2011

Expression and Regulation of Osteopontin in the Diabetic Heart

Abigail Goodman
Loyola University Chicago

Recommended Citation
https://ecommons.luc.edu/luc_diss/218

This Dissertation is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Dissertations by an authorized administrator of Loyola eCommons. For more information, please contact ecommons@luc.edu.

Creative Commons License
This work is licensed under a Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License.
Copyright © 2011 Abigail Goodman
LOYOLA UNIVERSITY CHICAGO

EXPRESSION AND REGULATION OF OSTEOPONTIN IN THE DIABETIC HEART

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PROGRAM IN CELL AND MOLECULAR PHYSIOLOGY

BY

ABIGAIL L. GOODMAN

CHICAGO, ILLINOIS

MAY 2011
ACKNOWLEDGEMENTS

I would like to thank my advisor and dissertation committee director, Dr. Leanne Cribbs for the help and guidance she provided while I worked to complete my dissertation in her lab. I would like to acknowledge the members of my dissertation committee: Dr. David Dostal, Dr. Karen Griffin, Dr. Ruben Mestril, and Dr. Allen Samarel for their helpful suggestions and critical analysis which lead to the successful completion of my dissertation. I am also grateful for the support of the Physiology faculty and staff. In addition, I would like to thank Dr. Florentina Pluteanu, Rekha Iyengar, Taehoon Kim, and Dr. Yevgeniya Koshman for their help with learning and understanding various new methods and techniques and their help developing the foundations for this project.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS........................................................................................................ iii

LIST OF TABLES ............................................................................................................... vii

LIST OF FIGURES ........................................................................................................... viii

LIST OF ABBREVIATIONS............................................................................................... x

ABSTRACT...................................................................................................................... xiii

CHAPTER ONE: INTRODUCTION ................................................................................. 1

CHAPTER TWO: REVIEW OF RELEVANT LITERATURE ........................................ 3
  A. Diabetes..................................................................................................................... 3
     1. Definition and Description.................................................................................. 3
     2. Classification of Diabetes Mellitus...................................................................... 4
     3. Diabetes and Cardiac Remodeling...................................................................... 5
  B. Myocardial Fibrosis................................................................................................. 7
  C. Osteopontin ........................................................................................................... 9
     1. Identification and Structure............................................................................... 9
     2. The OPN Promoter.............................................................................................. 10
     3. OPN Function and Regulation............................................................................. 12
  D. OPN in the heart...................................................................................................... 16
     1. Expression.......................................................................................................... 16
     2. Stimulation of OPN Expression in the Heart.................................................... 18
     3. Role of OPN in the Heart................................................................................... 21
  E. Angiotensin II........................................................................................................ 26
  F. Protein Kinase C..................................................................................................... 29
     1. Structure, Function, and Activation.................................................................. 29
     2. PKC in the Heart: Role in OPN Expression....................................................... 32
  G. Focal Adhesions.................................................................................................... 34
     1. Structure, Function, and Activation.................................................................. 34
     2. Osteopontin and Focal Adhesions.................................................................... 35
  H. Overview of Hypothetical Pathway for High Glucose-Induced OPN
     Expression in Cardiac Cells................................................................................ 36

CHAPTER THREE: SPECIFIC AIMS AND OVERVIEW OF PROJECT ............... 39
B. Results .................................................................................................................................. 114
C. Discussion .......................................................................................................................... 129

CHAPTER EIGHT: SUMMARY AND FUTURE DIRECTIONS .................................................. 134

REFERENCE LIST ................................................................................................................. 140

VITA ........................................................................................................................................ 161
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Rat reverse-transcriptase PCR primers</td>
<td>56</td>
</tr>
<tr>
<td>2. Antibodies used in Western blotting and immunostaining</td>
<td>58</td>
</tr>
<tr>
<td>3. Cardiac function parameters of the ZSF model of type 2 diabetes</td>
<td>79</td>
</tr>
<tr>
<td>4. Alterations in extracellular matrix and adhesion molecule mRNA expression in ZSF animals</td>
<td>81</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Analysis of fibrosis in WT and OPN KO mice</td>
<td>23</td>
</tr>
<tr>
<td>2. The hypothetical signaling pathway mediating high glucose induced upregulation of OPN</td>
<td>38</td>
</tr>
<tr>
<td>3. Increased weight of the ZSF model of type 2 diabetes</td>
<td>73</td>
</tr>
<tr>
<td>4. Blood glucose is increased in the ZSF model of type 2 diabetes</td>
<td>74</td>
</tr>
<tr>
<td>5. Blood pressure is not altered in the ZSF rat model</td>
<td>75</td>
</tr>
<tr>
<td>6. LV mass in the ZSF model of type 2 diabetes</td>
<td>76</td>
</tr>
<tr>
<td>7. LV wall thickness in the ZSF model</td>
<td>77</td>
</tr>
<tr>
<td>8. Cardiac characterization of the ZSF model of type 2 diabetes</td>
<td>78</td>
</tr>
<tr>
<td>9. Increased fibrosis in the LV of the ZSF model</td>
<td>80</td>
</tr>
<tr>
<td>10. Expression of OPN in Lean vs. ZSF rat heart</td>
<td>82</td>
</tr>
<tr>
<td>11. Regulation of OPN in response to high glucose in cardiac cells</td>
<td>83</td>
</tr>
<tr>
<td>12. Basal Expression of OPN in NRVM and NRVF</td>
<td>84</td>
</tr>
<tr>
<td>13. Regulation of OPN in response to high glucose in NRVM</td>
<td>85</td>
</tr>
<tr>
<td>14. Regulation of OPN in response to high glucose in NRVF</td>
<td>86</td>
</tr>
<tr>
<td>15. Localization of OPN-GFP in NRVF</td>
<td>87</td>
</tr>
<tr>
<td>16. Localization of OPN-GFP in NRVM</td>
<td>88</td>
</tr>
<tr>
<td>17. Ang II increases OPN expression in NRVM</td>
<td>98</td>
</tr>
<tr>
<td>18. Ang II increases OPN expression in NRVF</td>
<td>99</td>
</tr>
</tbody>
</table>
19. OPN expression in response to Ang II..............................................................100

20. Involvement of renin in high glucose-induced OPN expression in NRVM ..........101

21. Involvement of renin in high glucose-induced OPN expression in NRVF ..........102

22. Involvement of chymase in OPN expression in NRVM..................................103

23. Involvement of chymase in OPN expression in NRVF ..................................104

24. Involvement of the AT_1 receptor in OPN expression in NRVM......................105

25. Involvement of the AT_1 receptor in OPN expression in NRVF .......................106

26. PKC involvement in high glucose-induced OPN expression in NRVM ..........118

27. PKC involvement in high glucose-induced OPN expression in NRVF ..........119

28. PKC mediates Ang II-induced OPN upregulation in NRVM .........................120

29. PKC mediates Ang II-induced OPN upregulation in NRVF ............................121

30. Classical PKC isoforms mediate high glucose-induced OPN expression in NRVM ..................................................................................................................122

31. Classical PKC isoforms mediate high glucose-induced OPN expression in NRVF ..................................................................................................................123

32. PKC_ε increases OPN expression in NRVM ....................................................124

33. PKC_ε increases OPN expression in NRVF .....................................................125

34. Immunostaining of PKC_ε-induced increase of OPN expression in NRVF ..........126

35. PKC_ε mediates OPN expression in response to high glucose in NRVM ..........127

36. PKC_ε mediates OPN expression in response to high glucose in NRVF ..........128
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>AGT</td>
<td>Angiotensinogen</td>
</tr>
<tr>
<td>Ang I</td>
<td>Angiotensin I</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin II Receptor Blocker</td>
</tr>
<tr>
<td>AT1 receptor</td>
<td>Angiotensin type 1</td>
</tr>
<tr>
<td>β-gal</td>
<td>Beta-galactosidase</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>bpm</td>
<td>Beats per minute</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>caPKC(\epsilon)</td>
<td>Constitutively active PKC(\epsilon)</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>(C_T)</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>(\Delta C_T)</td>
<td>Difference in (C_T) values between the target and housekeeping gene</td>
</tr>
<tr>
<td>(\Delta \Delta C_T)</td>
<td>Difference in the (\Delta C_T) values</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DCM</td>
<td>Diabetic cardiomyopathy</td>
</tr>
<tr>
<td>dnPKC(\epsilon)</td>
<td>Dominant negative PKC(\epsilon)</td>
</tr>
</tbody>
</table>
ECM: Extracellular matrix
ERK: Extracellular signal-related kinase
FBS: Fetal bovine serum
GFP: Green fluorescent protein
HF: Heart failure
HR: Heart rate
kDa: Kilodalton
MI: myocardial infarction
MMP: matrix metalloproteinases
Moi: multiplicity of infection
mRNA: Messenger RNA
NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells
NRFV: Neonatal rat ventricular fibroblasts
NRVM: Neonatal rat ventricular myocytes
MAPK: Mitogen-activated protein kinase
OPN: Osteopontin
PBS: Phosphate buffered saline
PKC: Protein kinase C
PMA: Phorbol 12-myristate 13-acetate
qPCR: Quantitative real-time polymerase chain reaction
RACK: Receptor or activated C kinases
RAS: Renin angiotensin system
qRT-PCR: Quantitative reverse transcription polymerase chain reaction
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: Standard error of the mean

TGF-β: transforming growth factor-β
ABSTRACT

Diabetes leads to several alterations in cardiac structure, one of which is fibrosis of the ventricular myocardium. Hyperglycemia is thought to be an underlying problem in diabetes leading to a host of complications, including cardiac dysfunction. Patients with diabetes show enhanced myocardial dysfunction leading to accelerated heart failure referred to as diabetic cardiomyopathy (DCM). Myocardial fibrosis is a common underlying factor in most cardiac pathologies (R. D. Brown et al., 2005; R. D. Brown, Ambler, Mitchell, & Long, 2005), and leads to a stiffening of the cardiac tissue, decreased elasticity causing increased relaxation time, less contractile force and may lead to slowed electrical conduction and arrhythmias (Swaney et al., 2005). Osteopontin (OPN) is a small phospho-protein that has been implicated in processes of immunity and tissue remodeling. In the heart, the expression of OPN protein is increased after acute and chronic pathologies. It has been hypothesized that the increased expression of OPN plays a role in fibrillogenesis following certain pathologies, and that the upregulation of OPN coincides with a transition to heart failure. The role of OPN in the development and progression of dilated cardiomyopathy has been investigated, and a direct role for OPN in the progression to heart failure has been reported (M. Renault et al., 2010; Satoh et al., 2005; Stawowy et al., 2002). However, the mechanism behind the signaling pathways leading to OPN upregulation has yet to be determined.
The goal of this study is to determine if OPN is upregulated in cardiac cells in response to high glucose, and, if so, determine some of the mechanisms involved in high glucose mediated OPN expression in cardiac cells. In this study, I used microarrays and immunohistochemistry to determine that OPN is upregulated in the hearts of a rat model of type 2 diabetes (ZSF rats). This is significant because of OPN’s role in myocardial fibrosis and the associated functional effects increased cardiac fibrosis. Furthermore, the involvement of hyperglycemia, a contributor to diabetes, was examined in this study. I found that high glucose increased OPN expression in both neonatal rat ventricular myocytes (NRVM) and neonatal rat ventricular fibroblasts (NRVF). Additionally, I found that NRVM express more OPN mRNA than NRVF in unstimulated conditions. After it was determined that OPN was upregulated in the diabetic heart, a goal of this project became to elucidate the mechanism behind the upregulation of OPN in response to high glucose. I determined that the production of Angiotensin II (Ang II) is involved in the increased OPN expression in both NRVM and NRVF in response to high glucose. In NRVM chymase cleaves Ang I to form Ang II, inhibition of chymase in NRVM inhibits the high glucose mediated OPN expression. Renin cleaves angiotensinogen to form Ang I and inhibition of renin inhibits high glucose mediated OPN expression in NRVF. The Ang II type 1 (AT1) receptor appears to be the primary mediator of Ang II’s effect on OPN expression as inhibition of the AT1 receptor significantly inhibits the high glucose induced OPN expression. Ang II is a known activator of activator of Protein Kinase C (PKC), an intracellular multifunctional kinase. I hypothesize that high glucose induced Ang II leads to activation of PKC to mediate its effects on OPN expression. In particular, I show that PKCε and classical PKCs (α or β), are involved in the high
glucose-mediated upregulation of OPN mRNA expression. The results of this study further progress our understanding of signaling pathways leading to the detrimental cardiac effects of diabetes, including the structural and functional abnormalities that result from hyperglycemia, and identify key proteins involved in this cardiac remodeling that represent potential therapeutic targets for the management of diabetic cardiomyopathy.
CHAPTER ONE
INTRODUCTION

Diabetes contributes to more than 200,000 deaths a year. It is the seventh leading cause of death in America, with the risk of death for people with diabetes two fold greater than for non-diabetics of the same age. More than ten percent of Americans age 20 and over have diabetes and 800,000 new cases are diagnosed each year (CDC, 2005; National Institute of Diabetes and Digestive and Kidney Diseases, 2008).

Diabetics have an increased risk for and worse prognosis once diagnosed with cardiovascular disease (CVD) (Grundy et al., 1999). Due to the increased rate of cardiovascular disease, enhanced myocardial dysfunction and accelerated heart failure seen in diabetics it has been termed diabetic cardiomyopathy (DCM). This enhanced cardiomyopathy is thought to be caused, in part, by hyperglycemia. In diabetes cells are unable to take up glucose from the blood, either due to a lack of insulin or insulin resistance, leading to increased blood glucose (hyperglycemia) which causes vessel and organ damage over time. One approach to the development of new therapies for DCM is to elucidate the molecular mechanisms underlying changes in cardiovascular function, and then develop targeted intervention strategies to prevent or alleviate changes in gene expression that contribute to the pathological state. The first goal of this project was to examine alterations in the expression of mechanotransduction molecules in the LV of a model of type 2 diabetes hearts. Microarrays revealed that the expression of several
mechanotransduction molecules were altered in the type 2 diabetic hearts. One of these proteins, osteopontin (OPN) was increased in the hearts of diabetic animals; we chose to focus on OPN because of OPN’s involvement in fibrosis. Myocardial fibrosis is a common underlying factor in most cardiac pathologies (R. D. Brown et al., 2005). Cardiac fibrosis leads to a stiffening of the cardiac tissue, decreased elasticity causing increased relaxation time, less contractile force and may lead to slowed electrical conduction and arrhythmias (Swaney et al., 2005). The primary focus of this project was to determine the expression and regulation of OPN in response to high glucose in cardiac cells. I chose to focus on the regulation of OPN expression by the Angiotensin II (Ang II) and protein kinase C (PKC) pathways because of their involvement in cardiac fibrosis (Chintalgattu & Katwa, 2009; Schnee & Hsueh, 2000; Stawowy et al., 2005) and in OPN expression in other cell types (Beck & Knecht, 2003; Hsieh et al., 2006; Kelly, Chanty, Gow, Zhang, & Gilbert, 2005a; Kupfahl et al., 2000).

A literature review is presented in Chapter 2 as a means to provide some of the relevant background and supporting information. Chapter 3 provides a brief outline of the experimental hypothesis and describes the approaches used to test it. Chapter 4 gives a discussion of the methods used in this project. Chapters 5 through 7 describe the findings of the experiments within the context of the specific questions they were designed to answer. Lastly, Chapter 8 provides a discussion of the major findings of this study while attempting to explain their potential clinical implications and future directions of this work.
CHAPTER TWO

REVIEW OF RELATED LITERATURE

A. DIABETES

1. Definition and Description

Insulin is a key hormone central to the regulation of energy and glucose metabolism in the body. Insulin acts by causing cells in the liver, muscle, and fat to take up glucose from the blood for energy or storing it as glycogen in the liver and muscle. When insulin is absent, glucose is not taken up by body cells and the body begins to use fat as an energy source. The hyperglycemia observed in diabetes results from impairments in insulin secretion, insulin action, or a combination of both. Hyperglycemia in its own right then can cause long-term damage and dysfunction of various tissues and organs including the eyes, kidneys, blood vessels, and the heart. Some of the symptoms of hyperglycemia seen in humans include polyuria, polydipsia, weight loss, blurred vision, and susceptibility to infections. Furthermore, the long-term complications of diabetes include serious and life-threatening conditions such as loss of vision, peripheral sensory neuropathy leading to inadvertent injury and infection (usually the foot), and an increased occurrence of atherosclerotic, cardiovascular, peripheral arterial, and cerebrovascular disease. In addition, diabetes is the leading cause of new cases of blindness and kidney failure in the United States. Diabetic adult patients have
heart failure rates about 2 to 4 times higher than non-diabetic adults (Bauters et al., 2003; Kannel & McGee, 1979).

2. Classification of Diabetes Mellitus

Diabetes mellitus can be grouped into two different classes based on the condition of the patient at the time of the diagnosis. Type 1 diabetes (previously called insulin-dependent diabetes) is an immune-mediated type of diabetes. It results when the body’s immune system destroys the insulin-producing pancreatic \( \beta \)-cells, thus obliterating the body’s source of this key hormone. In order to survive, these patients must be administered insulin to mediate the cellular uptake of blood glucose. This form of diabetes accounts for approximately 5-10% of all diabetic patients.

Type 2 diabetes (previously called non-insulin-dependent diabetes) is the class of diabetes assigned to patients who exhibit insulin resistance and usually have some degree of insulin deficiency. Type 2 diabetes can encompass patients ranging from predominantly insulin resistant with modest insulin deficiency to predominantly insulin secretion deficient with modest insulin resistance. This form of diabetes accounts for 90-95% of individuals diagnosed with diabetes mellitus. Although many different causes of this type of diabetes are proposed, the specific etiologies are not clear; however, it is known that there is only mild destruction of the \( \beta \)-cells (25-50% decrease in \( \beta \)-cell mass) and this is not the primary cause of decreased insulin. Decreased insulin in type 2 diabetes seems to come primarily from a reduced ability of \( \beta \)-cells to produce insulin (Cnop et al.). Furthermore, a large portion of the patients diagnosed with type 2 diabetes are obese, and it has been shown that obesity can cause some level of insulin resistance.
on its own. Despite having potentially normal insulin levels, type 2 diabetics still exhibit the hallmark of hyperglycemia due to the resistance of cells to the action of insulin, preventing glucose uptake. A random blood glucose level of greater the 200 mg/dL constitutes a patient that warrants further testing (Diagnosis and classification of diabetes mellitus.2005). Type 2 diabetes can be controlled in many individuals with modifications to their diet and exercise routines. In fact, some type 2 diabetics do not need to be administered insulin to control their blood glucose levels based on how well they respond to diet and exercise. Due to the many complications that arise from hyperglycemia, many diabetics also need to take medications to control their blood pressure and cholesterol.

Other forms of diabetes also exist, but are not as prevalent as the main two categories described above. The causes of these other types of diabetes include genetic defects of the β-cells, genetic defects in insulin action, diseases of the exocrine pancreas, endocrinopathies, and drug- or chemical-induced diabetes.

3. Diabetes and Cardiac Remodeling

Hyperglycemia is thought to be an underlying problem in diabetes leading to a host of diabetic complications, including cardiac dysfunction. Diabetes is considered a major independent risk factor for cardiovascular disease (CVD) and patients with diabetes show enhanced myocardial dysfunction leading to accelerated heart failure referred to as diabetic cardiomyopathy (DCM) (National high blood pressure education program working group report on hypertension in diabetes.1994; Epstein & Sowers, 1992). DCM is a primary disease process which develops secondary to the hyperglycemia in patients with diabetes independent of vascular disease. DCM results in
structural and functional changes in the myocardium of patients, which ultimately leads to the development of heart failure. The structural remodeling of the myocardium results in functional changes in the heart, and some of these structural and functional changes include impaired relaxation, reduced chamber compliance, increased myocardial collagen deposition, and general left ventricular hypertrophy (Asghar et al., 2009).

People with diabetes are not only more likely to get CVD but their prognosis for survival once they have been diagnosed is worse than for people without diabetes (Grundy et al., 1999). Heart disease and stroke account for 65% of deaths among the diabetic population (American Diabetes Association), emphasizing the necessity of understanding the mechanisms leading to DCM. One approach to the development of new therapies for DCM is to elucidate the molecular mechanisms underlying changes in cardiovascular function, and then develop targeted intervention strategies to prevent or alleviate changes in gene expression that contribute to the pathological state. In diabetic patients, structural and functional changes in the heart develop independent of the typical cardiac risk factors of hypertension and atherosclerotic coronary artery disease (Kannel, Seidman, Fercho, & Castelli, 1974). Early stage DCM is associated with insulin resistance, hyperglycemia, metabolic changes, inflammation, changes in intracellular calcium homeostasis, and alterations in β-adrenergic function without noticeable changes in left ventricular (LV) structure (op den Buijs et al., 2005). These changes eventually lead to structural changes including myocyte hypertrophy, necrosis and apoptosis, fibroblast activation and proliferation, increased collagen and myocardial fibrosis with relatively minor changes in LV dimensions, and the appearance of diastolic dysfunction. In the late stage, concentric LV hypertrophy and more pronounced diastolic dysfunction
become apparent (Fang, Prins, & Marwick, 2004). Advanced DCM is also commonly associated with LV hypertrophy, LV myocyte hypertrophy (Levy, Garrison, Savage, Kannel, & Castelli, 1990) and increased LV fibrosis, which are also seen in hypertensive heart disease. Impaired function in DCM is usually attributed to compromised left ventricular diastolic function (Semeniuk, Kryski, & Severson, 2002), which includes impaired diastolic filling or decreased end diastolic volume while systolic function is less affected by diabetes. Although remodeling may initially be beneficial to maintain cardiac function in response to stress or injury through cellular hypertrophy, increased muscle mass and fibrosis; continued remodeling is usually adverse and is linked to LV dysfunction (impaired diastolic filling and/or decreased end diastolic volume) and eventual heart failure (Semeniuk et al., 2002)(Semeniuk et al., 2002). It is notable that although numerous epidemiological studies have established a clear association between diabetes and heart failure, the underlying pathological basis is only partially understood (Boudina & Abel, 2007).

**B. MYOCARDIAL FIBROSIS**

Myocardial fibrosis is a common underlying factor in most cardiac pathologies (R. D. Brown et al., 2005), and leads to a stiffening of the cardiac tissue, decreased elasticity causing increased relaxation time, less contractile force and may lead to slowed electrical conduction and arrhythmias (Swaney et al., 2005). In response to injury or stress fibroblasts become activated and differentiate to myofibroblasts. Myofibroblasts then mediate reparative remodeling via production of $\alpha$-smooth muscle actin, connective
tissue growth factor (CTGF), fibronectin, stress fibers and focal adhesion formation to form a fibrotic scar in an adaptive response to allow scar formation. This process can then finish with apoptosis of the myofibroblasts when the stress is removed (Carlson, Longaker, & Thompson, 2003). However, in situations of continued stress or injury, the myofibroblasts fail to undergo apoptosis and become an integral part of the pathology leading to cardiac fibrosis with excess production and secretion of extracellular matrix (ECM) proteins. Angiotensin II (Ang II) is well established as a paracrine early activator of fibroblasts in vascular smooth muscle and heart, and is a key stimulator of cardiac hypertrophic signaling (Mehta & Griendling, 2007). However, the mechanisms for its actions are only partially understood. It is thought that under conditions of stress, such as experienced in the diseased heart, Ang II is secreted by cardiomyocytes, thereby activating neighboring fibroblasts. The activated fibroblasts produce transforming growth factor-β (TGF-β), a cytokine that stimulates the production of numerous factors that mediate myofibroblast differentiation. Relevant to this proposal, a recent report by Lenga et al., identified one of these factors as osteopontin (OPN) (Lenga et al., 2008), a multifunctional extracellular matrix protein that is not normally found in blood vessels and heart, but is upregulated in these and other tissues in pathological conditions.

