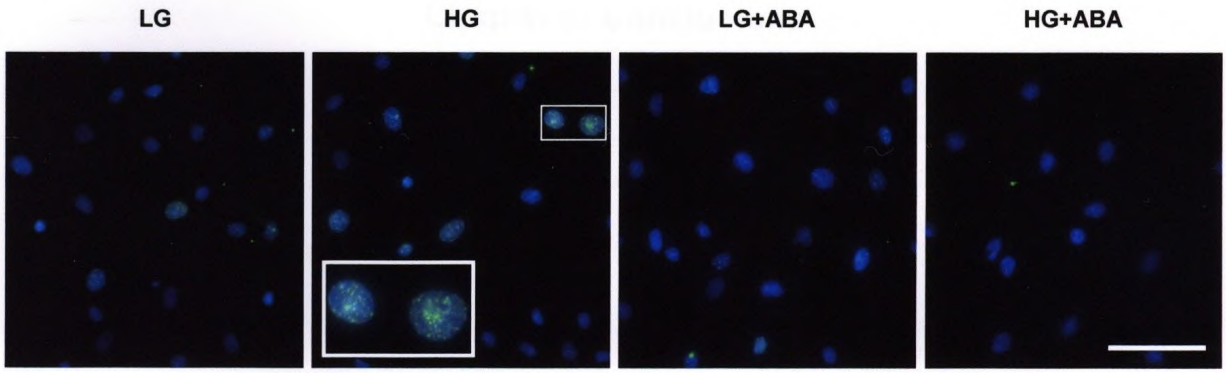

Cardiomyocyte Size (μm²)
700
600
500
400
300
200
100
0
*
*†
†‡
LG
HG
LG+ABA
HG+ABA

blocking glucose-induced cellular hypertrophy [Figure 3.11]. Interestingly, ABA reduced the cardiomyocyte size in cells exposed to low glucose [Figure 3.11]. No effect of glucose or ABA was seen on cell viability. Exposure of cells to L-glucose showed no demonstrable effects on these parameters.

DNA damage was also determined using cultured neonatal cardiomyocytes. These cells were stained for phosphor-H2A.X, a marker for double-stranded DNA breakage[139]. High glucose treatment results in increased nuclear staining in these cells compared to the cells subjected to low glucose indicating increased double-stranded DNA breaks [Figure 3.12]. Surprisingly, ABA treatment in both low and high glucose environments yielded less nuclear positivity than that of the cardiomyocytes subjected only to a low glucose environment [Figure 3.12]. These results indicate that PARP inhibition can limit overall double-straned DNA breaks as well as those induced by hyperglycemia, possibly as a result of oxidative stress, ultimately resulting in the prevention of glucose-induced cellular damage.

Figure 3.12

Immunofluorescent staining of neonatal cardiomyocytes showing DNA damage with phospho-H2A.X (granular green nuclear stain). Glucose-induced DNA damage was prevented with PARP inhibition [LG = 5 mmol/L glucose, HG = 25 mmol/L glucose, ABA = 3-aminobenzamide; bar represent 50μm, inset shows higher magnification].


