Role of heme oxygenase in cardiomyopathy in obese Zucker fatty rats

A Thesis

Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the Department of Physiology

University of Saskatchewan

Canada

By

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ABSTRACT

Visceral obesity, a serious health issue is implicated in insulin resistance and altered cardiac structure and function. Elevated inflammation, increased oxidative stress, insulin resistance, excessive extracellular matrix (ECM) deposition and tissue remodeling are among the possible mechanisms linking obesity with cardiomyopathy. Since, the cytoprotective role of the heme oxygenase (HO) system is well acknowledged, the present study investigated the effects of upregulation of the HO system by HO-inducer hemin on cardiomyopathy in obese Zucker Fatty (ZF) rats. This study also investigated the mechanisms by which HO improves insulin signaling, glucose metabolism and cardiac function in this model. HO modulates adiponectin and atrial natriuretic peptide (ANP). However, the interaction of these proteins with the HO system in ZF rats is unclear. ANP and adiponectin were also measured in this study.

My thesis work showed that treatment with hemin abated inflammation and oxidative stress by attenuating pro-inflammatory-M1 phenotype macrophage infiltration and suppressing cytokines/chemokines including TNF-α, IL-1β, IL-6, monocyte-chemoattractant protein-1, macrophage inhibitory protein-1α and endothelin-1. Furthermore, hemin treatment suppressed ECM/heart failure proteins such as osteopontin and osteoprotegerin, collagen IV, fibronectin and transforming growth factor-β, reduced cardiac hypertrophy and cardiac lesions and enhanced ANP, adiponectin, insulin sensitivity and HO-1 concentrations. Interestingly, hemin treatment improved several compromised echocardiographic and hemodynamic parameters including left-ventricular diastolic and systolic free wall thickness, mean-arterial pressure, arterial systolic pressure, arterial diastolic pressure and cardiac output. In contrast, the HO inhibitor, stannous mesoporphyrin nullified the effects of hemin.

In conclusion, my thesis data strongly suggest protective effects of hemin-induced upregulated HO system against impaired insulin signaling and cardiomyopathy in obese Zucker rats. The suppression of inflammatory/oxidative mediators, ECM and profibrotic proteins, heart failure proteins, left-ventricular hypertrophy, cardiac lesions and the concomitant increase in ANP and adiponectin levels are some of the mechanisms by which HO enhanced insulin signaling, improved glucose metabolism and cardiac tissue morphology and function in obese Zucker rats.

ACKNOWLEDGEMENTS

This thesis represents a milestone of journey towards Canada for more than five years, working together with many supportive people interdependently which made journey easier. I owe my gratitude to all those people who directly or indirectly supported me in this venture.

First and foremost I would like to express my deepest gratitude and respect to my supervisor Dr. Joseph Fomusi Ndisang who has supported me throughout my thesis with his patience and immense knowledge. His esteemed guidance, constructive criticism, incessant encouragement and constant support helped me to go-ahead with my research work. He is a great teacher who nourished my scientific writing skills and taught me how to think independently and enrich my growth as a student and researcher.

I would like to express my sincere thanks to my committee members, Dr. Linda Hiebert, Dr. Kailash Prasad, Dr. Paul Lee, Dr. Nigel West, Dr. Michel Desautels and Dr. Sean Mulligan for their insightful comments and guidance at different stages of my research. I would like to thank Dr. Veronica Campanucci who is always willing to help and give her best valuable suggestions. Thank you to Dr. Lynn Weber, for extending me the laboratory facilities of the Veterinary Medicine Laboratory for the successful completion of the echocardiographic analysis.

Special thanks to Dr. Kailash Prasad, Prof. Emeritus for his excellent guidance, care, unfailing help and valuable advice. I am really grateful to him for being helpful in personal life as well.

I am sincerely thankful to the people in our department: Lois Bradley and Corinne Howells for their assistance and care during all these years. I would like to thank the animal staff, University of Saskatchewan for their assistance and taking care of animals. I express my heartfelt thanks to our technician Dilip Singh and his wife Donna Singh for their assistance, friendship, care and providing a helping hand whenever needed.