It is clear that ventricular remodeling plays a key role in the progression of heart failure, and this is worsened in diabetes. However, the mechanisms leading to the exacerbated cardiac remodeling seen in diabetes are not entirely clear. Management of hyperglycemia is clearly beneficial in human type 2 diabetes with respect to end organ damage, suggesting that high blood glucose contributes to the pathogenesis of diabetic cardiomyopathy (Skyler).
Initial studies were designed to investigate the molecular basis underlying the ventricular remodeling and diastolic dysfunction observed in DCM. A common underlying factor in both hypertrophic heart failure and DCM is the structural remodeling of the left ventricle characterized by increased deposition of extracellular matrix and fibrosis. To examine the molecular alterations that occur in structural remodeling seen in diabetes preliminary extracellular matrix and adhesion molecule PCR microarray surveys of genes regulated in the ventricle of a rat model of type 2 diabetes revealed elevated mRNA levels of OPN, a matrix protein previously implicated in diabetic microvascular calcification and tissue fibrosis. OPN has also been shown to be upregulated in several other forms of cardiovascular disease, including atherosclerosis (C. M. Giachelli et al., 1993; Hirota et al., 1993; Shanahan, Cary, Metcalfe, & Weissberg, 1994), ischemia reperfusion (Trueblood, Xie, Communal, Sam, Ngoy, Liaw, Jenkins, Wang, Sawyer, Bing, Apstein, Colucci, & Singh, 2001), and heart failure (K. Singh et al., 1999).

C. OSTEOPONTIN

1. Identification and Structure

OPN is a phosphorylated glycoprotein of the extracellular matrix that is involved in cell signaling via integrin binding and its interaction with growth factors, cytokines, chemokines and proteases, and is involved in structural tissue remodeling (D. T. Denhardt, Noda, O'Regan, Pavlin, & Berman, 2001). OPN is also known as secreted phosphoprotein 1 (SPP1) and early T-lymphocyte activation protein 1 (ETA-1) highlighting its role in processes of inflammation and interactions with the immune system. The OPN protein is a negatively-charged acidic hydrophilic protein that is
synthesized in a variety of tissues and cells and then secreted into all body fluids. Mammals exhibit a high degree of sequence homology of the OPN coding sequence. Alternative splice sites are present, but no functional significance of these have been shown (Mazzali et al., 2002). The OPN protein is 314 amino acids long, with a predicted molecular weight of 32 kilo Daltons (kDa). However, post-translational modifications, including glycosylation and phosphorylation, cause it to have an apparent molecular weight ranging from 25-75 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Like the OPN mRNA, the OPN protein is well conserved among mammals (Kazanecki, Uzwiak, & Denhardt, 2007). OPN contains a central integrin binding arginine-glycine-aspartic acid (R-G-D) motif, where it has been shown to interact with $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins (Kazanecki et al., 2007). Interestingly, OPN has been shown to interact with $\alpha_5\beta_1$ and $\alpha_9\beta_1$ integrins in a manner independent of the R-G-D motif. It has been proposed that this interaction is mediated by a cryptic integrin binding motif S-V-V-Y-G-L-R that becomes accessible to integrins after OPN is cleaved by the protease thrombin. Thrombin cleavage expose an additional integrin binding site, it also produces functional chemotactic fragments of OPN (O'Regan et al., 1999). Thrombin cleavage of OPN has been shown to occur after activation of the blood coagulation pathway and is thought to occur at sites of tissue injury and in tumors (Senger, Perruzzi, Papadopoulos-Sergiou, & Van de Water, 1994). OPN can also bind to and signal through the CD44 receptor in an RGD independent manner (Weber, Ashkar, Glimcher, & Cantor, 1996).

2. The OPN Promoter
The predominant regulation of OPN is thought to be at the transcriptional level (D. T. Denhardt & Noda, 1998). The OPN promoter has several transcription factor recognition sites allowing regulation by many hormones, growth factors, and tumor promoters. A high glucose and glucosamine response element has been determined, which contains an E-box and GC rich region, both of which contribute significantly to the expression of OPN in response to high glucose and glucosamine in a PKC dependent manner (Asaumi et al., 2003). An E-box binding sequence (CAGGTG) is a reference to Myc and upstream stimulatory factor (USF) (and other bHLH leucine zipper family proteins) binding sites. Myc and USF have been shown to bind to the E-box binding sequence of the OPN promoter to induce transcription (Malyankar, Hanson, Schwartz, Ridall, & Giachelli, 1999; Wang, Chen, Seth, & McCulloch, 2003) with the involvement of an adjacent GC rich region (GGGCCG) to regulate OPN transcription. E-box elements also act as glucose response elements upon USF binding in other promoters including the acetyl-CoA carboxylase in cardiac fibroblasts (Makaula, Adam, & Essop, 2006), liver pyruvate kinase, spot-14 (Shih & Towle, 1992; Thompson & Towle, 1991) and fatty acid synthase (Foufelle et al., 1995; Shih & Towle, 1992). Never the less exactly how glucose enhances nuclear USF is unclear. A possible mechanism may be PKC dependent, as PKC phosphorylates USF1 in cardiac myocytes, leading to increased E-box binding on the α-myosin heavy chain promoter in response to hemodynamic changes (Xiao, Kenessey, & Ojamaa, 2002). The OPN promoter also contains a specific vitamin D response element (VDRE) that directly binds the vitamin D nuclear receptor (Noda et al., 1990). The Estrogen Related Receptor α (ERRα), an orphan nuclear receptor, also directly binds to the promoter at an unidentified location in a cell specific
manner (Vanacker, Delmarre, Guo, & Laudet, 1998). The promoter also contains functional sites for CRE, nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), AP-1, and the glucocorticoid response allowing regulation by CREB, UTP, and extracellular inorganic phosphate (Fatherazi et al., 2009; Jalvy et al., 2007; M. Renault et al., 2005).

3. OPN Function and Regulation

OPN is a multifunctional protein involved in many biological processes, some of which are described below. An integral aspect to the regulation of the various biological functions of OPN is the post-translational modification state of the protein, including phosphorylation, glycosylation, and proteolytic cleavage. For example, in vitro bone resorption can be stimulated to a greater extent by highly phosphorylated recombinant OPN compared to unphosphorylated recombinant OPN. In rat kidney cells, phosphorylated OPN forms a complex with the extracellular matrix protein fibronectin, indicating that phosphorylated OPN is an important part of the ECM. Furthermore, a reduction in the glycosylation of OPN has been shown to cause a decrease in the localization of OPN to the surface of cells, while proteolytic cleavage by thrombin enhances its adhesion properties and its interaction with various integrins (M. Singh, Foster, Dalal, & Singh, 2010).

Perhaps OPN’s most ubiquitous role is its involvement in the regulation of both physiological and pathological mineralization. The cells responsible for bone remodeling, osteoclasts and osteoblasts, both express OPN protein in normal bone tissue. Here, OPN plays a role in the inhibition of hydroxyapatite formation (Hunter, Kyle, &
Goldberg, 1994; Hunter, Hauschka, Poole, Rosenberg, & Goldberg, 1996). It appears that OPN is involved in the process of bone resorption as opposed to bone formation. The evidence for this comes from OPN knockout mice that do not show bone loss after ovariectomy as typically observed in WT mice (Yoshitake, Rittling, Denhardt, & Noda, ).

OPN has also been shown to exert an inhibitory effect on cell calcification. It is believed that the up-regulation of OPN protein seen in regions of dystrophic calcification is an attempt to prevent or at least limit the calcification of vascular tissues. Similarly, OPN synthesized in the kidney is released into the urine by epithelial cells in the Loop of Henle, distal convoluted tubule, and the papillary epithelium where it is proposed to play an important role in the prevention of renal stones (Hudkins et al., 1999; Kleinman, Beshensky, Worcester, & Brown, 1995). The majority of renal stones are comprised of calcium oxalate crystals, and OPN has been shown to inhibit the growth and aggregation of these crystals \textit{in vitro} (Shiraga et al., 1992; Worcester & Beshensky, 2006). Furthermore, OPN moves calcium oxalate crystallization to the calcium dihydrate phase. These crystals are much less adherent to the renal tubular epithelial cells than calcium oxalate in the monohydrate phase (Wesson & Worcester, 1996; Wesson, Worcester, Wiessner, Neil, & Kleinman, 1998). \textit{In vivo} studies have shown that urinary OPN, along with other molecules, is critical in the renoprotective function observed in animals, although the exact mechanism is still unclear (Hoyer, Asplin, & Otvos, 2001; Jono, Peinado, & Giachelli, 2000).

OPN has also been shown to play an important role in the inflammatory response. Epithelial and endothelial cells as well as non-resident macrophages and T cells may all express OPN during acute or chronic inflammation. However, it is still debated whether
OPN exerts pro-inflammatory or anti-inflammatory effects. It is known that OPN has a role in the recruitment of macrophages and T cells to sites of inflammation. OPN injection caused macrophage infiltration and this effect can be inhibited by administration of neutralizing anti-OPN antibodies (C. Giachelli, Lombardi, Johnson, Murry, & Almeida, 1998). This same method was used to show that, in addition to being expressed by T cells, OPN also causes the chemotaxis, stimulation, and proliferation of T cells. Interestingly, the fragments from thrombin-cleaved OPN seem to function better at these aspects than the native molecule (C. Giachelli et al., 1998; O'Regan et al., 1999).

In addition to the recruitment of leukocytes by OPN, the activation of these cells may also be influenced by OPN. OPN knockout mice are more susceptible to infection by bacteria (Nau et al., 1999), whereas OPN expression in humans contributes to our resistance to bacterial infection (Nau et al., 2000) by initiating cell-mediated immune responses. OPN can stimulate Th1 cytokine production and macrophage IL-12 production via β3 integrin ligation. Which in turn modulates activation of T cells. However, these facets of OPN function, along with the fact that serum OPN is increased in models of autoimmune disease, have also implicated the protein in the pathogenesis of autoimmunity (Cantor, 1995; Iizuka et al., 1998; Lampe, Patarca, Iregui, & Cantor, 1991; O'Regan, Hayden, & Berman, 2000).

In contrast, OPN also can function as a cell survival factor. Here, it may protect cells from apoptosis via the proposed mechanism of binding to the αvβ3 integrin on endothelial cells. This causes activation of NFκB, a pro-survival transcription factor, and thus protects cells from undergoing apoptosis (Scatena et al., 1998). This pathway has been proposed to be important in various cell types including tubular epithelial cells of
the kidney, vascular smooth muscle cells, and haemopoietic cells (Lin et al., 2000; Weintraub, Schnapp, Lin, & Taubman, 2000).

OPN has been hypothesized to contribute to tissue fibrosis by recruitment of macrophages and the production of TGF-β. However, more recent data indicate that OPN may have a more direct role (Ophascharoensuk et al., 1999). It is now believed that in the development of tissue fibrosis, OPN causes the recruitment of fibroblasts, stimulates their proliferation, and is involved in the modulation and secretion of matrix metalloproteinases by fibroblasts (G. Li, Chen, Kelpke, Oparil, & Thompson, 2000; Petrow et al., 2000; Takahashi et al., 2001). Several groups have used an OPN deficient mouse to further determine the effects of OPN in the heart in a variety of pathologies. OPN deficient mice show reduced fibrosis in myocardial fibrosis in models of Ang II infusion leading to cardiac hypertrophy, streptozotocin induced type 1 diabetes, and myocardial infarction (Collins et al., 2004; Matsui et al., 2004; Subramanian, Krishnamurthy, Singh, & Singh, 2007; Trueblood, Xie, Communal, Sam, Ngoy, Liaw, Jenkins, Wang, Sawyer, Bing, Apstein, Colucci, & Singh, 2001a). Osteopontin is also involved in fibrosis of the lungs (Schneider, Lindsay, Zhou, Molina, & Blackburn, 2010; Takahashi et al., 2001) and the kidney (Wolak et al., 2009; Yoo et al., 2006). OPN knockout models of pulmonary fibrosis show decreased collagen and MMP-2 expression in vivo (Berman et al., 2004). Aorta exogenously treated with OPN after acute injury in vivo show increased fibrosis, and transient increases in activated MMP-9, pro and activated MMP-2, and smooth muscle cell proliferation (Seipelt et al., 2005). Osteopontin is thought to modulate fibrosis and cardiac remodeling both by affecting cellular adhesion to the matrix (Collins et al., 2004; Lenga et al., 2008) and by regulating
components of the matrix including collagen, matrix metalloproteinase (MMP) (Kupfahl et al., 2000; Matsui et al., 2004; Z. Xie, Singh, Siwik, Joyner, & Singh, 2003). OPN increases collagen I, MMP-2 protein levels and secretion and TGF-β receptor production and migration (Lee, Seo, Park, Yoo, & Sohn, 2004; Samanna, Wei, Ego-Osuala, & Chellaiah, 2006). Additionally, OPN may further promote fibrosis causing the proliferation and infiltration of fibroblasts (Lenga et al., 2008; M. Renault et al., 2010).

In summary, the functions of OPN are highly varied, as it can act as a soluble cytokine and as a matricellular protein. Due to the various actions of OPN, it is involved in a number of physiological and pathological processes. It is currently of great interest in cancer, kidney disease, and idiopathic lung fibrosis due to its apparent regulatory actions on motility and the extracellular matrix (Oates, Barraclough, & Rudland, 1997).

**D. OPN IN THE HEART**

**1. Expression**

Cardiac myocytes express low levels of OPN under basal conditions (K. Singh, Balligand, Fischer, Smith, & Kelly, 1995; Trueblood, Xie, Communal, Sam, Ngoy, Liaw, Jenkins, Wang, Sawyer, Bing, Apstein, Colucci, & Singh, 2001a). However, the heart is comprised of many other cell types including fibroblasts, endothelial cells, and vascular smooth muscle cells. Cardiac endothelial cells, cardiac fibroblasts, and smooth muscle cells have all been shown to express OPN protein under basal conditions in primary cell culture (Liaw, Almeida, Hart, Schwartz, & Giachelli, 1994; K. Singh et al., 1995; Z. Xie, Singh, & Singh, 2004a). However, the expression of OPN is drastically increased in the heart under pathological conditions such as heart failure (HF) and myocardial infarction
(MI) (Graf et al., 1997; Sam et al., 2004; K. Singh et al., 1999; Subramanian et al., 2007; Szalay et al., 2009; Trueblood, Xie, Communal, Sam, Ngoy, Liaw, Jenkins, Wang, Sawyer, Bing, Apstein, Colucci, & Singh, 2001b; Williams et al., 1995). An increase in the expression of OPN has been associated with the transition to HF (K. Singh et al., 1999), and OPN protein levels increase in infarct and non-infarct regions of the heart following MI. In fact, OPN mRNA levels were increased nearly 40-fold in the infarcted region of the heart just 3 days after MI, and the level of OPN mRNA persisted above control animals for 28 days post-MI. Staining of these tissues for OPN protein indicated that it was mainly localized to the interstitium of the infarcted and non-infarcted regions (Trueblood, Xie, Communal, Sam, Ngoy, Liaw, Jenkins, Wang, Sawyer, Bing, Apstein, Colucci, & Singh, 2001b). Tissues from HF rats also showed that OPN mRNA was expressed primarily in non-myocytes, with it possibly originating from infiltrating macrophages and fibroblasts in the interstitial and perivascular space. This is a common theme in the heart, as OPN protein was also observed in the interstitium of rats with thermal injury (K. Singh et al., 1999), chronic myocarditis, or inherited cardiomyopathy in hamsters (Murry, Giachelli, Schwartz, & Vracko, 1994; Williams et al., 1995). In all cases it is proposed that the main source of the OPN protein is from infiltrating macrophages (Szalay et al., 2009). It has been shown that in left ventricular hypertrophy there is an associated increase in the expression of OPN, and this OPN is produced by cardiac myocytes (Graf et al., 1997; Z. Xie, Singh, & Singh, 2004b). More important to this study, in streptozotocin-induced diabetic cardiomyopathy an increase in the expression of OPN from cardiac myocytes was observed (Subramanian et al., 2007). OPN mRNA and OPN protein in cardiac myocytes were increased in human patients
with dilated cardiomyopathy, as determined by immunohistochemical analysis (Satoh et al., 2005; Stawowy et al., 2002). This increase in OPN expression by cardiac myocytes correlated with an impairment of left ventricular function in humans (Stawowy et al., 2002). These studies provide the critical evidence that increased levels of OPN mRNA and protein are associated with left ventricular remodeling in humans. Although observations indicate that infiltrating macrophages are the main source of OPN protein in particular pathologies, it has been proven that cardiac myocytes and fibroblasts are capable of expressing OPN under various pathologies and stresses (Graf et al., 1997; Satoh et al., 2005; Stawowy et al., 2002; Subramanian et al., 2007).

2. Stimulation of OPN Expression in the Heart

OPN expression appears to be differentially regulated by different factors depending on which cell types are examined in the heart in response to the various treatments. Glucocorticoids have been shown to increase OPN expression in cardiac myocytes and cardiac endothelial cells. However, a combination of interleukin-1β (IL-1β) and interferon-γ (INF-γ) can increase the expression of OPN in endothelial cells, but not the cardiac myocytes (K. Singh et al., 1995). Ang II has been shown to increase OPN expression in cardiac fibroblasts and endothelial cells (Z. Xie et al., 2001; Z. Xie, Singh, & Singh, 2004a). Further evidence for the role of Ang II in the regulation of OPN expression in the heart comes from studies showing that the expression of OPN in the myocardium of hypertensive rats with HF could be inhibited with the angiotensin converting enzyme inhibitor, captopril (K. Singh et al., 1999).
This complexity of the differential regulation of OPN gene expression could be due to variation in the signal transduction pathway that is activated by a specific treatment in a specific cell type. A common mechanism, however, is the Ang II-mediated increase in OPN expression in cardiac myocytes and cardiac fibroblasts appears to be the activation of extracellular related kinases 1/2 (ERK1/2) and production of reactive oxygen species (ROS) (Z. Xie et al., 2001; Z. Xie, Singh, & Singh, 2004a). In addition, ERK1/2 increases OPN expression in the mouse myocardium in the condition of chronic myocarditis (Szalay et al., 2009). Despite these advances, it still remains unclear as to what stimulus is responsible for the increased OPN expression seen in cardiac myocytes in conditions of cardiac hypertrophy and diabetic cardiomyopathy.

OPN contributes to a variety of pathologies, therefore the mechanisms regulating OPN has been investigated in multiple cell types. The regulation of OPN in other cells types should provided insight into mechanisms of OPN regulation in the heart and in cardiac cells. Ang II has been one of the most prominent regulators of OPN in arterial smooth muscle, and has been shown to increase OPN both in vitro (C. M. Giachelli et al., 1993) and in vivo (deBlois et al., 1996). In addition OPN knockout mice receiving Ang II infusion show reduced cardiac fibrosis and a lower heart to body weight ratio compared to wild type mice (Collins et al., 2004). Multiple lines of recent evidence suggest that OPN is a necessary component of the Ang II-dependent, pro-fibrotic signaling pathway, which has also been implicated in the pathology of DCM. It is clear that Ang II mediates the regulation of OPN in vascular remodeling and a similar association has been shown between the renin-angiotensin system (RAS) and OPN in fibrotic renal disease (Can et al., 2003). Ang II is a critical signaling molecule for
hypertrophic cardiac remodeling and fibrosis, and RAS is significantly activated in diabetes. Hyperglycemia has also been shown to increase Ang II levels in both NRVM and NRVF in a RAS dependent mechanism (V. P. Singh, Le, Bhat, Baker, & Kumar, 2007; V. P. Singh, Baker, & Kumar, 2008). Ashizawa et al. showed that NRVF stimulated with Ang II showed increased OPN mRNA and protein levels (Ashizawa et al., 1996) and OPN expression in response to Ang II has been observed in cardiomyocytes by Graf et al. (Graf et al., 1997). Recent compelling evidence begins to better define the relationship between high glucose, the RAS, and ECM production in the heart, and we propose that OPN is an important component of that profibrotic signaling pathway. Cardiac fibroblasts are the main contributors to the ECM, and in pathological settings Ang II induces proliferation and over production of ECM proteins, leading to adverse structural tissue remodeling, or fibrosis. In studies on cultured cardiac fibroblasts, high glucose treatment led to renin-dependent intracellular generation of Ang II via local, intracellular RAS (V. P. Singh et al., 2008). The objective of this project was to determine if there is a mechanistic connection between hyperglycemia, Ang II, and OPN. Based on these findings we hypothesize that OPN, which is not normally found in the heart but is upregulated in pathological conditions, is upregulated in response to hyperglycemia via an Ang II dependent mechanism.

PKC is also involved in OPN expression in other cell types including vascular smooth muscle cells, mesangial cells, and osteoblasts (Beck & Knecht, 2003; Kawamura et al., 2004; C. P. Sodhi, Batlle, & Sahai, 2000; Takemoto et al., 1999). Additionally, PKC was shown to be involved in the high glucose mediated OPN expression in aortic smooth muscle cells (Kawamura et al., 2004; Takemoto et al., 1999). There is some
indication in past literature of what PKC isoforms may be involved in OPN expression. PKCβ regulates OPN expression in the diabetic kidney (Ishii et al., 1996; Kelly, Chanty, Gow, Zhang, & Gilbert, 2005a) and the high glucose induced OPN expression in renal proximal tubular cells (Hsieh et al., 2006). Overexpression of PKCε in the heart leads to increased OPN expression in both the atria and ventricle in aged mice (Goldspink et al., 2004). PKCε and PKCδ regulate OPN expression in a model of skin carcinogenesis (Chang, Tucker, Hicks, & Prince, 2002). I hypothesize that OPN expression is regulated by PKC in cardiac cells in response to high glucose, additionally we will address which PKC isoforms are involved.

3. Role of OPN in the Heart

Expression of the OPN protein in the heart has been shown to play a role in the remodeling of the left ventricle and the extracellular matrix. Particularly, it was shown that post-MI, OPN is involved in the protection of the heart against left ventricular dilation (Krishnamurthy, Peterson, Subramanian, Singh, & Singh, 2009; Trueblood, Xie, Communal, Sam, Ngoy, Liaw, Jenkins, Wang, Sawyer, Bing, Apstein, Colucci, & Singh, 2001b). This shows that OPN plays a beneficial role in left ventricular remodeling post-MI, thus contributing to the strength and organization of the tissue. It is hypothesized by Trueblood et al. that OPN is beneficial in MI because of OPNs promotion of fibrosis surrounding the infarct area (Graf et al., 1997; Trueblood, Xie, Communal, Sam, Ngoy, Liaw, Jenkins, Wang, Sawyer, Bing, Apstein, Colucci, & Singh, 2001a). In support of this hypothesis, Figure 1 shows that mice lacking OPN protein show dramatically less fibrosis post-MI compared to their wild type counterparts (Trueblood, Xie, Communal,
Sam, Ngoy, Liaw, Jenkins, Wang, Sawyer, Bing, Apstein, Colucci, & Singh, 2001a). As shown in Figure 1, scanning electron microscopy revealed normal collagen content and fiber size in the myocardium of wild type sham mice. As shown in the wild type MI image there was an increase in the number and size of collagen filaments in the LV after MI in the wild type mice. In contrast, knock out (KO) sham mice had a disrupted fiber organization at baseline (KO-Sham). Post-MI these mice could not respond to the infarct by increasing fibrillar collagen between cells of the myocardium (KO-MI). Additionally, there was no significant increase in the collagen I mRNA in the myocardium of the KO-MI mice. However, collagen I mRNA was increased nearly 3-fold in the WT-MI mice (Trueblood, Xie, Communal, Sam, Ngoy, Liaw, Jenkins, Wang, Sawyer, Bing, Apstein, Colucci, & Singh, 2001a). These data suggest a critical role for OPN in the development of fibrosis following MI in the heart. Furthermore, OPN has been shown to be involved in the increased fibrosis seen in other models of myocardial remodeling such as streptozotocin-induced diabetic cardiomyopathy and Ang II-induced cardiac hypertrophy (Matsui et al., 2004; Subramanian et al., 2007).
It is also possible that OPN exerts its positive effects on fibrosis through inhibition of the proteins that degrade ECM proteins. Matrix metalloproteinases (MMPs) are a family of proteins that serve to digest the proteins of the ECM, and thus have an

Figure 1: Analysis of fibrosis in WT and OPN KO mice

Scanning electron microscopy of non-infarcted areas of left ventricle of wild-type (WT) and osteopontin knock-out (OPN KO) mice revealed differences in fibrosis. WT-Sham hearts exhibit normal collagen content and fiber size, whereas wild-type post-myocardial infarct (WT-MI) heart showed increased thin collagen filaments and numerous larger collagen fibers. The fibrillar collagen weave appeared reduced or disrupted in the KO group both in the KO-Sham and KO-MI heart. (Figure modified from Trueblood et al., 2001)
important role in the process of myocardial remodeling (Lindsey, Mann, Entman, & Spinale, 2003; Spinale, 2007). Lending support to this hypothesis, OPN inhibited the IL-1β-mediated increase in the expression and activation of MMP-2 and MMP-9 in rat cardiac fibroblasts. Interestingly, OPN alone had no effect on the activity of MMPs. However, when in the presence of interleukin 1 beta (IL-1β) OPN could act presumably through β3 integrins and eventually PKC-ζ to inhibit the activity of MMP-2 and MMP-9 (Z. Xie et al., 2003). In addition, trichrome stained sections of mouse myocardium to quantitatively measure fibrosis revealed reduced fibrosis post-MI in OPN-KO mice compared to WT mice, most likely due to the loss of inhibition of OPN on the MMPs (Krishnamurthy et al., 2009). Since the expression of IL-1β has been shown to be increased post-MI, OPN may lead to increased fibrosis, seen post-MI, by inhibiting the IL-2β-mediated increased MMP expression and activity (Ono, Matsumori, Shioi, Furukawa, & Sasayama, 1998; Z. Xie et al., 2003).