LG
HG
LG+ABA
HG+ABA

Chapter 4: Conclusions

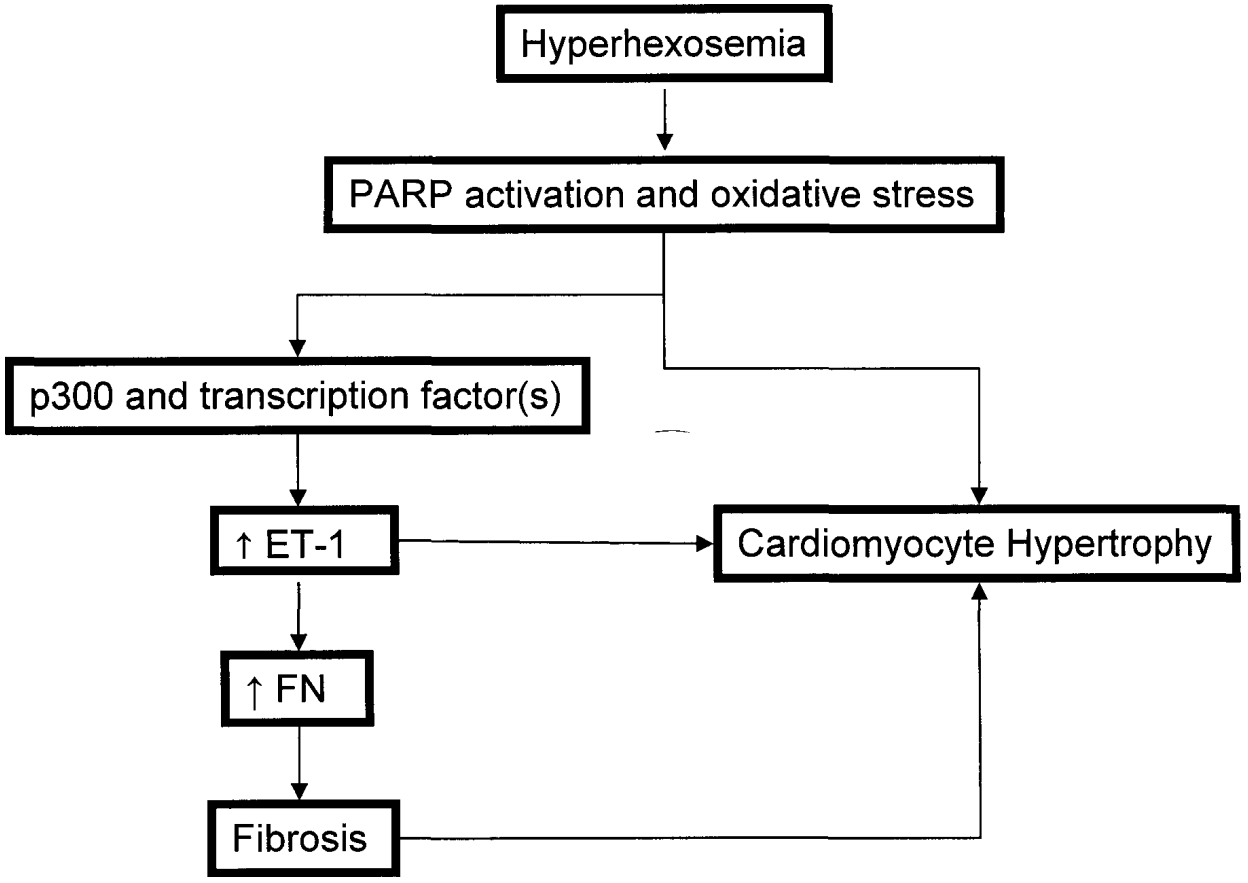
4.1 Discussion

In this study, we have shown that hyperhexosemia-induced oxidative stress and ECM protein expression along with cardiomyocyte hypertrophy is mediated through a PARP-dependent mechanism in diabetic cardiomyopathy. Furthermore, these changes may be associated with transcriptional co-activator p300. This is consistent with previous findings indicating that hyperhexosemia increases oxidative damage and that PARP-1 and p300 may interact to alter gene expression[92,142].

The novelty of this study lies in the elucidation of a specific pathway mediating hyperhexosemia-induced structural alterations in the heart. As well, our results from this and a previous study offer and exclude potential regulatory mechanisms for these PARP-mediated changes as shown by the p300 experiments and HDAC activity. These findings complement studies which implicate PARP-1 in hyperglycemia-induced functional modifications in the heart[134]. Additionally, our findings in conjunction with the findings from the available literature demonstrate a significant role for PARP-dependent increase in oxidative stress in the pathogenesis of diabetic cardiomyopathy. Furthermore, downstream effects, such as PARP-dependent upregulation of vasoactive factor ET-1 and ECM protein FN along with cardiac hypertrophy can detrimentally impair the function of the heart. This investigation elucidates, for the first time, a specific mechanistic pathway for the action of PARP in diabetes-induced structural alterations of the heart [Figure 4.1].

Figure 4.1

A schematic representation of PARP-1 activation mediating structural alterations in diabetic cardiomyopathy. Hyperhexosemia leads to increased oxidative stress and PARP-1 activation which either leads to cardiomyocyte hypertrophy or the recruitment of transcriptional co-activators, such as p300, and transcription factors. This causes the upregulation of ET-1 and FN leading to fibrosis. Fibrosis leads to functional defects in the heart which results in compensatory cardiomyocyte hypertrophy.


Hyperhexosemia
PARP activation and oxidative stress
p300 and transcription factor(s)
↑ ET-1
Cardiomyocyte Hypertrophy
↑ FN
Fibrosis

Due to the ability of PARP-1 to determine the fate of a cell, either to repair or to induce apoptosis[143], a deficiency in PARP-1 may prevent myocyte death and limit the development of compensatory cardiomyocyte hypertrophy seen in diabetic cardiomyopathy. The benefits of PARP inhibition in different pathological states are well documented. Mice deficient in PARP-1 are protected from STZ-induced diabetes[83,84], angiotensin II-induced cardiac hypertrophy[82] and LPS-induced endotoxic shock[81]. Pharmacological inhibition of PARP has also been proven to be beneficial in protecting against a variety of ischemia-reperfusion injuries[144-146] as well as MPTP-induced Parkinsonism[86] and preventing angiogenesis[147]. These findings indicate an important contributory role for PARP in the development of many diseases.