My sincere and heartfelt thanks goes to Dr. Subeer S. Majumdar, Scientist, NII and Dr. P.C. Pandey, Prof. Emeritus for their valuable guidance, moral support, care and providing me the reference letter anytime whenever needed.

Most importantly, none of this would have been possible without the love and patience of my family. This thesis is dedicated to my loving parents (Shri A.P. Tiwari & Smt. Urmila Tiwari) for their endless love, constant support and encouragement and for pushing me to do my best at every endeavor and special thanks to my mother for the millions of prayers she has prayed to keep me safe and well.

I would like to express my heart-felt gratitude to my sisters Vinita and Yogita for extending their moral support, love, laughter, kinship, excellent advice and always made time to listen me for hours to share my problems and helped me to overcome every difficult situation. Thanks for always stood beside me as a pillar and wonderful friends.

Thanks especially to my elder brother Dr.Anoop Tiwari for his valuable guidance, support, constructive criticism and who always stood beside me as a helping hand in every endeavor of life. My sincere thanks to Vivekji, Anshumanji, for their best wishes, support and encouragement. I have to give a special mention for the support and love given by my cute nephew Vrishank and nieces Varnika, Mihika, Tavishi & Kuhu that always made me to smile in tough situations during this journey.

I warmly appreciate the generosity and understanding of my in-laws Shri. J.P. Mishra & Late. Smt. Leela Mishra for their concern, support and strength all these years.

Above all, I would like to express my heart-felt gratitude to my husband, Manish. Thank you for being my steady, constant partner during these tumultuous years. Thank you for supporting me throughout all my studies. Without the motivation, support, help and guidance as of a good friend, this thesis would not have been possible. Thanks for everything.

Last but not the least; I would like to thank the people of Saskatoon, Canada for making my stay a wonderful experience and helping me go through everything without any hurdles.

Finally, I give my sincere thanks to the Almighty for his abundant grace and blessings on this project and giving me the opportunity and ability to do work.

DEDICATION

To my Mummy and Papa

Shri. A.P. Tiwari and Smt. Urmila Tiwari

A true blessing from God

Who groomed me and made me who I am today and

For their constant encouragement, blessings, unconditional love, care and support &

to the Almighty who made it all possible

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LIST OF ABBREVIATIONS

AP-1	Activating protein-1
ACE	Angiotensin converting enzyme
ANOVA	One way analysis of variance
ANG	Angiotensin
ANG II	Angiotensin-II
AT1	Angiotensin-II receptor subtype-1
CO	Carbon monoxide
cGMP	Cyclic guanosine monophosphate
cJNK	c-Jun-N-terminal-kinase
ET-1	Endothelin-1
eNOS	Endothelial nitric oxide synthase
EIA	Enzyme Immuno Assay
ECM	Extracellular matrix
FFA	Free fatty acids
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H_2O_2	Hydrogen peroxide
HO-1	Heme oxygenase-1
H&E	Haematoxylin and eosin
Hrs	Hours
iNOS	Inducible nitric oxide synthase
µl	Microliter
µm	Micrometer

μM	Micromole
mmHg	Millimetre of mercury
mM	Milimole
min	Minutes
MCP-1	Macrophage chemoattractant protein-1
MIP-1α	Macrophage inflammatory protein-1α
IL	Interleukin
IRS-1	Insulin-receptor-substrate-1
JAK-STAT	Janus kinase /signal transducers and activators of transcription
NO	Nitric Oxide
NOS	Nitric oxide synthase
NF-κB	Nuclear factor kappa B
$O2^{\bullet -}$	Superoxide
$ONOO^-$	Peroxynitrite
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol-3-kinase
PKB	Protein-kinase-B
PTP1B	Protein tyrosine phosphatase 1B
PAI-1	Plasminogen activator inhibitor-1
Px	Pixel
ROS	Reactive oxygen species
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate

RAAS	Renin-angiotensin-aldosterone system
SnMP	Stannous mesoporphyrin
sGC	Soluble guanylate cyclase
SD	Sprague-Dawley
SHR	Spontaneously hypertensive rat
SOD	Superoxide dismutase
SOCS	Suppressor of cytokine signaling
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor-alpha
ZDF	Zucker Diabetic fatty rat
ZF	Zucker fatty rat

CHAPTER 1

Introduction

Visceral obesity is an established risk factor for cardiovascular diseases (1-14). During obesity, elevated inflammation, increased oxidative stress, altered lipid metabolism, insulin resistance, impaired glucose metabolism, excessive extracellular matrix (ECM) deposition and tissue remodeling are among the possible mechanisms which are implicated in altered cardiac structure and function (13, 15-31) and links obesity with cardiomyopathy and other related-complications. Although, considerable efforts have been made in elucidating mechanisms implicated in obesity-related cardiovascular disease, new therapeutic strategies with novel mechanisms of action, are urgently needed. Since, the cytoprotective role of heme oxygenase (HO) system is well acknowledged (3-5, 32-54), an upregulated HO system can be explored as a novel paradigm against micro-vascular and macro-vascular complications associated with obesity-induced cardiomyopathy. In the present thesis the role of an upregulated HO system by HO-inducer hemin (a drug used against porphyria) (55, 56) on cardiomyopathy in obese Zucker fatty (ZF) rats was investigated and the mechanism by which HO improves insulin signaling, glucose metabolism and cardiac function was evaluated. The present study for the first time highlights the novel role of the HO system on macrophage polarization in the cardiac tissue of ZF rats. Importantly, my thesis work provides novel insights in the beneficial effects of concomitantly upregulating the HO system, atrial natriuretic peptide (ANP) and adiponectin by hemin against inflammation, insulin resistance, oxidative stress, pericardial adiposity and cardiac hypertrophy in obese ZF rats.

This thesis consist of four chapters. The first chapter is the general introduction that includes a brief description about obesity and the link between obesity, inflammation, insulin resistance, oxidative stress and cardiovascular disease. ANP, adiponectin and HO system has been discussed briefly. More detailed description are given in appendices. Appendix A, appendix B, appendix C are three of my first-authored published manuscripts. These manuscripts provide up-to-date and detailed knowledge of the scientific literature on obesity-related cardiomyopathy and nephropathy. In addition, the functional significance of the HO system and its downstream signaling molecules such as bilirubin, carbon monoxide (CO) and ferritin as potential therapeutic tools for effective management of cardio-metabolic diseases and related complications including cardiomyopathy have been acknowledged.

The results of this thesis has been published as two manuscripts (chapters 2 and 3). In these chapters, my role is described on the first page of the manuscripts. Chapter-2 explains the role of an upregulated HO system by the HO-inducer hemin on cardiomyopathy in ZF rats, an obese rat model characterized by insulin resistance and cardiomyopathy. This chapter also describes the mechanisms by which hemin therapy improves glucose metabolism and enhances cardiac function. Chapter-3 underscores how an upregulated HO system by hemin suppresses pericardial adiposity, abates inflammation, enhances insulin signaling and attenuates diabetic cardiomyopathy. Chapter-4 is the general discussion of the entire research results and it highlights the importance of my research in the context of cardiomyopathy co-morbid with obesity and insulin resistance as well as the potential therapeutic benefit of a hemin-induced upregulated HO system that may be explored as a novel alternative approach against cardiometabolic complications. In addition, chapter 4 includes the conclusions and future directions sections.

To fully address all the themes of my thesis, in the ensuing paragraphs below, an outline of obesity and the link between obesity, inflammation, insulin resistance, oxidative stress and cardiomyopathy are described in detail.