Multiple in vivo studies using OPN knockout mice suggest that OPN plays an essential role in cardiac remodeling (both reparative and eventually maladaptive) via its cellular and tissue actions. OPN knockout mice show impaired collagen production and reparative scar formation post-MI (Trueblood, Xie, Communal, Sam, Ngoy, Liaw, Jenkins, Wang, Sawyer, Bing, Apstein, Colucci, & Singh, 2001a), and reduced pressure overload-induced hypertrophy (Z. Xie, Singh, & Singh, 2004b). OPN knockout mice subjected to experimental diabetes show protection from cardiac fibrosis and dysfunction, suggesting that OPN is important in the development of diabetic cardiomyopathy (Subramanian et al., 2007). OPN has also been implicated in the progression of diabetic nephropathy and diabetic microvascular calcification that contributes to atherosclerosis
and hypertension (Bouvet, Peeters, Moreau, deBlois, & Moreau, 2007; Moe et al., 2002). More recent data have been accumulating that OPN has intracellular actions and localizations in addition to its actions as a secreted protein (Shinohara, Kim, Kim, Garcia, & Cantor, 2008). At the cellular level, OPN is required for differentiation of cardiac fibroblasts to myofibroblasts, an essential step in fibrotic tissue remodeling. Moreover, OPN has been shown to be necessary for the formation of mature focal adhesions, and is an integral structural component of these cellular complexes (Lenga et al., 2008). These functions are consistent with my overall hypothesis that increased OPN expression may play a major role in the cardiac remodeling and inflammation seen in the diabetic heart, and in other cardiac pathologies, contributing to LV dysfunction and eventual heart failure.

OPN can interact with fibronectin and collagen (Kaartinen, Pirhonen, Linnala-Kankkunen, & Mäenpää, 1999; Mukherjee et al., 2006), and it is proposed to be through these interactions that OPN can regulate the Ang II-induced growth of cardiac fibroblasts and their adhesion to these ECM proteins (Ashizawa et al., 1996; Collins et al., 2004). Cardiac fibroblast isolated from mice lacking OPN were shown to be less resistant to detachment and had impaired spreading compared to fibroblasts isolated from WT mice (Lenga et al., 2008).

OPN has a well-defined role in the increase and maintenance of the fibrosis at the site of MI, thus protecting the heart from dilation. Furthermore, the additional effects of OPN on the remodeling of the heart that are seen in cardiac fibroblasts (such as modulation of their growth and spreading) also contribute to the deposition of the ECM. Despite its protective effects outlined above, OPN-mediated increases in the fibrosis of
the ventricular myocardium can be detrimental to the heart. Diabetes leads to several alterations in cardiac structure, one of which is fibrosis of the ventricular myocardium. Fibrosis can be detrimental to cardiac function, by decreasing contractile force, slowing electrical conduction, and generating arrhythmias. Therefore, the goal of my research is to determine if OPN is upregulated in type 2 diabetes and the mechanisms leading to increased OPN expression in cardiac cells. This goal was addressed by investigating the regulation of OPN in the diabetic milieu and its regulation in isolated cultured cardiac cells.

E. ANGIOTENSIN II

Angiotensin II (Ang II) is an endocrine, autocrine, and intracrine hormone that is an integral part of the renin-angiotensin-aldosterone system (RAS). The RAS is a hormonal system that regulates blood pressure and fluid balance in the body. The kidneys secrete renin when the blood volume is low. Renin is produced in the kidneys in response to both decreased intra-renal blood pressure at the juxtaglomerular cells, or decreased delivery of Na\(^+\) and Cl\(^-\) to the macula densa. If more Na\(^+\) is sensed, renin release is decreased. Renin then can stimulate the production of Ang II by first acting on the precursor molecule angiotensinogen. Angiotensinogen is an \(\alpha\)-2-globulin that is produced constitutively and released into the circulation mainly by the liver and is the substrate molecule for the enzyme renin. Although human angiotensinogen is 452 amino acids long, only the first 12 amino acids are the important for activity. Renin cleaves the peptide bond between the tenth amino acid leucine (Leu) and the eleventh valine (Val) residues on angiotensinogen. The eleventh and twelfth amino acids are required for
proper cleavage. Angiotensin I is the ten amino acid molecule formed by the action of renin on angiotensinog en (angiotensin I sequence: Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu). Angiotensin I does not appear to have any known biological activity in the body, and exists solely as a precursor molecule to the active peptide, angiotensin II (Poe et al., 1984).

Ang I is converted to angiotensin II through removal of two C-terminal residues (His-Leu) by an enzyme known as angiotensin-converting enzyme (ACE). ACE, which is located predominantly in the capillaries of the lung, can also be found throughout the body. ACE is secreted by pulmonary and renal endothelial cells, therefore it has its highest density in the lung due to the high density of capillary beds there. ACE is a target for inactivation by ACE inhibitor drugs, which decrease the rate of angiotensin II production and in turn decrease the RAS system activity, and thus are prescribed in pathological conditions such as high blood pressure and heart failure.

One function of angiotensin II is arteriolar vasoconstriction, resulting in increased blood pressure. Ang II can also stimulate the secretion of the hormone aldosterone from the adrenal cortex. Aldosterone causes the kidney to increase the reabsorption of sodium and water. This increases the volume of fluid in the blood, which also increases blood pressure. If the renin-angiotensin-aldosterone system is too active, blood pressure will be elevated. There are many drugs that interrupt different steps in this system to lower blood pressure. These drugs are one of the main ways to control high blood pressure (hypertension), heart failure, kidney failure, and harmful effects of diabetes. Angiotensin II is degraded to angiotensin III by enzymes known as angiotensinases. These proteins are located in red blood cells and the vascular beds of most tissues. Ang II has a half-life in
circulation of approximately 30 seconds, whereas, in tissue, it may be as long as 15–30
minutes. Ang III retains the aldosterone stimulating ability of Ang II but has only 40% of
the vasoconstriction ability.

Several tissues, including the heart, also have a local RAS (Dostal, Rothblum,
Chernin, Cooper, & Baker, 1992; Dostal, Rothblum, Conrad, Cooper, & Baker, 1992;
Dostal, Rothblum, & Baker, 1994; Endo-Mochizuki et al., 1995; Pieruzzi, Abassi, &
Keiser, 1995). This local RAS can then produce Ang II intracellularly in both myocytes
and fibroblasts (V. P. Singh et al., 2007; V. P. Singh et al., 2008) or extracellularly in the
interstitial space (Danser & Deinum, 2005). Locally produced Ang II then can act as an
autocrine or paracrine effector by binding to cell membrane localized AT\textsubscript{1} and AT\textsubscript{2}
receptors. However, intracellularly produced Ang II is also able to act through an
intracellular mechanism (Baker et al., 2004; Baker & Kumar, 2006; Cook, Zhang, & Re,
2001; V. P. Singh et al., 2008; Tadevosyan et al.). Recently, localization of AT\textsubscript{1} and AT\textsubscript{2}
receptors on the nuclear envelope has been determined, elucidating a mechanism of
intracellular Ang II action (Tadevosyan et al.). Locally produced Ang II results in
cardiomyocyte growth, cardiac hypertrophy and fibrosis by regulating gene expression,
particularly TGF-\(\beta\), collagen and NFkB (Baker et al., 2004; Baker & Kumar, 2006; Cook
et al., 2001; Filipeanu, Henning, de Zeeuw, & Nelemans, 2001; V. P. Singh et al., 2007;
V. P. Singh et al., 2008).

The angiotensin receptors are a class of G protein-coupled receptors with
angiotensins as ligands. The AT\textsubscript{1} and AT\textsubscript{2} receptors share an amino acid sequence
identity of \(~30\)% and have a similar affinity for Ang II. The AT\textsubscript{1} receptor is coupled to
the G proteins G\textsubscript{q}, which stimulates membrane-bound phospholipase C beta, and G\textsubscript{i},
which inhibits the production of cAMP from ATP and thus activation of the AT\textsubscript{1} receptor leads to increased cytosolic Ca\textsuperscript{2+} concentrations. This mechanism is dependent on the IP\textsubscript{3}-dependent Ca\textsuperscript{2+}-release from internal stores such as the endoplasmic reticulum, and in turn triggers cellular responses such as stimulation of protein kinase C. Activation of AT\textsubscript{1} and G\textsubscript{q} also leads to production of diacylglycerol (DAG), another important activator of PKC activity. Some of the functional effects mediated by the AT\textsubscript{1} receptor include vasoconstriction, aldosterone synthesis and secretion, increased vasopressin secretion, cardiac hypertrophy, vascular smooth muscle cell proliferation, decreased renal blood flow, cardiac contractility, central osmo-control and extracellular matrix formation.

AT\textsubscript{2} receptors are not as abundant in the adult as the AT\textsubscript{1} receptors; however, they are more plentiful in the fetus and neonate. The AT\textsubscript{2} receptor remains enigmatic and has not been well-characterized. Effects mediated by the AT\textsubscript{2} receptor may include inhibition of cell growth, fetal tissue development, modulation of extracellular matrix, apoptosis, cellular differentiation, and potentially vasodilation and left ventricular hypertrophy.

F. PROTEIN KINASE C

1. Structure, Function, and Activation

To further define the mechanism regulating OPN expression in response to hyperglycemia, the role of Protein Kinase C (PKC) will be examined. Although Ang II is known to activate several downstream targets, I chose to concentrate on the involvement of PKC as it has been previously implicated in the regulation of OPN in other cell types.
It is my hypothesis that hyperglycemia increases Ang II leading to activation of PKC, causing increased OPN expression in cardiac cells.

PKC classically is activated in response to Ang II in a mechanism dependent on Gαq activation through the AT₁ receptor. However, Ang II has recently been reported to have an internal mechanism of PKC activation (V. P. Singh et al., 2008). There are three families of PKCs, classical, novel and atypical. PKC isoforms α, β₁, β₂ and γ make up the classical PKCs and require calcium and diacylglycerol (DAG) for activation. PKC δ, ε, η, and θ are novel PKCs and require DAG but do not require Ca²⁺ for activation. The atypical PKCs include the I, ζ, N1 and N2 isoforms and do not require either DAG or Ca²⁺ for activation. Upon activation PKCs are translocated to the membrane and can stay activated for extended periods after the original stimulus is gone. PKCs then phosphorylate a variety of target proteins that lead to actions including secretion, gene expression, proliferation, and muscle contraction. PKCs are comprised of two functional domains, the regulatory domain and the kinase domain. The regulatory domain is divided into a C1 and C2 domain; however, some isoforms only contain one or the other as described below. The C1 domain is the diacylglycerol (DAG) sensor, while the C2 domain is the Ca²⁺-sensing region. The dependence of PKCs on DAG and Ca²⁺ for activation relies upon which of these two domains are present in the mature protein. Classical PKCs contain both C1 and C2 domains. Novel PKCs contain only a functional C1 domain, therefore, these enzymes respond to only DAG and not the rises in intracellular [Ca²⁺]. Atypical PKCs have a C1 domain, although they are considered atypical because this domain does not bind DAG. Thus, atypical PKCs respond to neither DAG nor Ca²⁺. As reviewed above, all PKCs contain a C1 domain which
contains the binding pocket for DAG; however, the atypical PKC C1 domain has an impaired ligand-binding pocket that prevents it from interacting with DAG. The C2 domain is present in classical and novel PKCs where it serves to target these subclasses of enzyme to the membrane in a Ca\(^{2+}\)-dependent process. Novel PKCs, which do not respond to Ca\(^{2+}\), have a C2 domain that is deficient in the key amino acid residues for Ca\(^{2+}\) binding (Newton, 2010).

In general, the PKC enzyme is maintained in an inactive form in the cytosol due to binding of a pseudosubstrate domain, which is located in the regulatory region, to the substrate binding domain of the kinase region. DAG produced and tethered at the membrane along with free Ca\(^{2+}\) in the cytosol contribute to the translocation of the PKC enzyme to the membrane by these two activators interacting with the C1 and C2 domains, respectively. The energy for removal of the pseudosubstrate domain from the kinase core is provided by the enzyme binding to the membrane via the C1 and C2 domains. Phosphorylation of a key threonine residue in the activation Loop of the kinase region must take place before the kinase can become active and phosphorylate substrates (Newton, 2010). This threonine residue (a slightly polar, phosphorylatable amino acid) can be mutated to an alanine (small, neutral, non-phosphorylatable amino acid) residue to create a constitutively inactive or dominant negative recombinant PKC enzyme (Ping et al., 1999). A constitutively active PKC enzyme can be created by deleting amino acid residues 154-163 of the inhibitory pseudosubstrate domain of PKC (Wotton, Ways, Parker, & Owen). Generally, *in vitro* phosphorylation of the critical Threonine residue for activation of the PKC enzyme is accomplished by the phosphoinositide-dependent kinase-1 (PDK-1) (Newton, 2010).
For conventional PKCs, the majority of the enzyme is located in the cytosol. Occasionally, enzyme will collide with the membrane; however, it will not significantly interact with it. Once Ca\(^{2+}\) has bound to the C2 domain, collision with the membrane results in the C2 domain binding to the membrane. Here, PKC is tethered to the membrane but not activated. The PKC protein then can translocate in the membrane to allow the C1 domain to bind to its membrane-delineated ligand DAG. The energy of this high-affinity interaction is used to remove the pseudosubstrate domain from the substrate-binding core to allow substrate to bind here. Novel PKCs lack the C2 domain, consequently, their rate of translocation to the membrane fraction is decreased nearly an order of magnitude compared to conventional PKCs, which have both the C1 and C2 domains. The regulation of atypical PKCs is not well-defined; however, it is clear PDK-1 is required for the phosphorylation step (Newton, 2010).

2. PKC in the Heart: Role in OPN Expression

Six PKC isoforms are present in rat ventricular myocytes, PKC \(\alpha, \beta1, \beta2, \delta, \varepsilon, \) and \(\zeta\) (Disatnik, Buraggi, & Mochly-Rosen, 1994; Kohout & Rogers, 1993; Malhotra, Kang, Cheung, Opawumi, & Meggs, 2001), while ventricular fibroblasts express only PKC \(\alpha, \delta, \varepsilon, \) and \(\zeta\) (Borner, Guadagno, Fabbro, & Weinstein, 1992; Piacentini et al., 2000). PKC-\(\alpha\) is upregulated in end-stage heart failure (Belin et al., 2007), and contributes to ventricular dysfunction, concentric hypertrophy and alterations in calcium homeostasis (Braz et al., 2004), while PKC-\(\alpha\) knockouts showed increased myocardial contractility. PKC-\(\beta\) is also upregulated in heart failure (Inagaki et al., 2002) and is thought to be involved in
eccentric hypertrophy, the inflammatory response, and fibrosis. PKC-ε is involved in concentric hypertrophy, the inflammatory response and fibrosis while both ε and δ PKC isoforms have been shown to mediate myocyte death (Murriel, Churchill, Inagaki, Szweda, & Mochly-Rosen, 2004). *In vivo* PKC inhibition has been shown to help maintain a lower end-systolic dimension and increased fractional shortening, reduced fibrosis and collagen I levels in the LV, and increase the life span in Dahl hypertensive rats, which expire due to heart failure (Inagaki, Koyanagi, Berry, Sun, & Mochly-Rosen, 2008). Several of these actions have also been attributed to OPN and it is my hypothesis that PKC’s regulation of OPN contributes to these effects. In cultured adult rat cardiomyocytes PKC β1, β2, δ, ε, and ζ have been shown to be translocated in response to hyperglycemia in an Ang II dependent manner, however, only PKCζ did not require AT₁ receptor activation for translocation (Malhotra et al., 2001). This suggests that PKCζ is activated either through AT₂ or through an intracellular Ang II mechanism. Inhibition of PKCβ prevents the expression of OPN in rat kidney *in vivo*, and further it prevents TGF-β and phosphorylated Smad2 (which transmit TGF-β signals from the cell surface to the nucleus to regulate transcription) upregulation and fibrosis of the kidney (Kelly, Chanty, Gow, Zhang, & Gilbert, 2005b). PKC regulation of OPN has also been documented *in vitro* in rat aortic smooth muscle cells (C. P. Sodhi, Phadke, Batlle, & Sahai, 2001), mesangial cells (C. P. Sodhi et al., 2000), and osteoblast like cells (MC3T3-E1) (Beck & Knecht, 2003).
G. FOCAL ADHESIONS

1. Structure, Function, and Activation

Focal adhesions are site of tight adhesion to the extracellular matrix developed by cells. They provide a structural link between the actin cytoskeleton and the ECM. Focal adhesions regulate motility, inform the cell about the condition of the ECM (such as signaling force transduction) and regulate cell to cell and cell to ECM adhesion (Fraley et al., 2010). Focal adhesions are formed around a transmembrane core of an α-β integrin heterodimer, which binds to a component of the ECM on its extracellular region, constitutes the site of anchorage of the actin cytoskeletons to the cytoplasmic side of the membrane and mediates various intracellular signaling pathways. Because integrins do not contain actin-binding or enzymatic activities, all of the structural and signaling events are presumably mediated by proteins associated with the integrin cytoplasmic tails and molecules they recruit. There are three main groups of focal adhesion components based on their location, extracellular, transmembrane, and cytoplasmic. Extracellular components include collagen, fibronectin, heparin sulfate, laminin, proteoglycan, vitronectin. Transmembrane components include integrins, LAR-PTP receptor, layilin, syndecan-4. The majority of components are intracellular and include but are not limited to actin, paxillin, vinculin, protein tyrosine kinases (Focal adhesion kinase, Pyk2), protein Serine/Threonine kinases (ILK, PKC), protein phosphatases and paxillin (Lo, 2006).

Focal adhesions contain a bundling of actin filaments and aggregation of integrins in the plane of the membrane (Ezratty, Bertaux, Marcantonio, & Gundersen, 2009). The aggregation of integrins activates focal adhesion kinase and leads to the assembly of a
multicomponent signaling complex (Burridge & Chrzanowska-Wodnicka, 1996). Focal adhesions are temporary attachments that serve as points of traction for motile cells. They are built up on the leading edge of motile cell and broken down on the trailing edge. The contraction of focal adhesion associated actin stress fibers is thought to propel the cell body forward. As the cell migrates, integrin clustering induces the formation of small focal adhesions (also referred to as focal contacts) at the front of the cell. Some of these nascent focal adhesions mature into larger focal adhesions, whereas others are rapidly turned over (Ezratty et al., 2009).

2. Osteopontin and Focal Adhesions

Focal adhesions are built around integrins that are bound to the ECM. OPN is an ECM protein that binds to integrins to exert its effects, and the functions of OPN overlap significantly with the function of focal adhesions. OPN expression is associated with migration, matrix formation, adhesion to matrix and cell survival. Additionally OPN is required for the formation of mature focal adhesions and increases adhesion in several cell types that in dependent on integrins (Ding et al., 2002; Lenga et al., 2008). In osteoblasts where integrins are down regulated steady state OPN mRNA levels are increased (Lim, Taylor, Li, Vogler, & Donahue, 2005). In addition OPN effects the signaling molecules in focal adhesions; OPN induces FAK phosphorylation and ILK dephosphorylation to mediate migration (J. Li, Han, Wen, & Li, 2007).
The signaling pathway diagrammed in Figure 2 illustrates my hypothetical pathway for high glucose-induced OPN expression in cardiac cells. In my studies, a normal glucose concentration is considered 5 mM, as mammals maintain blood glucose levels between 3.6 and 5.8 mM. To stimulate the high glucose seen in diabetes a concentration of 25 mM glucose was used as uncontrolled diabetes can lead to fasting blood glucose levels of 22 mM (Khan, Safdar, Ali Khan, Khattak, & Anderson, 2003). I hypothesize that high glucose leads to Ang II production and action through the internal mechanism determined by Singh et al. High glucose, through a yet to be determined mechanism, stimulates the production of angiotensinogen (AGT) in both NRVM and NRVF. The enzyme renin then converts the AGT to Ang I, and this reaction can be inhibited pharmacologically by a renin inhibitor, aliskiren. Ang I is then converted to Ang II by chymase in myocytes or ACE in fibroblasts, and this pathway can be inhibited by a chymase inhibitor, chymostatin, or an ACE inhibitor, captopril or benzapril. High glucose leads to increased Ang II levels intracellularly in both NRVM and NRVF by this method (V. P. Singh et al., 2007; V. P. Singh et al., 2008). Angiotensin II, which can be secreted by the cell or produced extracellularly, can then bind to the AT1 or AT2 receptors to cause activation of PKC via autocrine or paracrine pathways. Ang II can also increase PKC activation in an AT1 independent mechanism (V. P. Singh et al., 2007; V. P. Singh et al., 2008). Whether Ang II is acting intracellularly or extracellularly, the result is still the activation of the enzyme PKC. I hypothesize that PKC or downstream effectors can then act at the OPN promoter to induce expression of the OPN protein. In response to
high glucose, OPN protein is produced and secreted by the cell into the myocardium where it can affect cardiac remodeling and myocardial fibrosis through its actions previously described. OPN may localize to focal adhesions to exert its effects on cardiac structure. The specific aims of this project are intended to determine if cardiac OPN expression is increased in type 2 diabetes and to elucidate the pathway leading to increased OPN expression in cardiac cells.
Figure 2: The hypothetical signaling pathway mediating high glucose induced upregulation of OPN. As explained in the text, high glucose activates PKCs through Ang II actions either intracellularly or extracellularly. I propose here a hypothetical pathway whereby high glucose causes PKC activation through either the traditional AT$_1$ receptor activation or through an intracellular production and action of Ang II leading to PKC activation. Activation of PKC leads to increased promoter activity of OPN, thus increasing OPN protein expression. OPN is proposed to then localize to focal adhesions were it affects cardiac remodeling. Experimentally relevant inhibitors of the various factors I propose to be a part of this signal transduction pathway are also shown. (Figure adapted from Singh, V.P. et al., AJP 2008)
CHAPTER THREE

SPECIFIC AIMS AND OVERVIEW OF PROJECT

Diabetes and cardiac pathology is a widespread and significant disease in populations throughout the world and determining alterations in gene expression leading to cardiac pathology and the mechanism behind these alterations leads to potential therapeutic advances.