One interesting phenomena observed in our study is the increase in basal mRNA levels of HO-1, ET-1, FN, ANP and p300 in the $PARP^{-/-}$ mice fed a normal rodent diet. The exact reasons for this are not clear. It is well-established that the deletion on one gene may alter regulatory mechanisms and may lead to compensatory effects as a result. An investigation into the specific regulatory machinery with respect to PARP-1 is complex and is beyond the scope of this project. However, to address this issue we carried out our investigation in other models of diabetic complications, e.g. STZ-induced diabetic rats and isolated cardiomyocytes.

PARP-1 plays a variety of roles in the cell; it can repair DNA damage, mediate gene expression and induce cell death[142,143,148]. This and previous studies have demonstrated a role for PARP activation in the retina[120], peripheral

nerves[126,130] , kidney[126] and heart[116,130] in diabetes. The enzymatic activity of PARP-1 may contribute to an overall increase in oxidative stress by creating a redox imbalance through its consumption of NAD+[99]. As previously proposed, this may explain the overall reduction of oxidative stress in the hearts of the hyperhexosemic animals when PARP is inhibited and suggests that PARP activation may be a primary contributor to the increased levels of oxidative stress.

This notion is further strengthened upon analysis of double stranded DNA breakage. Previously, Du *et al* found that 30mM glucose increases DNA damage[115]. Our findings indicate that PARP inhibition may in fact be beneficial in circumstances of PARP-1 overactivation and is in support of previously conducted research[116]. However, it is in contrast to other findings which suggest that PARP inhibition increases double-stranded DNA breaks[149]. This discrepancy may lie in the cause of the DNA damage, oxidative stress-induced versus ionizing radiation-induced and whether or not PARP-1 is overactivated. These findings indicate that different mechanisms of DNA damage may lead to different tissue responses. Diabetic cardiomyopathy, due to hyperglycemia, is a slow chronic process while radiation may cause acute injury. Further investigation is necessary to elucidate the exact mechanism by which PARP inhibition may stimulate or prevent DNA strand breakage. One cannot exclude the possibility that ABA, a weak inhibitor of PARP and scavenger of free radicals[150], may play a role in preventing this hyperglycemia-induced double-stranded DNA damage seen in the cardiomyocytes and decreased staining

positivity of oxidative stress markers 8-OHdG and NT in the rats. However, our studies included $PARP^{-/-}$ mice which exhibited similar staining patterns of the aforementioned markers as the ABA-treated rats. This further strengthens our notion that inhibition of PARP-1 is the primary reason a reduction in hyperhexosemia-induced oxidative stress is seen rather than a confounding result due to the non-specific actions of the inhibitor used.

We have previously shown that p300 is upregulated in the heart and in the retina of diabetic animals and in endothelial cells exposed to high glucose levels[65]. We have also demonstrated that p300 regulates the expression of ECM protein FN in the context of diabetes[65]. p300 directly interacts with the p65 subunit of NFκB to regulate the transcription of multiple genes[151]. In diabetic retinopathy, NFκB has been shown to be regulated by PARP-1[120] possibly through p300. It has been reported that both PARP-1 and p300 interact to form a complex which regulates gene expression by binding onto promoter regions[92]. Furthermore, it has been suggested that the presence of the PARP-1 protein itself is what mediates its co-activator function on NFκB and not its DNA bindings nor its enzymatic activity[94]. Our lab has shown that activation of transcription factors NFκB and AP-1 mediates the expression of FN in all target organs of diabetic complications[63]. In addition, PARP-1 has been shown to regulate NFκB transcriptional activation *in vivo*[92] and that this regulation is through p300[142]. Our study showed that PARP inhibition attenuated the hyperhexosemia-induced upregulation of p300 in both rodent models. We also found that FN levels were reduced when PARP is inhibited, showing the same trends as p300. These

findings suggest that PARP mediates its hyperhexosemia-induced transcriptional effects through p300 and may utilize other transcription factors, such as NFκB, to regulate hyperhexosemia-induced upregulation of FN. The possibility of other coexisting transcription factors such as CREB binding protein should also be considered and cannot be excluded.