Main Hypothesis: I hypothesize that OPN expression is upregulated in the diabetic heart by high glucose. I further hypothesize that this high glucose mediated upregulation is through an Ang II and PKC dependent pathway resulting in cardiac remodeling in the diabetic heart.

A. SPECIFIC AIM 1:

To determine alterations in gene expression of adhesion and extracellular matrix proteins that are involved in cardiac remodeling of an animal model of type 2 diabetes.

Rationale: Cardiac function is dependent upon the structure and components of the cardiac tissue. As such cardiac remodeling is particularly important to the continued function of the heart, and several physical alterations occur in damaged hearts. To this end the structural and functional changes that occur in a disease process and the
molecular alterations leading to these changes are of particular interest. There is not currently an ideal model of type 2 diabetes, the leading type of diabetes, suitable for cardiac research. The following experiments were undertaken to determine if the ZSF model would be a suitable model for cardiac research in type 2 diabetes. Further we wanted to determine alterations in gene expression that could be contributing to the cardiac remodeling seen in type 2 diabetes. Upon determining alterations in gene expression we focused on OPN expression because of its involvement in remodeling in other tissues and its upregulation in several cardiac pathologies. High glucose is known to be an underlying problem in diabetes leading to gene alteration so we chose to look at the expression of OPN in response to high glucose.

**Experiments:**

1. To better define the ZSF rat model and progression of diabetes body weight, blood pressure and blood glucose were monitored monthly from 8 to 40 weeks. Using transthoracic echocardiography, left ventricular catheterization, and trichrome staining the cardiac effects of type 2 diabetes were determined.

2. Extracellular and adhesion molecule microarrays were used to determine alterations in LV gene expression at the mRNA level in ZSF animals compared to control animals. Expression of OPN in response to high glucose was determined using RT-PCR and western blotting.

3. Localization of OPN to focal adhesions was studied by overexpressing OPN-GFP in NRVM or NRVF and examining if OPN colocalized with focal adhesion by immunostaining with paxillin to determine focal adhesions.
B. SPECIFIC AIM 2:

To evaluate the involvement of Angiotensin II in the regulation of OPN expression in response to high glucose stimulation.

Rationale: Ang II is increased in response to diabetes and hyperglycemia in the heart and cardiac cells (V. P. Singh et al., 2007; V. P. Singh et al., 2008) and Ang II infusion increases OPN expression in the heart (Collins et al., 2004). The classical method of Ang II action is through the angiotensin receptors, however intracellular actions of Ang II in cardiac cells has been described (Baker et al., 2004; Baker & Kumar, 2006; V. P. Singh et al., 2007; V. P. Singh et al., 2008; Tadevosyan et al., ). The existence of relatively selective Ang II pathway inhibitors permits the testing of whether Ang II is involved in OPN expression and if it is acting through the extracellular angiotensin receptors or through an intracellular pathway.

Experiments

(1) Isolated NRVM or NRVF were treated with Ang II to determine if Ang II could increase OPN expression. OPN expression was determined at the mRNA level using RT-PCR.

(2) Isolated NRVM or NRVF were treated with a renin inhibitor or a chymase inhibitor prior to treatment with high glucose to determine if the production of Ang II was involved in the high glucose mediated upregulation of OPN. OPN expression was determined at the mRNA.
(3) Isolated NRVM or NRVF were treated with an angiotensin receptor blocker (ARB) prior to treatment with high glucose to determine if Ang II was acting through the AT1 receptor to mediate high glucose induced OPN upregulation. OPN expression was determined at the mRNA level.

C. SPECIFIC AIM 3:

To identify the involvement of PKC in the regulation of OPN by high glucose in cardiac cells.

Rationale: PKC activity is increased in the cardiac tissue of a type 1 diabetic model (Liu et al., 1999; Subramanian, Krishnamurthy, Singh, & Singh, 2006) and high glucose causes activation of PKC in an Ang II dependent manner. This suggests that Ang II increases OPN expression in a PKC dependent manner. PKC has been shown to increase OPN in several cell types however the isoforms involved have not been determined. Some evidence has been provided by the PCKε overexpressing mouse which shows high levels of OPN mRNA expression in the atria and ventricles.

Experiments

(1) Isolated cultured NRVM or NRVF were treated with a general PKC inhibitor prior to treatment with high glucose or Ang II, OPN expression was then determined at the mRNA and protein level.

(2) A classical PKC inhibitor was applied to cultured NRVM or NRVF prior to stimulation with high glucose and OPN expression was determined at the mRNA
level to determine if classical PKCs are involved in the high glucose mediated OPN expression.

(3) Constitutively active PKCε or dominant negative PKCε was overexpressed in cultured NRVM or NRVF using an adenovirus to determine if PKCε could increase OPN expression and if PKCε was involved in the high glucose mediated expression of OPN. OPN expression was measured at the mRNA level.
CHAPTER FOUR

MATERIALS AND METHODS

A. REAGENTS

1. Materials and Culture Media for Cell Culture Experiments

Dulbecco’s Modified Eagle Medium (DMEM) was obtained from HyClone/Thermo Scientific or Cellgro/Mediatech (Herndon, VA). PC-1 tissue culture medium was obtained from Lonza (Basel, Switzerland). PC-1 medium is formulated in a specially modified DMEM/F12 base and contains a complete HEPES buffering system with insulin (15 µg/ml), T₃ (0.5 nM), fatty acids, and proprietary proteins (at concentrations of less than 530 µg/ml). 500 ml of PC-1 medium was completed by adding 1 vial of PC-1 supplement and 5 ml of Antibiotic-Antimycotic solution (100x). Antibiotic-Antimycotic solution used in PC-1 medium was obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS), penicillin-streptomycin, and trypsin used for tissue culture and cell propagation were obtained from Cellgro/Mediatech (Herndon, VA). All other chemicals used were reagent grade.

2. PKC Adenoviruses

Constitutively active PKCε adenovirus (Adv-caPKCε) was kindly provided by Dr. Allen Samarel. The PKCε enzyme was made constitutively active by deletion of amino acid residues 154-163 of its pseudosubstrate domain. Dominant negative PKCε
adenovirus (Adv-dnPKCε) was kindly provided by Dr. Allen Samarel with the consent of Dr. PeiPei Ping (University of California Los Angeles, Los Angeles, CA) (Ping et al., 1999). The PKCε enzyme was made dominant negative by mutating the ATP binding site (amino acid 436 (K to R)) and its pseudosubstrate domain (amino acid 159 (A to E)) thereby destroying the construct’s kinase activity but maintaining the enzyme in an active conformation. The nuclear encoded β-gal adenovirus was used to control for the effects of viral infection was kindly provided by Dr. Allen Samarel. All viruses were used at a multiplicity of infection (MOI) of 10 in NRVM and 100 in NRVF for up to 48 hours.

3. Antibodies

Osteopontin polyclonal antibody was obtained from Millipore (Billerica, MA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody was used at a dilution of 1:2,000 for Western blotting. Horseradish peroxidase-conjugated goat-anti-rabbit antibody was obtained from Bio-Rad and used at dilution of 1:10,000 for Western blotting. Horseradish peroxidase-conjugated goat-anti-mouse antibody was obtained from Sigma Aldrich and was used at a dilution of 1:5,000 for Western blotting.

4. Drug Treatments

The experiments utilized a combination of the following drugs; the angiotensin type 1 receptor blocker, Candesartan (AstraZeneca, 1 µM), the renin inhibitor aliskiren (Novartis, 50 µM), and the chymase inhibitor chymostatin (Sigma, 10 µM), the general PKC inhibitor GF109203X (Calbiochem, 1 µM), and the classical PKC inhibitor Go 6976 (Calbiochem, 1 µM). The drug concentrations used were based on previously
published reports (Frazier et al., 2007; V. P. Singh et al., 2007; V. P. Singh et al., 2008; Zhou et al., 2002).

**B. ZSF TYPE 2 DIABETIC MODEL**

Male ZSF obese diabetic rats, their lean controls, and Wistar Kyoto Controls were obtained at 6 weeks of age from Charles River Laboratories (GMI, Charles River). The ZSF rats were placed on the recommended diabetogenic diet (Purina no. 5008) the control and lean animals were placed on the normal diet. All animal protocols were approved by the Loyola University Chicago Institutional Animal Care and Use Committee. At 6 weeks of age radiotelemeters were implanted subcutaneously under Ketamine-xylazine anesthesia (100 mg/kg + 7 mg/kg) as previously described (Henze et al., 2008) to monitor blood pressure. Blood pressure and weight were measured weekly. BP was measured in awake animals maintained in their cages for one hour in the absence of human interaction. Blood glucose measurements were performed using the FreeStyle Blood Glucose Monitoring System (TheraSense) and transthoracic echocardiography was performed under the inhalation anesthesia, isoflurane, monthly starting at 18 weeks of age. Both long and short axis views were taken during transthoracic echocardiography to allow us to derive the left ventricular mass, end diastolic volume, end systolic volume, cardiac output, and ejection fraction, giving an indication of relative cardiac function. For a more accurate measure of cardiac function, LV catheterization was performed using a Millar pressure volume catheter just prior to euthanasia at 30 or 40 weeks to measure pressure and volume within the left ventricle. The data were analyzed using Dataquest A.R.T. 4.0 to obtain peak systolic pressure, LV end-diastolic pressure, +dP/dt, -dP/dt, and
the time-constant for isovolumic relaxation (tau), as well as pressure volume loops following inferior vena cava compression. The heart was excised upon euthanasia weighed and frozen in liquid nitrogen for subsequent analysis. To get measure of blood volume in µL in the ventricle blood volume was calibrated on one animal from each group (control, lean, ZSF). Blood volume was calibrated using the calibration cuvettes from Millar. Blood was obtained from the vena cava just prior to euthanasia and immediately placed into cuvettes of a known size and kept and 37 C. Volumes were measured using the Millar catheter to get a standard curve. Ventricular blood volume was assessed by correlating the catheter volume reading to the standard curve using the PVAN software.

After euthanasia at 40 weeks the hearts were excised and the heart was trimmed of RV and atria and the LV was weighed. The tibiae were removed from the animal and were cleaned, dried and measured. The LV weight was then normalized to the tibia length (LV weight/tibia length) to adjust for the size of the animals.

C. RNA ISOLATION FROM RAT LEFT VENTRICULAR TISSUE

Total RNA was isolated from LV tissue using the Chomczynski and Sacchi method published previously (Chomczynski & Sacchi, 1987). LV tissue was frozen in liquid nitrogen upon excision as mentioned previously. Frozen tissue was weighed and placed in 1 ml of Solution D (4 M guanidine thiocyanate, 0.025 M sodium citrate, pH 7, 0.5% sarcosyl) per 100 mg of tissue and homogenized. 10% 2M sodium acetate, pH 4, was added and mixed gently. An equal amount of water saturated phenol was added to the sample and mixed. Chloroform-iso-amyl alcohol was added at a volume of 20% of
the original sample size, and sample was vortexed. Sample was stored on ice for 15 minutes, then centrifuged at 10,000 rpm for 15 min at 4°C. The aqueous phase was transferred to a new tube and an equal amount of isopropanol was added, the sample was stored at -20°C for 18 hours to allow for RNA precipitation. Samples were then centrifuged at 10,000 rpm for 15 min at 4°C. The pellet was dried and dissolved in 400 µL of Solution D. 1.5 volumes of isopropanol was added to the sample and mixed. The sample was stored at -20°C for 18 hours to allow for RNA precipitation then centrifuged at 14,000 rpm for 10 min at 4°C. The pellet was washed with 500 µL of cold 75% ethanol then centrifuged for 5 min at 4°C and the ethanol removed. The sample was washed with ethanol (as just described) three times. The sample was then air dried and dissolved in RNase free water. Samples were stored at -80°C until use.

**D. cDNA SYNTHESIS**

cDNA was synthesized from RNA isolated from rat left ventricular tissue and cultured neonatal rat ventricular myocytes and fibroblasts. RNA was isolated as described (see sections C. and K.) and reverse transcribed using the iScript kit (Bio-Rad, Hercules, CA). RNA was quantified using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and 1 µg of total RNA was combined with 4 µl of 5X iScript reaction mix (which contains oligo(dT) and random hexamer primers), 1 µl of iScript reverse transcriptase and nuclease free water to get a total reaction volume of 20 µl. Samples were gently mixed and incubated at 25°C for 5 minutes, 42°C for 30 minutes, then 5 minutes at 85°C. The resulting cDNA was stored at -20 °C until further use.
E. MICROARRAYS

Extracellular Matrix and Adhesion Molecule Oligonucleotide or RT-PCR Microarrays from SuperArray were carried out according to the manufacturer’s protocol. Oligonucleotide microarrays were analyzed using the RT²Profiler PCR Array Data Analysis. RT-PCR Microarrays were analyzed using Relative Quantity (RQ) calculated using the ∆∆Ct method, where a value of 1 represents no change in expression.

F. IMMUNOHISTOCHEMISTRY

A cryostat was used to slice frozen LV tissue into 14 micrometer sections and fixed with 4% paraformaldehyde for 5 min. Sections were rinsed twice with PBS then permeabilized with 0.5% Triton X-100 in PBS for 15 min then washed with PBS for five minutes 2 times. Sections were then blocked for one hour using 0.1% Triton X-100 and 1% goat serum in PBS. OPN primary antibody (Santa Cruz) was applied to the sections at a dilution of 1:400 in blocking buffer for one hour. Sections were then rinsed 2 times with 0.1% Triton X-100 PBS for 5 minutes. Alexa fluor 488 goat anti-mouse (Invitrogen) was used as a secondary antibody. It was applied to the sections at a dilution of 1:400 in blocking buffer for one hour. Sections were then rinsed twice in 0.1% Triton X-100 PBS for 5 min. Phalloidin conjugated to Alexa Fluor 568 (Invitrogen) was used at a dilution of 1:40 in blocking buffer for 20 minutes. Coverslips were rinsed in 0.1% Triton X-100 twice for five minutes. Slides were rinsed twice with 200 proof ethanol then air dried. Slides were mounted with ProLong Gold Antifade reagent with DAPI (Invitrogen).
G. TRICHOME STAINING

LV sections were Masson trichrome stained in parallel using a Trichrome Staining Kit from Sigma-Aldrich according to the manufacturer’s instructions. Briefly, a cryostat was used to section frozen LV tissue into 14 micrometer sections and fixed with 4% formaldehyde for 5 min then sections were rinsed twice with PBS. Slides were covered in 56°C Bouin’s Solution in a 56°C incubator for 15 minutes, slides were then submerged in cooled tap water. Slides were rinsed section side down with running tap water. Slides were submerged in working Weigert’s Iron Hematoxylin Solution for 5 min then rinsed with deionized water. Slides were submerged in Biebrich Scarlet-Acid Fuchsin for 5 min then rinsed with deionized water. Slides were submerged in working phosphotungstic/phosphomolybdic acid solution for 5 min, then submerged in aniline blue solution for 5 min. Slides were then moved into 1% acetic acid for 2 min. Slides were rinsed and dehydrated using two 5 minute 200 proof ethanol rinses, cleared with xylene and mounted. All compared slides were done in parallel.

H. CELL ISOLATION PROTOCOL

All animal protocols were approved by the Loyola University Chicago Institutional Animal Care and Use Committee. Neonatal ventricular myocytes were isolated from the hearts of 2-day old rats by the collagenase digestion method by Rekha Iyengar (Samarel & Engelmann, 1991). Neonatal rat pups, at postnatal day 1 to 2 were decapitated and the heads immediately frozen in liquid nitrogen to lessen animal suffering, bodies were washed with 70% ethanol, and the chest cavity opened to expose
the heart. The beating hearts were rapidly removed and placed into an ice cold, CO$_2$-equilibrated perfusion buffer without collagenase or Ca$^{2+}$. Collected hearts were trimmed free of atria and major blood vessels, washed in fresh 37° C perfusion buffer and minced with an alcohol-cleaned, previously unused razor blade. About 6-10 hearts were placed into each 25 ml Erlenmeyer flask containing 5 ml of enzyme/calcium free perfusion buffer and 5 ml of perfusion buffer with collagenase and calcium for the first 15 min of incubation. The perfusion buffer containing 0.1 mM CaCl$_2$ was pre-warmed overnight in a 5% CO$_2$ tissue culture incubator and the next morning, 0.5 mg of collagenase/ml (collagenase was obtained from Worthington, Lakewood NJ) was added to make enzyme digestion perfusion buffer. Tissue fragments were shaken at 100 to 1200 rpm for 15 to 23-minute incubations at 37°C. Cells, cell debris, and residual red blood cells that were released during the first 15-minute incubation were aspirated off and discarded. Fresh enzyme digestion buffer (8-10 ml) was added and incubated for 15 min. Tissue fragments were triturated several times with a large sterile, large bore plastic pipette, and incubated for an additional 7 min. After 22 minutes of digestion, tissue fragments were allowed to settle by gravity for 0.5 min, and all released cells were collected from the supernatant using a transfer pipette.

Released cells were filtered through a sterile 40 mm nylon mesh that was washed with 3 ml of enzyme free buffer. Fresh enzyme containing buffer (8-10 ml) was added to the tissue fragments, this step was repeated two more times. The cells released after each dissociation were isolated by centrifugation (50 x g, 4 min at room temperature), and resuspended in 5 ml enzyme free perfusion buffer. The resuspension was added to a 50 ml collection tube containing 12.5 ml perfusion buffer, 90 µl fetal bovine serum, and 125
µl of 0.5 M EDTA and stored on ice. At the second collection another 125 µl of 0.5 M EDTA was added to the collection tube. Myocytes were pre-plated for 30 minutes in serum-free PC-1 medium to reduce non-myocyte contamination. The nonadherent NRVM were then plated onto gelatin coated dishes or glass coverslips at a density of 2.5 million cells per 60 mm² dish, 1.5 million cells per 35 mm² dish, or 0.15 million cells per coverslip and left undisturbed in a 5% CO₂ incubator for 24h. Unattached cells were removed by aspiration, washed twice in PBS, and the attached cells were maintained in DMEM containing penicillin streptomycin. NRVF were obtained from cells that adhered during preplating and were cultured in 25 mM glucose DMEM + 10% FBS +1% non-essential amino acids and penicillin streptomycin.

I. CELL CULTURE

Cell culture dishes for NRVM were coated using 0.2% gelatin, 1 ml for each well of 6 well plate or 2 ml for each 60mm plate, for 20 minutes. The remaining gelatin solution was aspirated. NRVM were plated onto the gelatin coated dishes in undiluted PC-1 medium and left undisturbed in a 5% CO₂ incubator for 24 hours. Unattached cells were then removed by aspiration. The cell layer was gently washed with PBS. Media was changed to low glucose DMEM containing penicillin-streptomycin for glucose studies or high glucose DMEM with penicillin-streptomycin for all other studies. Cells were then left in growth medium overnight and experiments started the following day. Myocytes were then maintained in serum-free culture medium for up to 96 hours. NRVF were used on the 3rd passage, except for the PKC translocation studies in which NRVF were used on the 4th passage. NRVF were passaged by removing the cells from the
culture dish with 2 mL 0.5% trypsin. When cells had begun to round up the culture plate was rinsed with 6 mL 10% FBS in PBS. Cells were collected in a 15 mL conical tube and centrifuged at 1,000 RPM for 5 minutes. The supernatant was then aspirated off and the NRVF were re-suspended in 25 mM DMEM containing penicillin streptomycin + 10% FBS +1% non-essential amino acids and plated on uncoated cell culture plates. At the 3rd or 4th passage NRVF were plated on uncoated cell culture dishes or glass coverslips and were maintained in 25 mM DMEM containing penicillin streptomycin + 10% FBS +1% non-essential amino acids until confluent. Media was changed to low glucose DMEM containing penicillin-streptomycin + 0.5% FBS for glucose studies or high glucose DMEM with penicillin-streptomycin + 0.5% FBS for all other studies. Cells were then left in growth medium overnight and experiments started the following day.

**J. CELL CULTURE EXPERIMENTS**

NRVM were plated on gelatin coated plates in PC-1 (Lonza) + 1% antibiotic/antimycotic (MediaTech). NRVF at the third passage were plated in fibroblast medium (25 mM DMEM with penicillin-streptomycin, 10% fetal bovine serum (FBS, heat inactivated) and 1% non-essential amino acids).

**1. Angiotensin II Studies**

24 hours after plating cells were gently rinsed with PBS and medium was changed to 25 mM glucose DMEM + 1% penicillin streptomycin with 1µM Ang II or 25 mM glucose DMEM + 1% penicillin streptomycin + 0.5% FBS with 1µM Ang II (Sigma) for
NRVM or NRVF respectively. Cultures were collected in RNeasy Plus lysis buffer for qRT-PCR or Hunter’s Buffer for SDS-PAGE and Western blotting.

2. High Glucose studies

24 hours after plating cells were gently rinsed with PBS and medium was changed to 5 mM glucose DMEM (MediaTech) with 1% penicillin streptomycin for NRVM or 5 mM glucose DMEM with 1% penicillin streptomycin with 0.5% FBS for NRVF. After 24 hours cultures were incubated with an inhibitor or vehicle (amount corresponding to volume of drug added to medium) for one hour prior to the addition of 20 mM glucose or mannitol. Cultures were collected after 18 hours in RLT plus lysis buffer for mRNA analysis or at 24 hours in hunter’s buffer for protein analysis, samples were stored at -80°C.

K. RNA ISOLATION FROM CULTURED CELLS

Total RNA was isolated from cultured NRVM and NRVF using the Qiagen RNeasy Plus kit. Cultured cells were washed twice with PBS then scraped in 350 µl of RNeasy Plus lysis buffer containing 0.1% β-mercaptoethanol. RNeasy Plus (Qiagen) was used to isolate RNA from each lysate according to the manufacturer’s instructions. The samples were stored at -70°C until use.
L. RT-PCR

1. General Real-Time PCR Conditions

SYBR Green technology was used to quantify the mRNA expression of OPN. SYBR Green is a DNA intercalating dye that emits fluorescence when bound to DNA. During the PCR cycle, as DNA accumulates, the fluorescent signal is produced in proportion to the DNA concentration. Due to the fluorescent nature of the detection, at some point (known as the threshold cycle or C\textsubscript{T}) the signal increases above a background signal. The earlier a signal is detected (i.e. the lower the C\textsubscript{T}), the higher the abundance of the original target mRNA. When the target signal is compared to a signal emitted by a housekeeping gene (in this case, 18S ribosomal RNA), an index of relative abundance is determined (i.e. the difference between the target and housekeeping gene signal is known as the ΔC\textsubscript{T}). When compared between test and control treatments, the relative indexes can be compared and is called a ΔΔC\textsubscript{T} analysis. By comparing the ΔC\textsubscript{T} from each treatment (i.e. High glucose treated versus control (mannitol treated)), changes in expression at the mRNA level can be assessed.

All quantitative PCR (qPCR) reactions were performed in 96-well plates using an ABI 7300 Real-Time PCR system (ABI, Foster City, CA). Each reaction consisted of the following components: 12.5 µl of 2X Maxima\textsuperscript{TM} SYBR Green qPCR Master Mix (Fermentas Life Sciences, Burlington, Ontario, Canada) 0.2 µM of forward and reverse primers (rat OPN and mammalian 18s RNA), 50 ng template RNA and nuclease free water to reach a total volume of 25µl and each reaction was performed in triplicate. Once each reaction was added to the plate, the entire plate was centrifuged at 4000 x g for 4 minutes to pull all reaction constituents to the bottom of each well and remove any
bubbles. The plate was then placed into the ABI 7300 system and the reaction parameters were set. The qPCR reaction conditions listed in order were as follows; (1) the initial pre-treatment step lasted 2 min at 50°C, (2) the initial denaturing cycle lasted 10 min at 95°C, (3) the PCR cycle had two stages (95°C for 15 s followed by 60°C for 1 min) that was repeated 40 times.

2. Quantitative PCR Data Analysis

All qPCR data were analyzed using the Sequence Detection Software v1.3 that is associated with the ABI 7300 qPCR System. The threshold detector was set to lie within the linear portion of the exponential phase. Once $C_T$ values were determined for all replicates of both target and house-keeping genes, $\Delta C_T$ values were generated based on averages of triplicates.

<table>
<thead>
<tr>
<th>Table 1: Rat reverse-transcriptase PCR primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>----------</td>
</tr>
</tbody>
</table>
| Mammalian 18S | (+) CGCCGCTAGAGGTGAAATTC  
| | (-) TTGGCAAATGCTTTCGCTC |
| Rat OPN   | (+)AGTGTTTGTCTTTGGCTTGTT  
| | (-)TCAGCCAAGTGGCTACAGCAT |

**Table 1: Rat reverse-transcriptase PCR primers.** Listed above are the primer sequences used to assess the presence of OPN mRNA from isolated NRVM and NRVF. Positive (+) sequences represent the 5’ to 3’ sense strand while the negative (-) sequences represent the 5’ to 3’ antisense strand. Primer sequences for OPN were adapted from Tajouri et al. (Tajouri, Mellick, Tourtellotte, Nagra, & Griffiths, 2005), primer sequences for mammalian 18S were adapted from Madani et al. (Madani, De Girolamo, Muñoz, Li, & Sweeney, 2006), and both were synthesized by Integrated DNA Technologies.
M. OPN QUANTIFICATION IN NRVM VS. NRVF

To determine the amount of OPN in NRVM vs. NRVF a standard curve was generated. RNA was isolated from NRVM treated with high glucose. RNA was reverse transcribed to produce cDNA using iScript as outlined previously. This cDNA was amplified by PCR using the rat OPN primers, the PCR product (125 bp) was then isolated on an agarose gel to isolate and extracted using Qiaquick gel isolation kit (Qiagen). The DNA obtained from the agarose gel was ligated into a plasmid and transformed into bacteria. The bacteria were grown up and DNA was isolated using a Qiagen plasmid purification kit (the work up to this point was done by Dr. Leanne Cribbs). The amount of plasmid was quantified, and using a serial dilution between the ranges of 0.1 ng to 0.0000000001 ng of OPN target cDNA, a standard curve qRT-PCR was run. To determine OPN mRNA expression in untreated NRVM (n=8) or NRVF (n=8) equal amounts of RNA (1 µg) were used to produce cDNA using iScript from 8 different cell preparations. Equal amounts of cDNA (corresponding to 50 ng template RNA) from each sample were used in the qRT-PCR reaction. The Ct of OPN in NRVM or NRVF was extrapolated to the standard curve previously described to assess the relative expression of OPN in NRVM vs. NRVF.

N. IMMUNOCYTOCHEMISTRY

NRVM or NRVF were plated on gelatin-coated or uncoated glass coverslips (NRVM were plated at a density of 1.5x10^5) in a 24 well plate, respectively. After treatment period coverslips were washed twice with PBS and fixed using 4% paraformaldehyde in PBS for 15 min. Coverslips were rinsed twice with PBS then
permeabilized with 0.5% Triton X-100 in PBS for 15 min then washed again with PBS for five minutes 2 times. Sections were then blocked for one hour using blocking buffer (0.1% Triton X-100 and 1% goat serum in PBS). Table 2 shows primary antibodies that were used in immunostaining and western blotting and indicates where the antibodies were obtained and the dilution of the primary antibody used. Primary antibody was applied to the sections at the dilution indicated in table 2 in blocking buffer for one hour at room temperature. Coverslips were then rinsed 2 times with 0.1% Triton X-100 PBS for 5 minutes. Primary binding was detected with Alexa Fluor secondary antibodies diluted in blocking buffer for 1 hour at room temperature. Sections were rinsed twice in 0.1% Triton X-100 PBS for 5 min. Coverslips were rinsed with 200 proof ethanol two times for 5 minutes and allowed to air dry. Coverslips were mounted using ProLong gold antifade reagent with DAPI (Invitrogen). Fluorescently labeled cells were then viewed with a Zeiss Microscope.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Western Blot Dilution</th>
<th>Immunostain Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH mAb</td>
<td>Fitzgerald Industries</td>
<td>1:5000</td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor 568 Phalloidin</td>
<td>Molecular Probes/ Invitrogen</td>
<td>1:40</td>
<td></td>
</tr>
<tr>
<td>Paxillin mAb</td>
<td>BD Transduction Laboratories</td>
<td>1:400</td>
<td></td>
</tr>
<tr>
<td>OPN pAb</td>
<td>Millipore</td>
<td>1:500</td>
<td>1:1000</td>
</tr>
<tr>
<td>OPN mAb</td>
<td>Santa Cruz Biotechnology</td>
<td>1:400</td>
<td></td>
</tr>
<tr>
<td>MPIIIB10, mAb (supernatant)</td>
<td>Developmental Studies Hybridoma Bank</td>
<td>1:50</td>
<td></td>
</tr>
<tr>
<td>SP1.D8, PAb (concentrate)</td>
<td>Developmental Studies Hybridoma Bank</td>
<td>1:500</td>
<td></td>
</tr>
</tbody>
</table>
O.  OPN-GFP IMMUNOLOCALIZATION

NRVM or NRVF were plated on glass coverslips in PC-1 medium with 1% antimycotic/antibiotic or fibroblast medium (25 mM glucose DMEM with 1% non-essential amino acids, 1% penicillin streptomycin, and 10% fetal bovine serum (FBS)), respectively. After 24 hours cells were gently rinsed with PBS and medium was changed to 5 mM glucose DMEM with 1% penicillin streptomycin for NRVM for 5mM glucose DMEM with 1% penicillin streptomycin and 0.5% FBS for NRVF and were infected with an adenovirus encoding GFP-OPN, 10 and 100 MOI respectively. After 24 hours glucose or mannitol was added for 24, 36, or 48 hours. Cells were fixed with 4% paraformaldehyde for 15 minutes then washed twice with PBS for 5 minutes. Cells were stained as above except paxillin (BD Transduction Laboratories) mouse monoclonal antibody was used as a primary antibody and was detected with Alexa Fluor 568 goat anti mouse secondary antibody (Invitrogen). DAPI was used to stain nuclei. Immunostaining was visualized using a Zeiss microscope.

P.  HARVESTING NRVM AND NRVF FOR PROTEIN QUANTITATION

NRVM or NRVF were washed with phosphate buffered saline (PBS) on ice then harvested in Hunter’s Buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1mM EGTA, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 0.1 mM deoxycholic acid, 1% Triton X 100, 0.1% SDS, 10% glycerol, 1X halt protease inhibitor cocktail (Pierce)) and lysed by sonication. The membranes were pelleted by centrifugation at 4°C for 10 minutes at 135,000 x g max. Samples were stored at -80°C until further use.
Q. PKC TRANSLOCATION

NRVM were plated on collagen coated plates as previously described. 24 hours after plating cells were gently rinsed with PBS and medium was changed to 5 mM glucose DMEM with 1% penicillin streptomycin. After 24 hours cultures were gently rinsed with PBS and either 5 mM glucose + 20 mM mannitol or 25 mM glucose DMEM with 1% penicillin streptomycin for 18 hours. NRVM were also treated with Phorbol 12-myristate 13-acetate (PMA) as a positive control. NRVM were treated with PMA for one hour. NRVM were collected in homogenization buffer (50 mM Tris 8.3, 1 mM Ethylenediaminetetraacetic acid, and 1X halt protease inhibitor cocktail (Pierce)) that contains no detergent and lysed by sonication. Samples were then centrifuged for at 50,000 RPM for 10 minutes. The supernatant was removed and pellet was washed with homogenization buffer, the sample was then centrifuged again. The supernatant was removed and the pellet was suspended in Hunter’s buffer. Samples were stored at -80°C.

R. SDS-PAGE AND WESTERN BLOTTING

For quantitative analysis of specific proteins, crude protein extracts were separated on SDS-PAGE gels. Samples were prepared containing equal amounts of protein (20 µg of total protein for NRVM, 50 µg of membrane fraction protein for PKC translocation studies, 80 µg of total protein for NRVF), as assessed by BCA protein assays, in Laemmli sample buffer (6.25 mM Tris-HCL, pH6.8, containing 0.01% bromophenol blue, 8% SDS, 5% β-mercaptoethanol, 19% glycerol). The samples were denatured by heating to 100°C for 5 min then briefly centrifuged to collect their total volume at the bottom of the tube. Samples were loaded onto 10% SDS-PAGE gels in
addition to lanes containing molecular weight standards. Precision Plus Protein Dual Color Standards were obtained from Bio-Rad (Hercules, CA). The gels were run in SDS gel running buffer (25 mM Tris, 192 mM Glycine, pH 8.3, containing 0.1% SDS) at 35 mA per gel for 2.5 hours until the 25 kDa molecular weight band had migrated to the bottom of the gel. The separated proteins were then transferred to nitrocellulose paper and analyzed via Western Blotting techniques detailed below.

S. WESTERN BLOT ANALYSIS

For quantitative analysis of specific proteins, samples were prepared in Laemmli Sample buffer. Equal amounts of protein were separated by 10% SDS-polyacrylamide gels as detailed above. Western blotting was performed by electro-transfer of the resolved proteins to nitrocellulose (Bio-Rad) in transfer buffer at 120 mA overnight at 4°C in transfer buffer (192 mM glycine, 25 mM Tris-HCl, and 20% methanol). Following transfer, the nitrocellulose was blocked using 5% non-fat dry milk (NFDM, Sanalac) reconstituted in TBST (150 mM sodium chloride, 10 mM Tris-HCl, 0.05% Tween-20, pH 8.0) for 1 hour at room temperature on a rocking platform. The primary antibodies were diluted in 5% NFDM reconstituted in TBST at a dilution of 1:500 for Anti-osteopontin and 1:2000 for anti-GAPDH were added to the nitrocellulose and allowed to incubate overnight at 4°C with shaking. After the primary antibody incubation, the nitrocellulose was washed two times with TBST for 10 minutes. Horseradish Peroxidase (HRP) Conjugate secondary antibody was diluted 1:10,000 for goat-anti-rabbit (Bio-Rad) or 1:5,000 for goat-anti-mouse (Sigma Aldrich) in 5% NFDM in TBST and then added to the nitrocellulose to incubate for 2 hours. The nitrocellulose was washed two times for
10 minutes with TBST. Antibody binding was visualized by using LUMIGlo enhanced chemi-luminescence (ECL) reagents (KPL) to the blots for 1 minute. Visualization of proteins was done by exposing the nitrocellulose to x-ray film (BioMax XAR film, Kodak) for periods of 2 to 30 seconds in a darkroom. Films were developed using an automatic film developer. The band intensity was quantified by scanning the blots and processing the scanned image using Un-Scan-It gel digitizing software (Silk Scientific Inc., Orem, Utah). Results of the densitometric analysis were used to calculate the ratio of OPN protein to GAPDH in each sample. Ratios were then compared to control treatment to get relative expression levels.

T. STATISTICS

SigmaStat (Systat Software, Inc., Point Richmond, CA) was used for all statistical analyses. ANOVA was used for comparisons between the three different animal groups. Paired Student’s t-tests were used for comparisons of RT-PCR data using the dCt values. Comparisons among quantified SDS-PAGE/ western blotting data were done using Student’s t-tests. Differences associated with p-values <0.05 were considered statistically significant and are represented on all figures. The specific statistical tests are indicated in each figure legend.
CHAPTER FIVE

DETERMINE THE ALTERATIONS IN GENE EXPRESSION IN ADHESION AND EXTRACELLULAR MATRIX PROTEINS THAT ARE INVOLVED IN CARDIAC REMODELING OF AN ANIMAL MODEL OF TYPE 2 DIABETES

A. INTRODUCTION

The ZSF rat model exhibits several factors of metabolic syndrome and type 2 diabetes including obesity, hyperglycemia, insulin resistance, moderate hypertension, and severe dyslipidemia. We chose the ZSF animal because it exhibits moderate hypertension in addition to other contributors to diabetes including hyperglycemia which have been thought to work synergistically to lead to enhanced cardiovascular disease, as seen in the human type 2 diabetic population (National high blood pressure education program working group report on hypertension in diabetes, 1994; Epstein & Sowers, 1992). The ZSF animal is a model of diet induced type 2 diabetes, which is commonly seen in the human population. Several past studies have used a streptozotocin induced type 1 diabetic model, however because the etiology and the progression of type 1 and type 2 diabetes are different limited information from this model can be applied to type 2 diabetes. There is also the Zucker Diabetic Fatty (ZDF) model of type 2 diabetes, however the ZDF type 2 diabetic model does not present with hypertension. Therefore, the synergistic effects of hypertension and diabetes that is thought to lead to DCM will not be present in the ZDF model. Given that the ZSF animals have mild hypertension in
addition to the other diabetic symptoms I wanted to further characterize the ZSF rat model particularly with respect to cardiac remodeling in type 2 diabetes. It has been very difficult for researchers to determine what factors of diabetes are responsible for particular changes because of the large number of variables present in most diabetic patients. To separate the added effects of hypertension combined with diabetic factors from just the effects of hypertension, I examined three groups of animals. A control group (Wistar Kyoto) which have no predisposition towards hypertension or diabetes, a lean control group which have a predisposition towards hypertension and are heterozygous for the leptin receptor mutations but fed a normal diet, and the ZSF model of type 2 diabetes. The ZSF rats are a cross of rat strains with two separate leptin receptor mutations (fa and fa<sup>cp</sup>). The two strains crossed to produce the ZSF rats were the lean female ZDF rat (+/fa) and the lean male SHHF rat (+/fa<sup>cp</sup>), which are derived from the obese spontaneously hypertensive rat carrying the corpulent fa<sup>cp</sup> gene. The ZSF animals were fed a high fat “western” diet and the leptin receptor mutation prevents the animal from feeling sated. These factors lead to obesity and eventually metabolic syndrome in the ZSF animals. Both the lean control and the ZSF animals are reported to show mild hypertension, thus comparisons between the lean control group and the ZSF group will help separate the effects of hypertension and the synergistic effects of hypertension combined with other contributors to diabetes and metabolic syndrome. To examine the general characteristics of these animals I looked at weight, blood glucose, and hypertension. I chose to examine the weight of these animals as obesity is often a factor in diabetic patients and because this a model of diet induced diabetes. Furthermore, blood glucose is increased in diabetes as diabetes is a body’s inability to
regulate blood glucose, and increased blood glucose is one of the main criteria for the
diagnosis of diabetes. Hypertension is also a factor in many diabetic patients and mild
hypertension has been reported in the ZSF model. Hypertension was measured using
blood pressure (BP) radiotelemetry in order to determine if there were significant
differences between the ZSF, lean control, and control rats. BP telemetry allowed us to
measure BP in awake animals over an extended time period in absence of human
presence.

It is my hypothesis that the ZSF group will progress toward diastolic dysfunction
compared to lean and control animals. 50 to 75% of diabetics have diastolic dysfunction
depending on glycemic control and diagnostic methods used. Diastolic dysfunction in
diabetic is often characterized by concentric LV hypertrophy and impaired relaxation of
the ventricle, but with normal ejection fraction (Asghar et al., 2009). Echocardiography
was used to assess cardiac function monthly to monitor the progression of cardiac
alterations. Because echocardiography is a non-invasive procedure it allows for serial
measurements of LV wall thickness, end diastolic and end systolic volumes. From these
measurements other indices of cardiac function can be determined such as ejection
fraction, hypertrophy or thinning of the LV wall and if this is concentric or asymmetrical.
Just prior to euthanasia, cardiac function was assessed using a pressure volume catheter.
Pressure volume catheterization is an invasive method that measures LV pressure and
volume simultaneously. By changing preload during while assessing the pressure and
volume in the LV a measure of contraction and relaxation that is independent of pre-load
and after-load can be determined. This method was used to get a more in-depth measure
of cardiac function. Upon euthanasia at 30 weeks the LV was excised and weighed. LV
weight was normalized to tibia length to correct for the large size of the ZSF animals as suggested by Yin et al. (Yin, Spurgeon, Rakusan, Weisfeldt, & Lakatta, 1982). Normalized LV weight was measured to determine if ZSF animals showed LV hypertrophy.

In DCM myocardial collagen deposition is one of the primary pathological processes responsible for reduced elasticity of the myocardium (van Heerebeek et al., 2008), thus I expected to see increased fibrosis in the LV of the ZSF animals. Trichrome staining was used to assess fibrosis in the LV. Trichrome staining differentially stains muscle/cytoplasm, nuclei, and collagen.

Cardiac remodeling is crucial element in the progression of DCM. Remodeling is regulated by molecules of the ECM and molecules involved in adhesion of cells to the ECM, such as collagen, fibronectin, and MMPs. To assess changes in gene expression that may be leading to cardiac remodeling in DCM adhesion molecule and extracellular microarrays were carried out on mRNA isolated from LV tissue. I expected to see alterations in several genes in the ZSF animals compared to control, but particularly increases in genes that would lead to increased fibrosis, such as collagen and fibronectin.

Osteopontin was consistently upregulated in LV of ZSF animals compared to controls and is involved in fibrosis of several tissues and in cardiac remodeling following pathology (Collins et al., 2004; Kupfahl et al., 2000; Matsui et al., 2004; Schneider et al., 2010; Subramanian et al., 2006; Takemoto et al., 1999; Takemoto et al., 2000). Immunohistochemistry was used to assess OPN expression in cardiac tissue and cultured NRVM or NRVF were used to determine if cardiac cells could be contributing to the increased OPN expression seen in the LV of the ZSF animals. To determine if
unstimulated NRVM or NRVF expressed more OPN, qRT-PCR was used to determine the expression of OPN mRNA.

Hyperglycemia is thought to be an underlying factor leading to a host of diabetic complications. High glucose increases production of collagen by fibroblasts (Asbun, Manso, & Villarreal, 2005) and increases OPN expression in a variety of tissues (Hsieh et al., 2006; Takemoto et al., 1999). Thus I chose to look at the effects of high glucose on OPN expression in cardiac cells, although there are other diabetic factors that may contribute to OPN expression in the diabetic heart. Isolated cultures of NRVM or NRVF were used to determine if high glucose increased OPN expression in cardiac cells. qRT-PCR and Western blotting were used to determine OPN expression in at the mRNA and protein level, respectively. 25 mM glucose was used for high glucose studies; this is a particularly high level of glucose seen clinically in uncontrolled diabetes (Hanas & Fox, 2008; Owens, 2006; Zabihi, Wentzel, & Eriksson, 2008). 5 mM glucose was used in the control samples and is in the physiological range of blood glucose levels for mammals (Standards of medical care in Diabetes–2006.). Mannitol (20 mM), a sugar that cells are only able to take up very slowly thereby reducing the energy that can be obtained from this sugar (Boland & Garland, 1993; Mattila, Svanberg, Makinen, & Knuuttila, 1996), was used in conjunction with the normal glucose level (5 mM) in control samples as an osmotic control.

To examine the role of OPN in remodeling we determined if OPN localized to sites of focal adhesions. Because of the large overlap in function of focal adhesions and OPN, such as cell adhesion and migration, I hypothesized that OPN localized and was an integral part of focal adhesions. OPN null mice show reduced focal adhesion number,
leading to a rounder morphology, reduced adhesion, and reduced size (Lenga et al., 2008). OPN binds to integrins and CD44 receptors which are part of focal adhesions and regulates the phosphorylation of focal adhesion kinase, an integral component of focal adhesions (Chen et al., 2009; J. Li et al., 2007). As mentioned previously OPN is involved in cell adhesion and migration. Immunostaining was used in conjunction with adenoviral expression of OPN-GFP to determine if OPN co-localized to focal adhesions in cardiac cells.

The results presented below provide a phenotypic cardiac characterization of the ZSF rat model of diabetes; examine the regulation of OPN by high glucose in cardiac cells, and the localization of OPN in cardiac cells.

B. RESULTS

1. Characterization of the ZSF Rat Model of Type 2 Diabetes

As shown in Figure 3, the group averages ± standard error of the mean (SEM) of weight for control, lean, or ZSF rats show that the ZSF animals have increased weight compared to the control rats, however there was not a significant difference between the weights of the ZSF animals compared to the lean animals. The ZSF rats exhibited increased blood glucose levels compared to both control groups (Figure 4). Blood pressure was measured weekly from 6 weeks of age to euthanasia at 30 weeks by radiotelemetry. However, there was no statistically significant difference in the systolic blood pressures between the three groups of animals (Figure 5). There was not a significant difference in the LV normalized weight (Figure 6) and LV wall thickness (Figure 7) in the ZSF animals. A more precise measure of cardiac function was
determined at 30 weeks using LV pressure volume catheterization. I did not see any significant changes in the left ventricular pressure volume loops (Figure 8). Particularly the end diastolic pressure volume relationship (EDPR), which is a measure of relaxation, or in the end systolic pressure volume relationship (ESPVR), which is a measure of contractility, that are independent of pre-load or after-load. Negative and positive change in pressure over change in time (negative dP/dT and positive dP/dT, respectively) were also not altered. These are measures of the rate of contraction and active relaxation. The only significant differences in indices of cardiac function were increased end systolic pressure in the ZSF compared to control animals and increased end diastolic pressure in the ZSF animals compared to lean animals (Table 3). Increased end diastolic pressure is indicative of diastolic dysfunction. Additionally, trichrome staining revealed apparent increased collagen expression in the LV of the ZSF model compared to control (Figure 9).

2. Molecular alterations in the cardiac tissue of the ZSF rat

Following the functional cardiac characterization of the ZSF rat model, the molecular alterations were assessed. An extracellular matrix and adhesion molecule microarray was used to determine what genes were up- or down-regulated in ZSF animals compared to controls. The data summarized in Table 4 show alterations in some select genes. Osteopontin was consistently upregulated in the LV of the ZSF animals. Selectin and vitronectin are also glycoproteins involved in cell adhesion and spreading. While selectin is upregulated, vitronectin in decreased in the ZSF animals. Hyaluronan and proteoglycan link protein 1 (Hapln 1) is involved in cell to ECM adhesion (Spicer,
Joo, & Bowling) and is increased in the ZSF animals. Additionally, there are alterations in the expression of MMPs, which are involved in the degradation of the ECM. MMP 9 is involved in the degradation of type IV and V collagens, and MMP 7 is involved in proteoglycan and fibronectin cleavage. Both MMP7 and 9 are down regulated in these hearts. MMP12 degrades elastin and MMP16 is cleaves thereby activating MMP2 and both are increased in the ZSF hearts (Nagase & Woessner). Frozen sections (14 µm) from LV tissue of Lean and ZSF animals were processed for immunohistochemistry and stained using a mouse monoclonal OPN antibody detected with a goat anti-mouse Alexa Fluor 488 secondary antibody (green). Nuclei are stained with DAPI (blue) and myofilaments with phalloidin conjugated with Alexa Fluor 568 (red). These immunohistochemistry experiments confirmed that OPN was expressed in the LV of the ZSF animals (Figure 10). However, we were unable to determine what cell type was contributing to the OPN expression in the LV of the ZSF animals from these images.

3. Regulation of OPN in cardiac cells by diabetic factors

After determining that OPN was increased in the LV of the ZSF diabetic rat, I evaluated the regulation of OPN in response to high glucose. Mixed primary cultures NRVM and NRVF were serum starved, then treated with control or high glucose medium for 48 hours. Cells were fixed and stained with phalloidin (red) to distinguish NRVM, an OPN antibody detected with Alex Fluor 488 (green, and this channel is shown in grayscale in bottom panels for better OPN distribution), and the nuclear stain DAPI (blue). As shown in Figure 11, both NRVM and NRVF show cytoplasmic OPN
expression in response to high glucose. Once I had determined that both NRVM and NRVF expressed OPN in response to high glucose, I aimed to determine the expression of OPN in unstimulated NRVM and NRVF. These results would allow me to determine which cell type had a greater basal OPN expression. Isolated cultures of NRVM or NRVF were processed for qRT-PCR. Equal amounts of total RNA were used in the cDNA synthesis reaction. Equal amounts of the cDNA synthesis reaction were then used to perform the qRT-PCR. OPN mRNA expression was extrapolated from the shown standard curve created using known amounts of OPN plasmid. NRVM had significantly increased basal expression of OPN mRNA (Figure 12). To determine if high glucose increased OPN mRNA, isolated cultures of NRVM or NRVF were treated with control (5 mM glucose +20mM Mannitol) or high glucose (25 mM glucose) medium. Both NRVM (Figure 13) and NRVF (Figure 14) show increased OPN mRNA expression as assessed by qRT-PCR and increased OPN protein expression measured by SDS-PAGE and Western blotting in NRVM.