The action of HATs, such as p300, are counteracted by HDACs[152]. It is of interest to note that in our studies HDAC activity levels were increased in the diabetic rats and the WT galactose-fed mice. This was unexpected since there was increased gene expression of FN and ET-1 seen in these animals. However, this finding suggests that HDACs may in fact be attempting to counteract the elevated levels of HATs such as p300 through increased deacetylation of lysine residues in hyperhexosemic conditions even with PARP inhibition which either signifies a more complex regulatory relationship between HATs and HDACs. The exact role of HDACs in the hyperhexosemic heart requires further investigation. However, our results suggest that HATs such as p300 may be of greater importance in this PARP-dependent pathway in the diabetic heart. But one cannot exclude the possibility that HDACs may be exerting their effects upstream of PARP-1 and thus changes in their activity would not be evident with PARP blockade.

4.2 Future Directions

Since PARP-1 has been shown to interact with a variety of transcription factors[81,153,154], it would be interesting to determine whether specific ones mediate the structural alterations seen in diabetic cardiomyopathy. This would allow for the development of more targeted therapeutic modalities. As shown in previous studies done by our lab, both NFκB and AP-1 may be involved in this signalling pathway[63,64]. However, further experiments must be done to definitively pinpoint the exact action hyperglycemia-induced PARP activation may exert on these and other transcription factors in the heart. Experiments which elucidate the transcriptional co-activators involved would also be of importance.

As oxidative stress appears to play a significant role in this pathogenetic mechanism, a strong antioxidant should be tested to determine whether these PARP-mediated alterations can in fact be prevented. These experiments will also determine whether it is PARP itself or the increase in oxidative stress that underlies these hyperglycemia-induced effects. Due to the important role that PARP plays in normal physiology, potent inhibition of this nuclear enzyme does not appear to be a feasible option. Rather, treatment options should be designed to counteract NAD+ depletion, if this is in fact the disease-inducing step. Perhaps another option for treatment would be to decrease the amount of PARP protein production in a cell specific way. This will allow the cell to respond normally to the hyperglycemia-induced stress but without depleting intracellular co-factors. Furthermore, downstream effects would be prevented.

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Appendix 1: Ethics Approval

Western

August 22, 2007

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2011

Dear Dr. Chakrabarti:

Your Animal Use Protocol form entitled:
Vasoactive and Cardioactive Factors in Diabetic Cardiomyopathy
Funding Agency CIHR - Grant #THDC; HSFO - Grant #THG1

has been approved by the University Council on Animal Care. This approval is valid from **August 22, 2007 to August 31, 2008.** The protocol number for this project is **2007-055-08 and replaces 2003-075-06.**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 1 YR.

Species	Strain	Other Detail	Pain Level	Animal # Total for 1 Year
Mouse	PPAR gamma (-/-)	M/F	D	3 breeding prs
Mouse	PPAR gamma (-/-)	M/F	D	60
Mouse	NFKB P50 (-/-)	M/F	D	3 breeding prs
Mouse	NFKB P50 (-/-)	M/F	D	60
Mouse	AP-1 (-/-)	M/F	D	3 breeding prs
Mouse	AP-1 (-/-)	M/F	D	60
Mouse	PPAR gamma wild type	M/F	D	3 breeding prs
Mouse	PPAR gamma wild type	M/F	D	60
Mouse	NFKB P50 (-/-) wild type	M/F	D	3 breeding prs
Mouse	NFKB P50 (-/-) wild type	M/F	D	60
Mouse	AP-1 (-/-) wild type	M/F	D	3 breeding prs
Mouse	AP-1 (-/-) wild type	M/F	D	60
Mouse	B6	M/F	D	100
Rat	Sprague Dawley	Male	D	100
Rat	Zucker fatty	Male	D	50
Rat	Zucker lean	Male	D	50
Rat	Sprague Dawley	neonates	D	200

STANDARD OPERATING PROCEDURES
Procedures in this protocol should be carried out according to the following SOPs. Please contact the Animal Use Subcommittee office (661-2111 ext. 86770) in case of difficulties or if you require copies.
SOP's are also available at http://www.uwo.ca/animal/acvs

310 Holding Period Post-Admission
320 Euthanasia
321 Criteria for Early Euthanasia/Rodents
330 Post-Operative Care/Rodent
343 Surgical Prep/Rodent/Recovery Surgery
360 Blood Collection/Volumes/Multiple Species

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol - S. Chakrabarti, W. Lagerwerf
Approval Letter - W. Lagerwerf