4. Localization of OPN in NRVM or NRVF

Immunostaining was used to determine if OPN localized to sites of focal adhesions. NRVM or NRVF were infected with adenoviral OPN-GFP and treated with control or high glucose medium. Focal adhesions were identified by using an antibody to paxillin, which is localized to focal adhesions (M. C. Brown, Perrotta, & Turner, 1996; Turner, Pietras, Taylor, & Molloy, 1995). Localization to focal adhesions was determined using an antibody to paxillin conjugated to Alex Fluor 568 (red), OPN-GFP is shown in green, and the nuclear stain DAPI is shown in blue. These results show OPN
does not localize to focal adhesions (Figure 15 and 16). OPN localization was altered upon glucose stimulation having a diffuse cytoplasmic localization in fibroblasts and an aggregated cytoplasmic localization in NRVM.
**Figure 3: Increased weight of the ZSF model of type 2 diabetes.** The data presented in this figure are the group averages ± SEM for control (Wistar Kyoto, n=5), Lean (n=4), or ZSF (n=5) rats. The ZSF animals have an increased weight compared to control animals at 40 weeks (one way-ANOVA and post hoc Holm-Sidak).
**Figure 4: Blood Glucose is increased the ZSF model of type 2 diabetes.** The data presented in this figure are the group averages ± SEM for control (Wistar Kyoto, n=5), Lean (n=4), or ZSF (n=5) rats. Blood glucose was measured at 40 weeks by one touch ultra blood glucose monitoring system, blood was obtained from a foot prick. Blood glucose is significantly increased in ZSF animals compared to control and lean animals (one way-ANOVA and post hoc Holm-Sidak).
**Figure 5: Blood pressure is not altered in the ZSF rat model.** The data presented in this figure are the group averages ± SEM for control (Wistar Kyoto, n=5), Lean (n=4), or ZSF (n=5) rats. Systolic blood pressure was measured at 40 weeks by radiotelemetry. There is not a significant difference between the three groups (one way-ANOVA and post hoc Holm-Sidak).
Figure 6: LV mass in the ZSF model of type 2 diabetes. The data presented in this figure are the group averages ± SEM for control (Wistar Kyoto, n=5), Lean (n=4), or ZSF (n=5) rats. After euthanasia at 40 weeks the hearts were immediately removed and the RV and atria were trimmed from the heart and the LV was weighed. The tibiae were removed from the animal after euthanasia and were cleaned, dried and measured. The LV weight was then normalized to the tibia length (LV weight/tibia length) to adjust for the size of the animals. Normalized LV weight was not significantly different between the groups (One way ANOVA and Holm Sidak Post Hoc).
Figure 7: LV wall thickness in the ZSF model. The data presented in this figure are the group averages ± SEM for control (Wistar Kyoto, n=5), Lean (n=4), or ZSF (n=5) rats. LV wall thickness was measured by echocardiography at 40 week just prior to euthanasia. Diastolic intraventricular septum (IVS, d) or diastolic left ventricular posterior wall (LVPW, d) thickness were not significantly different between the groups (One way ANOVA and Holm Sidak Post Ho), only lean and ZSF are shown.
Figure 8: Cardiac Characterization of the ZSF model of type 2 diabetes. Pressure volume loops were measured with a Millar pressure volume catheter in the LV. The vena cava was occluded to reduce preload and slowly released to gradually increase the preload. The table shows the group averages ± SEM of the end systolic pressure volume relationship (ESPVR) and the end diastolic pressure volume relationship (EDPVR) for control (Wistar Kyoto, n=6), Lean (n=7), or ZSF (n=5) rats. ESPVR or EDPVR values were not statistically significant different between groups (one way ANOVA).

<table>
<thead>
<tr>
<th>Group</th>
<th>ESPVR</th>
<th>EDPVR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.045 ± 0.186</td>
<td>0.0205 ± 0.0174</td>
</tr>
<tr>
<td>Lean</td>
<td>0.836 ± 0.205</td>
<td>0.0374 ± 0.0081</td>
</tr>
<tr>
<td>ZSF</td>
<td>0.701 ± 0.232</td>
<td>0.0695 ± 0.0213</td>
</tr>
</tbody>
</table>
Table 3: Cardiac Function Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Lean</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate (bpm)</td>
<td>215.8 ± 32.3</td>
<td>211.6 ± 38.7</td>
<td>192.0 ± 26.5</td>
</tr>
<tr>
<td>End-Systolic Volume (µL)</td>
<td>135.9 ± 18.0</td>
<td>135.3 ± 18.2</td>
<td>146.1 ± 12.0</td>
</tr>
<tr>
<td>End-Diastolic Volume (µL)</td>
<td>193.4 ± 12.1</td>
<td>229.3 ± 26.6</td>
<td>201.5 ± 24.7</td>
</tr>
<tr>
<td>End-Systolic Pressure (mmHg)</td>
<td>90.1 ± 8.0</td>
<td>112.7 ± 9.8</td>
<td>138.6 ± 10.6*</td>
</tr>
<tr>
<td>End-Diastolic Pressure (mmHg)</td>
<td>8.4 ± 1.2</td>
<td>5.1 ± 0.2</td>
<td>12.2 ± 2.6*</td>
</tr>
<tr>
<td>Stroke Volume (µL)</td>
<td>95.2 ± 9.2</td>
<td>127.5 ± 19.0</td>
<td>110.9 ± 31.7</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>43.6 ± 4.7</td>
<td>48.0 ± 2.1</td>
<td>41.1 ± 6.5</td>
</tr>
<tr>
<td>+ dPdt (mmHg/sec)</td>
<td>5898.8 ± 437.1</td>
<td>7012.0 ± 1368.9</td>
<td>7901.4 ± 623.7</td>
</tr>
<tr>
<td>- dPdt (mmHg/sec)</td>
<td>-5122.0 ± 573.0</td>
<td>-4898.8 ± 1143.4</td>
<td>-7068.8 ± 923.3</td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>14.9 ± 1.8</td>
<td>21.3 ± 5.6</td>
<td>21.4 ± 7.5</td>
</tr>
</tbody>
</table>

Table 3: Cardiac Function parameters of the ZSF model of type 2 diabetes. The data presented in this figure are the group averages ± SEM for control (Wistar Kyoto, n=6), Lean (n=7), or ZSF (n=5) rats. The end systolic pressure was significantly increased in ZSF animals compared to control animals (p=0.041, one way ANOVA on ranks), and the end Diastolic pressure was significantly increased in ZSF animals compared to Lean animals (p=0.025, one way ANOVA on ranks).
**Figure 9: Increased Fibrosis in the LV of the ZSF model.** Frozen LV tissue was sliced to 14 µM, mounted on glass slides, and fixed in 4% paraformaldehyde. Slides were stained using the Masson’s trichrome method in parallel. Trichrome staining of LV tissue suggests increased collagen content in the ZSF animal compared to WKY control animals.
Table 4: Selected Microarray Results

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Fold Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hapln1</td>
<td>Hyaluronan proteoglycan protein 1</td>
<td>3.33</td>
</tr>
<tr>
<td>Mmp12</td>
<td>Matrix metalloproteinase 12</td>
<td>3.20</td>
</tr>
<tr>
<td>Mmp16</td>
<td>Matrix metalloproteinase 16</td>
<td>2.53</td>
</tr>
<tr>
<td>Selp</td>
<td>Selectin</td>
<td>3.74</td>
</tr>
<tr>
<td>Spp1</td>
<td>Osteopontin</td>
<td>2.18</td>
</tr>
<tr>
<td>Mmp7</td>
<td>Matrix metalloproteinase 7</td>
<td>-2.57</td>
</tr>
<tr>
<td>Mmp9</td>
<td>Matrix metalloproteinase 9</td>
<td>-2.18</td>
</tr>
<tr>
<td>Vtn</td>
<td>Vitronectin</td>
<td>-2.32</td>
</tr>
</tbody>
</table>

Table 4: Alterations in extracellular matrix and adhesion molecule mRNA expression in ZSF animals. The data presented in this figure are the average relative quantity of mRNA expression of selected genes in the ZSF rats (n=5) compared to the mRNA expression of that gene in lean animals (n=7). Extracellular matrix and adhesion molecule microarrays were used to assess gene expression in control, lean, and ZSF animals. OPN expression was upregulated in all ZSF animals compared to lean animals measured by microarrays.
**Figure 10:** Expression of OPN in Lean vs. ZSF rat heart. Frozen sections (14 µm) from LV tissue of Lean and ZSF animals were processed for immunohistochemistry and stained using a mouse monoclonal OPN antibody detected with a goat anti-mouse Alexa Fluor 488 secondary antibody (green). Nuclei are stained with DAPI (blue) and myofilaments with phallloidin conjugated with Alexa Fluor 568 (red).
**Figure 11: Regulation of OPN in response to high glucose in cardiac cells.** Primary cultured NRVM and NRVF were serum starved, then treated with control or high glucose medium for 48 hours. Cells were fixed and stained with phalloidin to distinguish myofibrillar structure (red), an OPN antibody (green, and shown in grayscale in bottom panels to better distinguish OPN distribution), and the nuclear stain DAPI (blue). M designates the myocyte and F designates the fibroblast.
Figure 12: Basal Expression of OPN in NRVM and NRVF. 

A. Known quantities of OPN DNA were used to make a standard curve using qRT-PCR. 

B. Isolated cultures of NRVM or NRVF were processed for qRT-PCR. OPN mRNA quantities were determined and NRVM and NRVF OPN mRNA expression was extrapolated from the standard curve. Unstimulated NRVM express significantly more OPN mRNA than NRVF (n=8, Student’s t-test).
Figure 13: Regulation of OPN in response to high glucose in NRVM. A. Isolated cultures of NRVM were exposed to control medium (5 mM glucose +20 mM mannitol) or high glucose medium (25 mM glucose) for 18 hours. OPN mRNA expression measured by qRT-PCR is significantly increased with high glucose treatment compared to control treatment (n=8, paired Student’s t-test on dCt values). B. Isolated cultures of NRVM were exposed to control medium (C) or high glucose medium (HG) for 18 hours. OPN protein expression measured by SDS-PAGE and Western blotting and was normalized to GAPDH as a loading control. OPN expression was quantitated using Un-Scan-It gel digitizing software. OPN protein expression is significantly increased with high glucose treatment compared to control treatment (representative blot and graph showing mean ± the SEM for all data, n=9, Student’s t-test on relative quantity).
Figure 14: Regulation of OPN in response to high glucose in NRVF. Isolated cultures of NRVF were exposed to control medium (5 mM glucose +20 mM mannitol) or high glucose medium (25 mM glucose) for 18 hours. OPN mRNA expression measured by qRT-PCR is significantly increased with high glucose treatment compared to control treatment (n=10, paired Student’s t-test on dCt values).
Figure 15: Localization of OPN-GFP in NRVF. Isolated NRVF were infected with an adenovirus expressing OPN-GFP for 24 hours. Cultures were then exposed to high glucose (25 mM glucose) or control medium (5 mM glucose + 20 mM mannitol) for 24 hours. Cells were fixed and immunostained using an anti-paxillin antibody detected with Alex Fluor 568 (red) and the nuclear stain DAPI (blue). OPN-GFP is shown in green. White arrows indicate focal adhesions. Both figures shown are from glucose treated samples due to increased focal adhesions. No colocalization of OPN with focal adhesions.
was observed, but there upon glucose stimulation OPN has a diffuse cytoplasmic localization.

**Figure 16: Localization of OPN-GFP in NRVM.** Isolated NRVM were infected with an adenovirus expressing OPN-GFP for 24 hours. Cultures were then exposed to high glucose (25 mM glucose) or control medium (5 mM glucose + 20 mM mannitol) for 24 hours. Cells were fixed and immunostained using an anti-paxillin antibody detected with Alex Fluor 568 (red) and the nuclear stain DAPI (blue), OPN-GFP is shown in green. White arrows indicate focal adhesions. Both figures shown are from glucose treated samples due to increased focal adhesions. No colocalization of OPN with focal adhesions was observed. OPN localization has an aggregated cytoplasmic localization after glucose localization.
C. DISCUSSION

Diabetes is characterized by high blood glucose and an inability to appropriately regulate the blood glucose levels. Type 2 diabetic patients often present with metabolic syndrome, which entails hyperglycemia, obesity, hypertension, insulin resistance and dyslipidemia. The ZSF rat model of type 2 diabetes expresses all of these contributors to type 2 diabetes. One of the goals of this study was to determine cardiac alterations due to the synergistic effects of hypertension and diabetes. However, the ZSF animals in this study did not have hypertension. These experiments still give insight into cardiac dysfunction in type 2 diabetes independent of blood pressure. All the changes we see are due to effects of obesity and hyperglycemia. Data presented in this chapter show the ZSF animals having blood glucose levels in excess of 300 mg/dL, confirming that the ZSF group in our study did develop hyperglycemia. Further, the ZSF have significantly increased weight. Type 2 diabetes is often associated with obesity in the patient population and is thought to be a factor contributing to the onset of diabetes.

At 40 weeks we see early signs of cardiac remodeling, including increased end diastolic pressure and apparent LV fibrosis. The increased end diastolic pressure is indicative of diastolic dysfunction. This is particularly significant because 47-75% of diabetic patients have some form of diastolic dysfunction. The ZSF model had preserved ejection fraction, which is commonly seen in DCM (Asghar et al., 2009), which would suggest that there is no systolic dysfunction. Passive relaxation is based on the physical
properties of the LV, particularly increased fibrosis which leads to a stiffening of the muscle and reduced passive relaxation. Active relaxation is caused by the binding of ATP allowing release of the myosin heads and muscle relaxation. The negative dP/dT or tau, measures of active relaxation, are not decreased in the ZSF animals. This suggests that the diastolic dysfunction in these animals is due to a reduced passive relaxation, not a problem with cellular relaxation. This reduced passive relaxation emphasizes the role of cardiac remodeling and fibrosis of the LV in DCM. Trichrome staining revealed apparent increased collagen expression in the LV of the ZSF animal, and correlates with the functional alterations in passive relaxation. Several alterations occurred in gene expression, one of these alterations was increased OPN expression, determined by extracellular matrix and adhesion molecule mRNA microarrays. Although several proteins were altered in the LV of the ZSF animals I chose to focus on the regulation of OPN because of OPN role in fibrosis and the increased fibrosis seen in DCM (Asghar et al., 2009; Collins et al., 2004; Matsui et al., 2004; Schneider et al., 2010; Subramanian et al., 2006) and that we saw in the LV of the ZSF model. Notably several molecules that interact with OPN were also altered. OPN down regulates MMP-9 expression and activity in cardiac fibroblasts (Z. Xie et al., 2003), and MMP-9 is reduced in the ZSF hearts. MMP-9 is involved in collagen digestion and its decrease may be involved in the increased collagen staining in the ZSF animals. Additionally, both MMP 7 and 12 can cleave OPN, although the functional significance of this is unknown. MMP-7, which also cleaves other ECM proteins including fibronectin, elastin and other proteoglycans, appears to be decreased, which may also be involved in some of the increased fibrosis
seen in Type 2 diabetes and in the ZSF model. MMP-12 is increased in the LV of the ZSF animals, and is involved in elastase degradation.

The increased LV OPN expression in the ZSF rats was visualized using immunohistochemistry. OPN is present in the LV of the ZSF animals; however, I was unable to determine what cell types were contributing to the OPN expression. It is thought that in certain pathologies the increased cardiac OPN expression may be coming from infiltrating immune cells, as immune cells express high levels of OPN and infiltrate cardiac tissue in diabetes (Sam et al., 2004; Trueblood, Xie, Communal, Sam, Ngoy, Liaw, Jenkins, Wang, Sawyer, Bing, Apstein, Colucci, & Singh, 2001a). Increased OPN expression seen in the LV of the ZSF animals could be from infiltrating immune cells or from resident cardiac cells. However, co-cultured NRVM and NRVF showed OPN expression in response to high glucose. This suggested that both NRVM and NRVF are capable of producing OPN and could contribute to the increased OPN expression seen in the LV of the ZSF animals. I initially assumed that NRVF would produce more OPN as fibroblasts are generally considered the major contributors to the extracellular matrix, and OPN is a secreted protein involved in extracellular matrix remodeling. However, NRVM had a ~4 fold higher basal expression of OPN mRNA. Neonatal cells express low levels of OPN in un-stimulated conditions possibly due to a role in cardiac remodeling during development. Whereas, adult cardiac myocytes and fibroblasts express a nominal amount or no OPN in physiologic conditions (Ashizawa et al., 1996; K. Singh et al., 1995; Z. Xie et al., 2001). The method I used to assess OPN mRNA expression assumed that the efficiency of the cDNA synthesis and qRT-PCR reactions were similar in both
cell types. This information should be further determined at the protein level, as it is possible while NRVM express more mRNA this does not accurately represent the protein expression, in the presence of an OPN stimulator, and in adult cells.

High glucose is thought to be an underlying factor leading to several symptoms of diabetes and tight glycemic control reduces the cardiac effects of diabetes (Asghar et al., 2009; Brownlee, 2001). Isolated cultures of NRVM or NRVF were treated with high glucose and OPN expression was determined at the mRNA and protein level. High glucose increased OPN expression in both NRVM and NRVF. Due to the greatly increased blood glucose in the ZSF animals this suggests that high glucose may contribute to the elevated OPN expression seen in the ZSF animal and that cardiac cells contribute to increased OPN production in the LV. This information further emphasizes the importance of glycemic control prior to overt cardiac alteration.

Lenga et al. had previously shown evidence that OPN localized to sites of focal adhesions in embryonic dermal fibroblasts in response to TGF-β1 leading to differentiation of myofibroblast. Contrary to my hypothesis, I saw no evidence that OPN localized to focal adhesions in NRVM or NRVF. This difference could be due to a difference in stimulation type and amount of stimulation (Lenga et al. used 10 ng/ml of TGF-β1 while we used 25 mmol glucose), the cell type, or the experimental design (Lenga et al. showed localization of OPN to collagen coated beads, while we looked at focal adhesions using paxillin antibody). Additionally, we looked at the localization of OPN at 24, 36 and 48 hours, while they assessed the localization after 1 hour of treatment. As OPN has been reported to interact with focal adhesion proteins and affects
focal adhesion formation and signaling it seems odd that OPN did not localize to focal
adhesions. It is possible that the experimental method we used was not sensitive enough
or the GFP tag on OPN may have prevented its correct localization. Localization of OPN
to focal adhesions could also be determined by co-immunoprecipitation of OPN or the
OPN-GFP construct with a component of focal adhesions. We saw that localization of
OPN was originally diffusely cytoplasmic, over time the OPN seemed to become more
concentrated into defined areas. These defined concentrations of OPN may be vesicles
transporting OPN out of the cell, as OPN is a secreted protein that can act as a
matricellular component. This may suggest a vesicular localization of OPN to allow for
secretion from the cell. This hypothesis could be further investigated by staining for
vesicles or Golgi and determining if OPN localized to these structures, which are both
involved in preparing molecules for secretion. Secretion of OPN could also be
investigated by imaging OPN-GFP in live cells over time or by detecting OPN protein in
the media using an enzyme linked immunosorbent assay or a radioimmunoassay.

The data presented in this chapter establish that OPN is upregulated in the LV of
an animal model of type 2 diabetes, and further that cardiac cells may contribute to the
increased OPN expression. Un-stimulated NRVM show greater expression of OPN than
NRVF, and OPN expression in both NRVM and NRVF is increased in response to high
glucose. The localization studies may suggest that OPN is secreted from both NRVM
and NRVF but does not appear to target to focal adhesions.
CHAPTER SIX

THE INVOLVEMENT OF ANGIOTENSIN II IN THE REGULATION OF OPN EXPRESSION IN RESPONSE TO HIGH GLUCOSE STIMULATION

A. INTRODUCTION

The focus of this chapter is determining the role of Ang II in OPN expression in NRVM and NRVF. To define the pathway leading to increased OPN expression and to determine if the mechanism leading to increased OPN expression was consistent in both NRVM and NRVF, isolated cultures of NRVM or NRVF were used for most experiments in this chapter. I initially aimed to determine if OPN expression could be mediated by Ang II. The reasons I chose to look at Ang II is that it is increased in the diabetic heart and has a significant role in diabetes induced organ damage (Connelly, Boyle, & Kelly, 2007). Ang II is increased in both NRVM and NRVF in response to high glucose (V. P. Singh et al., 2007; V. P. Singh et al., 2008), and Ang II increases OPN expression in other tissues (Natarajan, Scott, Bai, Yerneni, & Nadler, 1999; Ricardo, Franzoni, Roesener, Crisman, & Diamond, 2000). Additionally, both Ang II (Takeuchi, Nakamura, Cook, Pratt, & Dzau, 1990) and high glucose (Natarajan et al., 1999) increase AP-1 in vascular smooth muscle cells, and the osteopontin promoter contains an AP-1 binding site that has been shown to regulate increased expression of OPN mRNA (M. -. Renault et al., 2003). Initially I determined if Ang II was able to regulate OPN expression in both NRVM and NRVF by stimulating isolated cultures with Ang II. I further examined if
Ang II was a factor in the high glucose-mediated OPN upregulation. Both NRVM and NRVF have a complete renin-angiotensin system (RAS) and are able to produce Ang II (V. P. Singh et al., 2007; V. P. Singh et al., 2008). The first step in the RAS is the production of angiotensinogen (AGT). AGT is then hydrolyzed by renin to form Ang I. Ang I is cleaved by chymase in myocytes and angiotensin converting enzyme (ACE) in fibroblasts to form Ang II (V. P. Singh et al., 2007; V. P. Singh et al., 2008). To determine if Ang II production was necessary for OPN expression, a renin inhibitor was used to block the production of Ang I from angiotensinogen. A chymase inhibitor was used to block the cleavage of Ang I to form Ang II. I hypothesized that inhibiting renin would prevent OPN expression in both NRVM and NRVF, but that the chymase inhibitor would only inhibit OPN expression in NRVM, as ACE cleaves Ang I to form Ang II in NRVF.

The classical pathway of Ang II action is through the cell membrane localized Ang II receptors, AT$_1$ and AT$_2$. However, recently an internal mechanism of Ang II action has been shown to be mediated through nuclear membrane localized AT$_1$ and AT$_2$ receptors (Tadevosyan et al.). This may be the same mechanism through which Singh et al. showed that Ang II could activate PKC through an intracellular action (V. P. Singh et al., 2007; V. P. Singh et al., 2008). Involvement of the Ang II receptors would suggest that NRVM or NRVF secrete Ang II allowing it to act in an autocrine or paracrine function. I did not look at the regulation of OPN expression by the AT$_2$ receptor because it is expressed at low levels in adult cardiac cells and their involvement in cardiac pathology remains unclear, and their functions are often poorly reproducible (Senbonmatsu, Ichihara, Price, Gaffney, & Inagami, 2000). To determine if the AT$_1$
receptor was mediating the high glucose-induced upregulation of OPN mRNA, an AT$_1$ receptor blocker, candesartan, was used. OPN mRNA expression was measured by qRT-PCR.

B. RESULTS

1. Ang II Regulation of OPN Expression

Treatment of NRVM or NRVF with Ang II was used to test the hypothesis that Ang II increases OPN expression in cardiac cells. Isolated cultures of NRVM or NRVF were treated with 1 µM Ang II or control medium and processed for immunostaining or mRNA analysis. As shown in Figure 17, qRT-PCR results indicate Ang II increases OPN mRNA expression in NRVM. Similarly, qRT-PCR results from NRVF treated with Ang II for 18 hours show increased OPN mRNA expression (Figure 18). A primary mixed culture of NRVM and NRVF were serum starved then treated with control or Ang II (1 µM) medium for 48 hours. Cells were fixed and stained with phalloidin conjugated to Alexa Fluor-568 (red) to distinguish NRVM, an anti-OPN antibody (Santa Cruz) detected with Alexa Fluor-488 (green), and DAPI. Immunostaining shows OPN expression in control versus Ang II treated NRVM and NRVF. NRVF show a punctate cytoplasmic OPN staining, while NRVM show a diffuse cytoplasmic OPN localization. Control cells, however, show a nuclear OPN localization (Figure 19). This may suggest that OPN localization is altered upon stimulation.
2. Internal Ang II Production Regulates OPN Expression in Response to High Glucose

To determine if intracellular production of Ang II is necessary for OPN expression, a renin inhibitor (aliskiren) or a chymase inhibitor (chymostatin) was used to pretreat isolated cultures of NRVM or NRVF for 1 hour prior to the addition of control or high glucose medium. Renin hydrolyzes angiotensinogen to form angiotensin I in the RAS in both NRVM and NRVF, while chymase cleaves Ang I to form Ang II in NRVM. As shown in Figure 20, inhibition of renin does not block the upregulation of OPN mRNA in response to high glucose in NRVM. However, inhibition of renin in NRVF did prevent increased OPN mRNA expression in response to high glucose (Figure 21). Chymase cleaves Ang I to form Ang II in NRVM. Chymostatin, a chymase inhibitor, was used to pretreat isolated NRVM or NRVF prior to high glucose stimulation. Inhibition of chymase blocked the increased expression of OPN mRNA in NRVM in response to high glucose in NRVM (Figure 22). However, it did not significantly alter OPN mRNA expression in NRVF (Figure 23).

3. Angiotensin Receptor Involvement in OPN Expression

Candesartan, an AT1 receptor blocker, was used in conjunction with high glucose stimulation. OPN mRNA expression was measured by qRT-PCR. Candesartan inhibited OPN expression in response to high glucose in both NRVM (Figure 24) and NRVF (Figure 25).
Figure 17: Ang II increases OPN expression in NRVM. Cultured isolated NRVM were treated with control medium or medium containing 1 µM Ang II for 18 hours. OPN mRNA expression measured by qRT-PCR is significantly increased in response to 1 µM Ang II compared to control treatment (n=4; paired Student’s t-test on ΔC_Ts).
Figure 18: Ang II increases OPN expression in NRVF. Cultured isolated NRVF were treated with control medium or medium containing 1 µM Ang II for 18 hours. OPN mRNA expression was measured by qRT-PCR. OPN expression is significantly increased in response to 1 µM Ang II compared to control treatment (n=5; paired Student’s t-test on ∆Cₜₛ).
**Figure 19:** OPN expression in response to Ang II. Primary cultured NRVM and NRVF were serum starved, then treated with 1 μM Ang II or vehicle medium for 48 hours. Cells were fixed and immunostained with phalloidin conjugated to Alexa Fluor 568 (red) to distinguish NRVM, an OPN antibody detected with Alexa Fluor 488 (green, and shown in grayscale in bottom panels to better distinguish OPN distribution), and the nuclear stain DAPI (blue).
Figure 20: Involvement of renin in high glucose-induced OPN expression in NRVM. Isolated cultures of NRVM were treated with vehicle (DMSO) or a renin inhibitor (50 µM aliskiren) for one hour prior to the addition of high glucose (25 mM Glucose) or control (5 mM glucose + 20 mM mannitol) medium for 18 hours. OPN mRNA expression was measured by qRT-PCR. OPN expression was not significantly different between any of the groups (n=6, paired Student’s T-test on ΔC_Ts and one way ANOVA on ranks on relative quantity).
Figure 21: Involvement of renin in high glucose-induced OPN expression in NRVF. Isolated cultures of NRVF were treated with vehicle (DMSO) or a renin inhibitor (50 µM aliskiren) for one hour prior to treatment with high glucose (25 mM glucose) or control (5 mM glucose + 25 mM mannitol) medium for 18 hours. OPN mRNA expression was measured by qRT-PCR. OPN expression was significantly decreased in response to high glucose + aliskiren compared to high glucose (n= 7, paired Student’s t-test on ΔC_T).

![Graph showing OPN mRNA expression in NRVF under different conditions](image-url)
Figure 22: Involvement of chymase in OPN expression in NRVM. Isolated cultures of NRVM were treated with vehicle (DMSO) or a chymase inhibitor (10 µM chymostatin) for one hour prior to treatment with high glucose (25 mM glucose) or control (5 mM glucose + 20 mM mannitol) medium for 18 hours. OPN mRNA expression was measured by qRT-PCR. Chymostatin significantly decreased the expression of OPN mRNA in response to high glucose compared to high glucose treatment with no inhibitor (n=4, paired Student’s t-test on ΔC_T).
Figure 23: Involvement of chymase in OPN expression in NRVF. Isolated cultures of NRVF were treated with vehicle (DMSO) or a chymase inhibitor (10 µM chymostatin) for one hour prior to treatment with high glucose (25 mM glucose) or control (5 mM glucose + 20 mM mannitol) medium for 18 hours. OPN mRNA expression was measured by qRT-PCR. Chymostatin did not significantly decrease the expression of OPN mRNA in response to high glucose compared to high glucose treatment with no inhibitor (n=4, paired Student’s t-test on ∆CTs).
Figure 24: Involvement of the AT$_1$ receptor in OPN expression in NRVM. Isolated cultures of NRVM were treated with vehicle (DMSO) or an Ang II receptor blocker (1 µM candesartan) for one hour prior to treatment with high glucose (25 mM glucose) or control (5 mM glucose + 20 mM mannitol) medium for 18 hours. OPN mRNA expression was measured by qRT-PCR. Treatment with the Ang II receptor blocker significantly decreased the expression of OPN mRNA in response to high glucose compared to high glucose treatment with no inhibitor (n=6, paired Student’s t-test on ΔC$_T$s).
Figure 25: Involvement of the AT1 receptor in OPN expression in NRVF. Isolated cultures of NRVF were treated with vehicle (DMSO) or an AT1 receptor blocker (1 µM candesartan) for one hour prior to treatment with high glucose (25 mM glucose) or control (5 mM glucose + 20 mM mannitol) medium for 18 hours. OPN mRNA expression was measured by qRT-PCR. Treatment with the Ang II receptor blocker significantly decreased the OPN mRNA expression in response to high glucose compared to high glucose with no inhibitor (n=4, paired Student’s t-test on ΔC_Ts).
C. DISCUSSION

As stated previously, cardiac Ang II is increased in diabetes and additionally, Ang II regulates OPN expression in other tissues. This information supports the hypothesis that increased Ang II in diabetic hearts might mediate the increased OPN expression in the diabetic hearts. High glucose, a contributor to diabetes, increases Ang II in cardiac cells. Thus I propose that in diabetes high glucose leads to increased Ang II production and action which mediates increased OPN expression. Increased cardiac OPN expression then leads to remodeling and fibrosis and contributes to the diastolic dysfunction seen in diabetes. Ang II increased OPN expression in both NRVM and NRVF as shown in Figures 17 and 18, which corroborated with previous literature indicating increased OPN expression in cardiac myocytes in response to Ang II infusion (Collins et al., 2004; Matsui et al., 2004). Both techniques increase extracellular Ang II indicating a role for Ang II receptors in mediating OPN expression. In hyperglycemia circulating Ang II levels are reduced, indicating that locally produced tissue Ang II is critically important (Giacchetti, Sechi, Rilli, & Carey, 2005). Renin hydrolyzes angiotensinogen to angiotensin I in the renin angiotensin system (RAS). The renin inhibitor only significantly inhibited OPN production in response to high glucose in NRVF, not in NRVM. This verifies that OPN production in fibroblasts in response to high glucose is dependent on internal production of Ang II. There are several possibilities why renin may not inhibit OPN expression in NRVM. Aliskiren is a competitive inhibitor of renin...
that binds to the active site of renin, thereby inhibiting its ability to cleave angiotensinogen. However, in addition to renin’s actions by cleaving angiotensinogen it also mediates some of its effects through the (pro)renin receptor (Nguyen et al., 2002). The (pro)renin receptor binds both renin and prorenin, even in the presence of aliskiren. Through the (pro)renin receptor prorenin and renin induce activation of the extracellular signal-related kinase (ERK) pathway (Feldt et al., 2008) leading to increased TGF-β1, fibronectin and collagen (Huang et al., 2006). The (pro)renin inhibitor activates p38 in cardiomyocytes (Saris et al., 2006). Additionally, upon renin, or any component of the RAS, inhibition there is a compensatory rise in renin concentration (Nussberger, Wuerzner, Jensen, & Brunner, 2002). Thus it may be that increased OPN expression in NRVM can be mediated through the (pro)renin receptor. Cardiomyocytes have been documented to express the (pro)renin receptor, however I did not find any literature that examined the expression or actions of the (pro)renin receptor in cardiac fibroblasts. OPN is increased in response to p38 in several cell types, including vascular smooth muscle cells (Nerurkar et al., 2007; C. P. Sodhi et al., 2001; C. P. Sodhi et al., 2001), but does not increase OPN expression in cardiac fibroblasts (Z. Xie, Singh, & Singh, 2004). Differential expression of the (pro)renin receptor, downstream mediators or this receptor, or difference in OPN mediators between NRVM and NRVF may account for the inhibition of OPN expression by aliskiren in NRVF but not in NRVM. Clinically, prorenin is significantly increased in diabetic patients (Danser & Deinum, 2005), thus if OPN (or other fibrotic mediators) are regulated in part by the (pro)renin receptor in NRVM it would not be inhibited by any of the RAS inhibitors, and in fact may be exasperated by their use as inhibition of any component of the RAS pathway leads to
increased prorenin production. It is also possible that Ang II is not part of the high glucose mediated OPN expression seen in NRVM (although this seems unlikely as candesartan inhibits OPN expression), or the renin inhibitor was not able to enter NRVM at a high enough concentration to sufficiently inhibit renin activity. These possibilities could be further examined by inhibiting angiotensinogen production using short hairpin RNA (shRNA), this would help determine if I did not see reduced OPN expression due to inadequate renin inhibition by aliskiren. Additionally a (pro)renin inhibitor could be used independently and in conjunction with aliskiren to determine if the (pro)renin receptor was mediating OPN expression and if inhibition of both pathways further inhibited OPN expression, this would give us a better understanding if the (pro)renin receptor and Ang I production were involved in OPN expression.

Chymase cleaves Ang I to form Ang II in myocytes. Inhibition of this conversion using a chymase inhibitor, chymostatin, blocked the expression of OPN in response to high glucose in NRVM but not NRVF. As chymase is able to cleave several proteins, this data may suggest that OPN production may be reliant on the cleavage of another protein. However, while the high glucose mediated increased OPN expression is not inhibited by aliskiren, it is inhibited by the AT$_1$ receptor inhibitor, thus suggesting that Ang II is involved and chymostatin cleaves Ang I to produce this Ang II in NRVM. While the literature does not suggest any other method of angiotensinogen cleavage (other than the renin) this mechanism could be further examined by determining if Ang I expression is increased in response to high glucose in presence of a renin inhibitor and further if inhibition of chymase leads to an increased Ang I expression (as it is not being cleaved to form Ang II). Inhibition of chymase was not hypothesized to have an effect in
NRVF because fibroblasts use angiotensin converting enzyme (ACE) to convert angiotensin I to angiotensin II. In NRVF ACE cleaves Ang I to form Ang II, and my results that OPN expression is not inhibited in the presence of chymostatin in NRVF confirm that chymase is not involved in the cleavage of Ang I to form Ang II. Particularly as we have shown that OPN expression is dependent on the internal production of Ang I by renin in NRVF. This needs to be further verified using an ACE inhibitor in the presence of high glucose, as it is possible that Ang I is regulating OPN expression. However, taken with the results that the AT$_1$ receptor inhibits OPN expression in NRVF, the data suggests that Ang II, not Ang I, is the mediator of OPN expression. The classical method of Ang II action is through the AT$_1$ receptor. To determine if the angiotensin receptor was mediating the high glucose induced OPN expression, an AT$_1$ receptor inhibitor was used. The AT$_1$ receptor blocker inhibited OPN expression in both NRVM and NRVF and suggests that the AT$_1$ receptor is the primary mediator of Ang II increased OPN expression in response to high glucose as there is not a great difference between the inhibition seen with the renin or chymase inhibitor and the AT$_1$ receptor blocker. The information that AT$_1$ is involved in OPN production in NRVM further confirms that Ang II is being produced by NRVM. Recent literature has determined that AT$_1$ and AT$_2$ receptors are also present on the nuclear envelope (Tadevosyan et al.), as well as the cell membrane. To further determine if OPN expression is regulated by cell membrane or nuclear envelope localized AT$_1$ receptors, Ang II could be used to treat isolated nuclei and intact NRVM or NRVF and alterations in OPN expression between the two preparations could be assessed. If OPN expression was not altered in the whole cell preps it would suggest that nuclear AT$_1$ receptors...
completely mediated the increased OPN expression, however if no increases in OPN expression were detected in the nuclear preparation it would suggest that cell membrane localized AT₁ receptors mediate the increased OPN expression. Nuclear AT₁ receptors were suggested to regulate gene expression by regulating nuclear calcium signals, however, Singh et al. reported that intracellular Ang II to could activate PKC in a mechanism independent of AT₁ receptors. Whether these mechanisms are related will have to be determined, as it is possible that the inhibition of AT₁ receptors by candesartan present in the medium used by Singh et al. did not sufficiently inhibit nuclear localized AT₁ receptors, or that these are two completely separate mechanisms of intracellular Ang II action. In the context of OPN this could be further examined by inhibiting AT₁ receptor expression using shRNA and determining if OPN expression could be induced by high glucose, however since there was significant inhibition of OPN expression with candesartan this would only play a minor role in the high glucose induce OPN expression. Candesartan increased OPN expression in control cells in NRVF but not in NRVM. Pharmacological agents are a useful tool as they can be used to relatively specifically inhibit particular pathways; however, they also have the potential to activate other pathways. It is possible that Candesartan activates another pathway that increased OPN expression in control conditions in NRVF. As mentioned previously myocytes and fibroblasts may have slightly different mechanisms to regulate OPN expression and this may be why we do not see this increase in OPN expression in NRVM.

In this chapter I show that Ang II is a mediator of the high glucose induced upregulation of OPN, and that both Ang II production and action through the AT₁ receptor mediate this effect.
CHAPTER SEVEN

THE INVOLVEMENT OF PKC IN THE REGULATION OF OPN EXPRESSION IN RESPONSE TO HIGH GLUCOSE IN CARDIAC MYOCYTES AND FIBROBLASTS

A. INTRODUCTION

Having determined that Ang II was involved in the high glucose-induced OPN expression, I wanted to further elucidate the mechanism and signaling pathway leading to increased OPN expression. I decided to pursue the regulation of OPN by PKC because PKC is involved in the regulation of OPN expression in other tissues (Beck & Knecht, 2003; Hsieh et al., 2006; Kelly, Chanty, Gow, Zhang, & Gilbert, 2005a; C. P. Sodhi et al., 2000), high glucose and Ang II activate PKC in cardiac cells (Malhotra et al., 2001), and PKC mediates several fibrotic effects of Ang II (Stawowy et al., 2004). It is my hypothesis that high glucose increases OPN expression by increasing Ang II production and action. This leads to increased PKC activation, which mediates OPN expression. To determine if PKCs regulate OPN expression in response to high glucose a general PKC inhibitor was used in conjunction with high glucose stimulation to treat isolated cultures of NRVM and NRVF. The general PKC inhibitor inhibits both classical and novel PKCs, however, it does not inhibit atypical PKCs, of which only ζ is present in cardiac cells. I did not further examine the regulation of OPN by PKCζ as no previous data has implicated it in the regulation of OPN, and PKCζ has been shown to be mediated by OPN (Z. Xie et al., 2003). This would suggest that activation of PKCζ is secondary to the
upregulation of OPN. To determine if PKC was downstream of Ang II a general PKC inhibitor was used in conjunction with Ang II. If Ang II mediated OPN expression is inhibited by PKC it would suggest that Ang II is leading to increased activation of PKC which is then regulates OPN expression. If however, PKC did not inhibit Ang II mediated OPN expression it may suggest that PKC is not involved in OPN expression (which examined using a general PKC inhibitor in conjunction with high glucose), that PKC mediates OPN through a different pathway, or that it is upstream of Ang II.

To better determine what PKC isoforms regulate OPN expression we will selectively inhibit particular isoforms. Determining what PKC isoforms regulate OPN expression will help give a better idea of what stimuli would increase OPN expression and what the downstream effects of OPN might be. Very few stimuli are known to increase PKCα activation in NRVM (Goldberg, Zhang, & Steinber, 1997), additionally classical PKCs tend to be associated with pathological remodeling, where PKCε is often associated with a cardioprotective effect (Pass et al., 2001). The classical PKC β isoform regulates OPN expression in renal cells and the kidney (Hsieh et al., 2006; Kelly, Chanty, Gow, Zhang, & Gilbert, 2005a). To determine if classical PKCs (α, β1, β2 in NRVM, only α in NRVF) are involved in OPN expression a classical PKC inhibitor was used in conjunction with high glucose stimulation. PKCβ activation in response to high glucose has previously been determined (Malhotra et al., 2001), however the activation of PKCα in response to high glucose in NRVM had not yet been shown. As mentioned PKCα is not activated by many stimuli in NRVM, to determine if high glucose activated PKCα, I determined the membrane expression of PKCα in response to control or high glucose conditions. PMA was used as a positive control in these experiments. Upon activation
PKCs translocate from the cytosol to membrane, thus PKC translocation can be used as measure of PKC activation.

The role of PKCε was examined because Goldspink et al. had determined that PKCε overexpressing mice had increased cardiac OPN expression (Goldspink et al., 2004). Additionally, PKCε has been shown to be activated in response to high glucose and Ang II in adult cardiac myocytes (Malhotra et al., 2001; Stawowy et al., 2005), which would suggest that it mediates some of the downstream effects of high glucose, and we have determined that high glucose increases OPN expression. To determine if active PKCε could increase OPN expression in cardiac cells isolated NRVM or NRVF were infected with a constitutively active PKCε (caPKCε) adenovirus and OPN mRNA expression was determined. PKC enzyme can be made constitutively active by deletion of residues 154-163 of its inhibitory pseudosubstrate domain. To determine if PKCε was involved in high glucose mediated OPN expression a dominant negative PKCε was overexpressed in NRVM or NRVF using an adenovirus prior to the addition of high glucose medium. The dominant negative PKC enzyme was created by mutating the ATP-binding site and its pseudosubstrate domain, thereby destroying the construct's kinase activity but maintaining the enzyme in an active conformation. It can act as a competitive inhibitor by binding to substrates but is unable to phosphorylate them.

B. RESULTS

1. PKC Involvement in OPN Expression

To determine if PKC was involved in the high glucose-induced upregulation of OPN, isolated cultures of NRVM or NRVF were treated with a general PKC inhibitor
(GF109203X, 1 µM) prior to glucose stimulation. Real-time quantitative PCR was used to determine OPN mRNA expression in NRVM and NRVF and SDS-PAGE and Western Blotting was used to determine OPN protein expression in NRVM. The increased OPN mRNA expression in response to high glucose is abolished by treatment with the general PKC inhibitor in both NRVM (Figure 26) and NRVF (Figure 27). Osteopontin has reported molecular weights ranging from 25 to 75 kDa on SDS-PAGE gels due to alterations in glycosylation, phosphorylation and cleavage (D. Denhardt & Guo, 1993). The osteopontin antibody detected several bands on the Western blots, however I only used the 50, 56, 60 kDa bands for quantification purposes because the 50 and the 56 kDa band have been reported in muscle and brain cells and the 60 kDa band is the most commonly seen and quantified band (Kuykindoll, Nishimura, Thomason, & Nishimoto, 2000; Parrish & Ramos, 1997; Parrish, Weber, & Ramos, 1997). The inhibition of OPN expression in the presence of the general PKC inhibitor indicates that PKC mediated the high glucose induced OPN expression.

To examine the role of angiotensin II in the regulation of OPN a general PKC inhibitor (GF10203X, 1 µM) was used in conjunction with Ang II (1 µM). The general PKC inhibitor significantly decreased OPN mRNA expression in response to Ang II compared to Ang II treatment with no inhibitors in NRVM (Figure 28) and NRVF (Figure 29). This further suggests that Ang II increases OPN expression in a PKC mediated pathway.
2. The Role of Classical PKCs in the Regulation of OPN Expression

As mentioned previously, past studies have implicated PKCβ in OPN expression. In order to determine which PKC isoforms are involved in OPN expression, a classical PKC inhibitor was used in conjunction with high glucose treatment. Isolated cultures of NRVM or NRVF were pretreated with the classical PKC isoform inhibitor, Go 6976, prior to high glucose stimulation. OPN mRNA expression was measured using qRT-PCR. The classical PKC inhibitor decreases the relative quantity of OPN mRNA expression in response to high glucose in NRVM (Figure 30) and NRVF (Figure 31). This would suggest that classical PKCs (α or β in NRVM and α in NRVF) are involved in high glucose mediated OPN expression.

3. PKCε Mediates OPN Expression in Cardiac Cells

To investigate the role of PKCε in high glucose mediated OPN expression, constitutively active or dominant negative PKCε was overexpressed in isolated cultures of NRVM or NRVF. Isolated cultures of NRVM or NRVF were infected with an adenovirus encoding caPKCε (10 MOI for NRVM, 100 MOI for NRVF) or a control virus (nuclear encoded β-galactosidase, NE β-gal) and then cultured for 48 hours. OPN mRNA expression was determined using qRT-PCR. OPN localization and expression were also examined by immunostaining in NRVF. Overexpression of caPKCε increases OPN mRNA expression in both NRVM (Figure 32) and NRVF (Figure 33). Immunostaining shows a cytoplasmic localization of OPN (Figure 34). This indicates that PKCε can mediate OPN expression.
To determine if PKCε was involved in high glucose-induced OPN expression, I overexpressed dnPKCε using adenovirus. Isolated cultures of NRVM or NRVF were infected with an adenovirus encoding dnPKCε (NRVM were infected with 10 MOI, NRVF were infected with 100 MOI) or control virus (NE β-gal) for 30 hours prior to the addition of high glucose or control medium for 18 hours. OPN mRNA expression was measured using qRT-PCR. Overexpression of dnPKCε inhibits increased OPN mRNA expression in response to high glucose in NRVM (Figure 35) and NRVF (Figure 36). This suggests that PKCε mediates high glucose induced OPN expression in both NRVM and NRVF.
Figure 26: PKC involvement in high glucose-induced OPN expression in NRVM. 

A. Isolated cultures of NRVM were treated with vehicle (DMSO) or a general PKC inhibitor (1 μM GF109203X) for one hour prior to the addition of high glucose (25 mM glucose) or control (5 mM glucose + 20 mM mannitol) medium for 18 hours. OPN mRNA expression was measured by qRT-PCR. The general PKC inhibitor prevented the increase in OPN mRNA expression in response to high glucose compared to high glucose treatment with no inhibitor (n=5, paired Student’s t-test of ΔC_T).

B. Isolated cultures of NRVM were treated with vehicle or a general PKC inhibitor (1 μM GF109203X) for one hour prior to the addition of high glucose (HG) or control (C) medium for 24 hours. OPN protein expression measured by SDS-PAGE and Western blotting is significantly decreased in NRVM treated with high glucose + GF109203X compared to high glucose (representative blot and graph showing mean ± the SEM for all data) and quantitated using Un-Scan-It gel digitizing software (n=4, Student’s t-test on relative quantity).
Figure 27: PKC involvement in high glucose-induced OPN expression in NRVF. Isolated cultures of NRVF were treated with vehicle (DMSO) or a general PKC inhibitor (1 µM GF109203X) for one hour prior to the addition of high glucose (25 mM glucose) or control (5 mM glucose + 20 mM mannitol) medium for 18 hours. OPN mRNA expression was measured by qRT-PCR. The general PKC inhibitor prevented the increase in OPN mRNA expression in response to high glucose compared to high glucose treatment with no inhibitor (n=6, paired Student’s t-test of ΔC_T).
Figure 28: PKC mediates Ang II-induced OPN upregulation in NRVM. Isolated cultures of NRVM were treated with vehicle (DMSO) or a general PKC inhibitor (1 µM GF109203X) for one hour prior to the addition of Ang II (1 µM) for 18 hours. OPN mRNA expression was measured by qRT-PCR. The general PKC inhibitor prevented increased OPN expression in response to Ang II (n= 3).
Figure 29: PKC mediates Ang II-induced OPN upregulation in NRVF. Isolated cultures of NRVF were treated with vehicle (DMSO) or a general PKC inhibitor (1 µM GF109203X) for one hour prior to the addition of Ang II (1 µM) for 18 hours. OPN mRNA expression was measured by qRT-PCR. The general PKC inhibitor prevented increased OPN expression in response to Ang II (n= 3).
Figure 30: Classical PKC isoforms mediate high glucose-induced OPN expression in NRVM. Isolated cultures of NRVM were treated with vehicle (DMSO) or a classical PKC inhibitor (1 µM Go 6976) for one hour prior to treatment with high glucose (25 mM glucose) or control (5 mM glucose + 20 mM mannitol) for 18 hours. OPN mRNA expression was measured by qRT-PCR. The classical PKC inhibitor prevented increased OPN expression in response to high glucose in NRVM (n=5, paired Student’s t-test of ΔC_{T5}).
Figure 31: Classical PKC isoforms mediate high glucose-induced OPN expression in NRVF. Isolated cultures of NRVF were treated with vehicle (DMSO) or a classical PKC inhibitor (1 µM Go 6976) for one hour prior to treatment with high glucose (25 mM glucose) or control (5 mM glucose + 20 mM mannitol) for 18 hours. OPN mRNA expression was measured by qRT-PCR. The classical PKC inhibitor prevented increased OPN expression in response to high glucose in NRVF (n=5, paired Student’s t-test of ΔC_T).
Figure 32: PKCε increases OPN expression in NRVM. Isolated cultures of NRVM were infected (10 MOI) with an adenovirus overexpressing caPKCε or a control virus (NE β-gal) for 48 hours. OPN mRNA expression was measured by qRT-PCR. Overexpression of constitutively active PKCε increased OPN expression in NRVM (n=5, paired Student’s t-test of ΔCₜₛ).
Figure 33: PKCε increases OPN expression in NRVF. Isolated cultures of NRVF were infected (100 MOI) with an adenovirus overexpressing caPKCε or a control virus (NE β-gal) for 48 hours. OPN mRNA expression was measured by qRT-PCR. Overexpression of constitutively active PKCε increased OPN expression in NRVF (n=4, paired Student’s t-test of ΔC_{75}).
Figure 34: Immunostaining of PKCε-induced increase of OPN expression in NRVF. Isolated cultures of NRVF were infected (100 MOI) with an adenovirus overexpressing caPKCε or a control virus (NE β-gal) for 48 hours. Cells were fixed and processed for immunostaining. Cells were stained with an anti-OPN antibody MPIIB10 (DSHB), detected with Alexa Fluor 488 (green) and the nuclear stain DAPI (blue).
Figure 35: PKCε mediates OPN expression in response to high glucose in NRVM. Isolated cultures of NRVM were infected (10 MOI) with an adenovirus overexpressing dnPKCε or a control virus (NE β-gal) for 30 hours prior to the addition of high glucose (25 mM glucose) or control medium (5 mM glucose + 20 mM glucose) for 18 hours. OPN mRNA expression was measured by qRT-PCR. Overexpression of dnPKCε inhibited increased OPN expression in response to high glucose treatment compared to high glucose treated with the control virus (n=5, paired Student’s t-test of ΔC_T).
Figure 36: PKCε mediates OPN expression in response to high glucose in NRVF. Isolated cultures of NRVF were infected (100 MOI) with an adenovirus overexpressing dnPKCε or a control virus (NE β-gal) for 30 hours prior to the addition of high glucose (25 mM glucose) or control medium (5 mM glucose + 20 mM glucose) for 18 hours. OPN mRNA expression was measured by qRT-PCR. Overexpression of dnPKCε inhibited increased OPN expression in response to high glucose treatment compared to high glucose treated with the control virus (n=7, paired Student’s t-test of ΔC_T).

![Graph showing relative OPN mRNA expression in control, high glucose, control + dnPKCε, and high glucose + dnPKCε conditions.](image)
C. DISCUSSION

Several studies provide evidence that PKC is involved in OPN expression in a variety of tissues (Beck & Knecht, 2003; Hsieh et al., 2006; Kelly, Chanty, Gow, Zhang, & Gilbert, 2005a; C. P. Sodhi et al., 2000), and my findings presented in Chapter 7 broaden this range to include cardiac cells, both myocytes and fibroblasts. Particularly, I found that PKC mediates high glucose induced OPN upregulation in NRVM and NRVF.

My findings that a general PKC inhibitor blocked the increased OPN expression in response to high glucose in cardiac cells determined that PKC mediates high glucose induced expression of OPN as shown in Figures 26 and 27. As mentioned in the Results section several bands on the Western blots were detected by the OPN antibody I used. To further determine which of these bands are OPN, and thus give a better quantification and information about which OPN post translational modifications were present mass spectrometry would need to be done on each of these. This could help in determining alterations in glycosylation and phosphorylation states of OPN in response to different stimuli or conditions. High glucose is suggested to lead to excess glycation of proteins, it would be interesting to determine if OPN expressed in response to high glucose was more highly glycosylated than when induced with other stimuli and if this had functional effects. As ECM proteins modified by glycation interact abnormally with other matrix proteins and integrins (Brownlee, 2001).

The general PKC inhibitor also blocks the upregulation of OPN in response to Ang II. I showed in Chapter 6 that Ang II is involved in OPN expression in response to high glucose through production of Ang II and the AT₁ receptor. That inhibition of PKC blocks the Ang II mediated OPN expression suggests that PKC is downstream of Ang II
in the pathway regulating OPN expression. This lends credence to my hypothesis that high glucose increases Ang II production which then activates PKC leading to increased OPN expression. To further verify that PKC is downstream of Ang II an ARB or cell appropriate Ang II production inhibitor could be used in conjunction with PMA, a potent PKC activator, I would expect to see no decrease in OPN expression.

It is important to note that many PKC inhibitors stimulate MAPK cascades (Heidkamp, Bayer, Martin, & Samarel, 2001) and as mentioned previously OPN expression is regulated by MAPK, particularly p38 in several cell types. That inhibitors of one pathway often activate others must be kept in mind when interpreting any results obtained with the use of pharmacological agents.

The regulation of OPN by PKC has been determined previously in other tissues. Six PKC isoforms are present in rat ventricular myocytes, PKC α, β1, β2, δ, ε, and ζ (Malhotra et al., 2001), while ventricular fibroblasts express only PKC α, δ, ε, and ζ. As mentioned the role of PKCζ was not examined in these studies, use of the general PKC inhibitor used (GF109203X), which completely blocked OPN expression, but does not inhibit PKCζ, further suggests that PKCζ is not involved in OPN expression.

I did examine the role of classical PKCs and PKCε, as both have been implicated in the regulation of OPN expression in various tissues (Goldspink et al., 2004; Kelly, Chanty, Gow, Zhang, & Gilbert, 2005a; C. P. Sodhi et al., 2001). Since NRVF only express one classical PKC isoform, α, this would suggest that PKCα regulates OPN expression in NRVF. However, NRVM express two classical PKC isoforms, α and β, so further work would need to be done to determine which classical isoform is regulating OPN expression. This could be further determined by using a PKCβ inhibitor, such as
ruboxistaurin, or by decreasing PKCα or β expression using shRNA. It would also be interesting to examine cardiac OPN expression in response to injury in the presence of a PKCβ inhibitor or in rats lacking PKCα or β. Additionally, adult myocytes and fibroblasts could be harvested from rats lacking PKCα or β and exposed to high glucose of Ang II to determine if OPN expression was increased. Translocation, suggestive of activation, of PKCβ and ε has previously been shown in adult rat ventricular myocytes (Malhotra et al., 2001). However, the activation of PKCα in response to high glucose had not been previously shown. I show in chapter 7 that PKCα is activated in response to high glucose in NRVM. This would suggest that it is able to participate in the high glucose mediated OPN expression. Malhotra et al. had previously reported that in adult cardiomyocytes PKCα is not activated in response to high glucose. This discrepancy may be due to the cell type, so we would want to further determine if PKCα could be regulated in adult cardiac myocytes as this may help determine what PKC isoforms are involved in OPN expression in diabetes as we saw OPN expression in LV of adult ZSF rats. Additionally, it is important to determine what PKC are activated in response to high glucose in fibroblasts, as PKCα is the only classical PKC present I would assume it was activated.

Due to the observation by Goldspink et al. that overexpression of constitutively active PKCε increased OPN expression in the atrium and ventricles of aged mice I looked at the role of PKCε. To verify that PKCε could induce OPN expression in cardiac cells we overexpressed constitutively active PKCε in isolated cultures of NRVM or NRVF. As shown in Figures 32 and 33, overexpression of constitutively active PKCε increased OPN production in both NRVM and NRVF, with a much greater increase in OPN
expression in NRVF. This may suggest that the majority of OPN expression from cardiac cells seen in the heart of the PKCε overexpressing mouse was primarily from fibroblasts. The large increase in OPN expression in response to PKCε may be due to the lack in an eventual decrease in PKC signaling. Usually active PKCs are degraded or become inactivated over time or PKCs become desensitized to the stimulus. However, in the viral expression there is never a suppression of PKC activation as new active PKCs are constantly being produced. Thus we may see such a large difference in OPN expression in NRVM vs. NRVF based on the amount of time it takes for the active PKC to be degraded or PKC targets to be down regulated. This effect may also have been seen due to differential expression of caPKCε in NRVM vs. NRVF.

Once we had determined that PKCε regulated OPN expression we wanted to further determine if PKCε was necessary for mediating the high glucose induced OPN upregulation. Dominant negative PKCε was overexpressed using an adenovirus in conjunction with high glucose in isolated cultures of NRVM or NRVF. As shown in Figures 35 and 36, overexpression of dominant negative PKCε abolished the high glucose induced OPN expression but does not decrease OPN expression in the control samples (Control +dnPKCε). This suggests that PKCε is involved only in the high glucose mediated OPN expression and not the basal expression.

Overexpression of PKCε (~ 2-fold) does not seem to cause activation or subcellular localization of PKCα (Takeishi et al., 2000). Some of the downstream effects of PKCs are modulated by receptors for activated C kinase (RACK); PKCε preferentially binds to RACK2 and PKCβ to RACK 1. There does not appear to be much overlap under physiologic conditions or even when PKCε is overexpressed by 2-fold. However,
overexpression of PKCε by 4-fold has been shown to also associate with RACK1 (Pass et al., 2001). This suggests that results obtained using overexpression of caPKCε or dnPKCε may have also influenced downstream targets of other PKC isoforms. To further investigate this PKCε could be reduced using a specific shRNA in conjunction with high glucose, and determining if OPN expression is decreased, as I saw with the dnPKCε. Additionally, the expression of PKCε in the caPKCε or dnPKCε could have been determined by Western blotting, giving an indication if we had overexpressed PKCε to 4 fold or greater than the enogenous level it may be associating with RACK1. Association of RACK1 with PKCε could be determined by immunoprecipitating RACK1 and western blotting for PKCε and the reciprocal experiment of immunoprecipitating PKCε and western blotting for RACK1.

The data presented in this chapter show that PKC is an integral mediator of OPN expression. However, further work will need to be done to determine if which PKC isoforms are involved.
Diabetic patients show enhanced myocardial dysfunction leading to accelerated heart failure referred to as diabetic cardiomyopathy (DCM). Diabetes leads to several alterations in cardiac structure, one of which is fibrosis of the ventricular myocardium. One approach to the development of new therapies for DCM is to elucidate the molecular mechanisms underlying changes in cardiovascular function, and then develop targeted intervention strategies to prevent or alleviate changes in gene expression that contribute to the pathological state. The first goal of this project was to determine expression alterations in cardiac genes involved in cardiac remodeling in type 2 diabetes. A type 2 diabetic model, the ZSF rat had diastolic dysfunction and appeared to have increased LV fibrosis at 30 weeks. One of the genes increased in the LV of this model was OPN. OPN is a small phospho-protein that has been implicated in processes of immunity and tissue remodeling, particularly in fibrosis. Cardiac OPN expression is upregulated in several cardiac pathologies. Upregulation of OPN coincides with a transition to heart failure and it is hypothesized that increased OPN expression is involved in the fibrotic remodeling seen in cardiac pathologies.

Hyperglycemia is thought to be an underlying problem in diabetes leading to a host of complications, including cardiac dysfunction. Although several factors contribute to the cardiac pathology seen in diabetes I looked at the involvement of high glucose in
regulating OPN expression in cardiac cells. High glucose increased OPN expression in both NRVM and NRVF. I also determined that un-stimulated NRVM express more OPN mRNA than NRVF.

I examined the regulation of high glucose induced OPN expression by Ang II and PKC because of their involvement in fibrosis and OPN expression in other cell types. Ang II production and action through the AT\(_1\) receptor were shown to be involved in high glucose induced OPN expression in both NRVM and NRVF. Additionally, I show that Ang II leads to activation of PKC\(\alpha\), and that PKC\(\varepsilon\) and a classical PKC (\(\alpha\) or \(\beta\)) appear to be involved in OPN expression. The results of this study further progress our understanding of signaling pathways leading to increased cardiac OPN expression, which may contribute to the detrimental cardiac effects of diabetes.

An important component that has yet to be thoroughly addressed in cardiac cells is the molecular and physical changes brought about by increased OPN expression. OPN knockout mice have been subjected to a variety of pathologies and consistently show reduced fibrosis. Recently a cardiac OPN overexpressing mouse was produced. OPN overexpressing mice died prematurely, with a half life of 12 weeks, and only about 15% of OPN overexpressing mice survived to 30 weeks. OPN overexpressing mice had electrocardiographic abnormalities and heart block, without atrial or ventricular arrhythmias. The cardiac structure was significantly altered in cardiac OPN overexpressing mice, which showed dilated cardiomyopathy, reduced myocyte concentration, and greatly increased LV fibrosis. There was a significant increase in the concentration of T-cells, neutrophils, and cytokines. With an increase in the cytotoxic
proteins, perforin and granzyme, involved in cardiomyocyte death were increased (M. Renault et al., 2010). OPN knockout and overexpression models have shown the importance of OPN in the heart and emphasize the need to understand the effects of OPN on other signaling pathways in the heart. Particularly, examining OPN’s effects on MMPs, collagen, and fibronectin, determining which integrins OPN is binding and the downstream effects, and cardiomyocyte apoptosis or survival.

There has been information in the renal and vascular literature that the phosphorylation and glycosylation state of the OPN protein have an effect on its activity. It is well documented that the thrombin cleaved form of OPN has increased chemotactic ability and that thrombin cleavage exposes a cryptic binding site allowing OPN to interaction with more integrins and increasing its binding affinity with integrins (D. T. Denhardt et al., 2001). These variations in OPN phosphorylation, glycosylation, and cleavage need to be further examined in the heart and how the posttranslational modifications affect OPN’s function. Determining if particular stimuli lead to different OPN products could help us gain a better understanding of the functions of OPN and the different OPN products. An in-depth understanding of the different phosphorylation, glycosylation and cleavage products could allow for selection of certain effects of OPN by altering the phosphorylation, glycosylation, and cleavage of OPN.

Additionally, more insight into the regulation of OPN expression could be gained by determining the effect of various stimuli on the promoter. This would allow us to determine if OPN expression is regulated at the transcriptional level or the post transcriptional level. I determined that OPN mRNA is regulated in cardiac cells;
however it could be through the production of new mRNA or through stabilization of existing OPN mRNA. Our laboratory is currently working on an adenoviral reporter construct to do this. Dr. Claude Desgranges (INSERM, France) kindly provided us with a plasmid containing the OPN promoter directly followed by the sequence coding for firefly luciferase. This construct would allow one to determine OPN promoter activity in various treatment conditions (i.e. high glucose, Ang II application, PKC activation) by measuring the production of luciferase using a luciferase assay kit and a luminometer. The light emitted can be quantified and that correlates with the amount of luciferase present which would be indicative of OPN promoter activation. I would expect factors that increase OPN mRNA to increase OPN promoter activation and thus luciferase production, suggesting that regulation of OPN is at the promoter level. Although, there is a report of OPN mRNA stabilization in response to treatment, most reports suggest that OPN expression is regulated at the transcriptional level (Manji, Ng, & Martin, 1998; Renault et al., 2003).

In addition to Ang II and PKC, TGF-β has also been shown to increases OPN expression in vascular smooth muscle and renal epithelial cells (C. M. Giachelli et al., 1993; Noda, Yoon, Prince, Butler, & Rodan). However, there is conflicting results about whether TGF-β regulates OPN or if OPN regulates TGF-β (Vetrone et al., 2009). The interaction of TGF-β and OPN will need to be further determined in cardiac tissues. If TGF-β does regulate OPN expression in cardiac tissue it may be through a parallel pathway or part of the pathway we describe in this study, particularly as Ang II is involved in TGF-β expression. As mentioned previously OPN expression is also
mediated by MAPK in other cell types. It remains to be seen how these pathways interact to regulate OPN expression.

Further, the regulation of OPN expression by PKC isoforms needs to be further determined, I saw regulation of OPN expression by both classical and novel PKC isoforms. I suggested that this work be followed up using shRNA to knockdown specific PKC isoforms. Additionally, how PKC activation is leading to increased OPN expression is still unknown. Additionally PKC increases AP-1 and OPN has an AP-1 site in its promoter, so it may be beneficial to examine the role of AP-1 in the high glucose mediated OPN expression.

It is important to note that most of the work done throughout this project was conducted in neonatal rat ventricular myocytes and fibroblasts. A lot of these studies could not be conducted in isolated adult cells due to the reduced time frame. However it may be beneficial to do some experiments in adult cells to confirm that the regulation of OPN is the same in neonatal and adult cells. Further experiments in co-cultures of myocytes or fibroblasts or by culturing cells with medium other cells had previously conditioned to examine if multiple cell types interact to regulate OPN expression or its effects.

Clinically both ARBs and ACE inhibitors have been beneficial in the treatment of cardiovascular disease and diabetes. Some of this beneficial effect may be due to the inhibition of OPN production, as Ang II increased OPN expression in an AT$_1$ dependant manner and production of Ang II in NRVF requires ACE. My data would suggest that ARBs would inhibit OPN expression more thoroughly as AT$_1$ receptor mediated OPN
expression in both NRVM and NRVF. Use of Ang II production inhibitors (renin, chymase, or ACE inhibitor) would not inhibit OPN production to the same extent as ARBs because of the differential pathways of Ang II production in myocytes and fibroblasts. Several groups have suggested that inhibition of OPN expression is not beneficial in animal models with particular pathologies and increased OPN expression is important for regulating compensatory fibrosis to prevent systolic dysfunction or cardiac rupture (Matsui et al., 2004; Trueblood, Xie, Communal, Sam, Ngoy, Liaw, Jenkins, Wang, Sawyer, Bing, Apstein, Colucci, & Singh, 2001). Additionally, OPN prevents calcification in bone, urinary stones, and vessels (Speer et al.) this may be beneficial in particular pathologies. This may also be why OPN was increased so significantly in the atria, which had significant calcification, of the PKCε overexpressing mouse. Examining OPN expression in the diabetes (or other cardiac pathologies) with Ang II production inhibitors or ARB treatment would help define the best course of treatment for particular pathologies.

An interesting point that I have not previously mentioned is the role of PKC and OPN in the development and progression of diabetes. Both PKC and OPN have been implicated in mediating insulin resistance (Davidoff, Davidson, Carmody, Davis, & Ren, 2004; Kiefer et al.; Nomiyama et al., 2007). OPN deficient mice or neutralization of OPN prevents insulin resistance in response to obesity (Kiefer et al.; Nomiyama et al., 2007). This may suggest that in patients prone to diabetes or pre-diabetics regulating OPN expression or PKC activity may be a potential target for preventing diabetes. This is particularly important because conditional transgenic mice over expressing OPN for
only the 11 weeks after birth had increased cardiac fibrosis at 30 weeks of age, although OPN was no longer overexpressed (M. Renault et al., 2010). This suggests that the structural alterations caused by increased OPN expression can not be reversed, thus treatment would be more beneficial if it came before overt signs of cardiac dysfunction.


Diagnosis and classification of diabetes mellitus.(2005). *Diabetes Care, 28*(suppl 1), s37-s42.


Standards of medical care in Diabetes–2006. *Diabetes Care, 29*(suppl 1), s4-s42.


Tadevosyan, A., Maguy, A., Villeneuve, L. R., Babin, J., Bonnefoy, A., Allen, B. G.,
et al. Nuclear-delimited angiotensin receptor-mediated signaling regulates
cardiomyocyte gene expression. *Journal of Biological Chemistry*, 285(29), 22338-
22349.

examination of MS candidate genes identified as differentially regulated in multiple
sclerosis plaque tissue, using absolute and comparative real-time Q-PCR analysis.

(2001). Role of osteopontin in the pathogenesis of bleomycin-induced pulmonary
fibrosis. *American Journal of Respiratory Cell and Molecular Biology*, 24(3), 264-
271.

Transgenic overexpression of constitutively active protein kinase C ε causes

Takemoto, M., Yokote, K., Nishimura, M., Shigematsu, T., Hasegawa, T., Kon, S., et al.
(2000). Enhanced expression of osteopontin in human diabetic artery and analysis of
its functional role in accelerated atherogenesis. *Arterioscler Thromb Vasc Biol*,
20(3), 624-628.

Takemoto, M., Yokote, K., Yamazaki, M., Ridall, A. L., Butler, W. T., Matsumoto, T., et
al. (1999). Enhanced expression of osteopontin by high glucose in cultured rat aortic
smooth muscle cells. *Biochemical and Biophysical Research Communications*,
258(3), 722-726.

II can regulate gene expression by the AP-1 binding sequence via a protein kinase C-
dependent pathway. *Biochemical and Biophysical Research Communications*,
172(3), 1189-1194.

Thompson, K., & Towle, H. (1991). Localization of the carbohydrate response element of
the rat L-type pyruvate kinase gene. *Journal of Biological Chemistry*, 266(14), 8679-
8682.

Exaggerated left ventricular dilation and reduced collagen deposition after


Xie, Z., Singh, M., & Singh, K. (2004a). ERK1/2 and JNKs, but not p38 kinase, are involved in reactive oxygen species-mediated induction of osteopontin gene expression by angiotensin II and interleukin-1β in adult rat cardiac fibroblasts. *Journal of Cellular Physiology, 198*(3), 399-407.


VITA

Abigail Goodman was born in Chicago Illinois, the first daughter of Patricia and Chris Goodman. After graduating from Barrington High School in 2001, she attended Ball State University in Muncie, IN. Following graduation from Ball State University, Abigail entered the Ph.D. program at Loyola University Chicago in the Department of Cell and Molecular Physiology. She joined the lab of Leanne Cribbs in 2006 and spent the next four years studying the regulation and expression of OPN in cardiac cells. Abigail’s initial projects were aimed at determining alterations in mechanotransduction signaling in a model of type 2 diabetes. Abigail was awarded an American Heart Association Predoctoral fellowship in 2007 for her project titled “Altered Mechanotransduction Signaling in Type 2 Diabetes and Diabetic Cardiomyopathy”. Abigail’s dissertation project was to determine the regulation of OPN expression in cardiac cells. Abigail has accepted a position in the 2014 class at Chicago College of Osteopathic Medicine.