1.1 Obesity

1.1.1 Prevalence of obesity

Obesity is a serious health problem growing rapidly worldwide. The prevalence of obesity is increasing at an alarming rate and has almost doubled in the last few decades (1, 11, 15, 27, 57-65). It is estimated that over the world more than one billion people are obese (61, 62, 65, 66). The World Health Organization reported that obesity is growing at a fast pace, with nearly 35% of the population being obese globally (61, 67). Approximately, the worldwide prevalence of obesity between 1980 and 2008 has risen from 4.8% to 9.8% in men and from 7.9% to 13.8% in women (60, 61) respectively. Although obesity has escalated globally, epidemiological data from North America indicates that the prevalence of obesity has reached epidemic proportions in the USA compared to other parts of the world. For example, in a related survey in the USA, it was shown that approximately, 26 % of adults are obese (60, 61, 63), whereas in South East Asia, the situation is less dramatic as only 3% of adults are considered obese (60). Obesity has increased in every segment of the population including children. The global burden of childhood obesity between 1990 and 2010 has risen from 4.2% to 6.7% respectively (60, 61). Approximately, 43 million children are obese and it is expected that this total may rise to over 60 million by 2020 (61).

The global rise in prevalence of obesity in both adults and children is a matter of great concern since the major health consequences of obesity include an increased risk for

cardiometabolic complications and associated comorbidities such as type-2 diabetes, insulin resistance, hypertension, dyslipidemia and renal diseases (1, 11, 14, 15, 27, 57-60, 62, 64, 65, 68, 69) **(Figure 1-1).** Moreover, the increased prevalence of obesity is an independent risk factor for cardiovascular disease and heart failure (1, 15, 60, 63-65, 69, 70). Epidemiological study showed that approximately 32%–49% of individuals with heart failure are obese (63) displaying that obesity is associated with significant cardiovascular morbidity and mortality in both men and women (1, 11, 57, 59). Thus, obesity has emerged as a major global public health challenge.

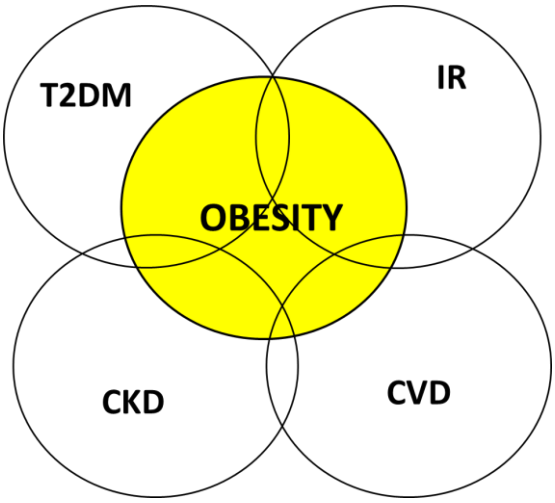

Figure 1-1: Consequences of obesity. Adapted and modified from *Med Clin N Am 97 (2013) 59–74.*

Abbreviations: Type-2 diabetes (T2DM), Insulin resistance (IR), Chronic kidney disease (CKD) and Cardiovascular disease (CVD).

1.1.2 Adipose tissue - a dynamic endocrine organ

Adipose tissue plays important regulatory roles in energy homeostasis (7, 71-73). The primary function of adipose tissue is to store excess nutrients in the form of triglycerides and to release free fatty acids (FFA) during nutritional deprivation (7, 71-73). Due to its ability to generate heat

via non shivering thermogenesis as well as insulator properties, adipose tissue also functions as a thermoregulator (74). However, with the discovery that adipocytes can produce and secrete the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) (75, 76) as well as the hormone leptin, that plays a role in appetite and energy homeostasis, adipose tissue is now recognized as an active endocrine organ that regulates metabolism (1, 5, 77). Adipose tissue serves as an important endocrine gland that secrets different bioactive molecules including adipokines (cytokines and chemokines) as well as lipokines (lipid mediators) (1, 5, 77), which function in an autocrine, paracrine or systemic manner and regulate important physiological functions related to lipid and glucose homeostasis (75, 78-81). Moreover, adipokines are involved in the regulation of crosstalk between adipose tissue and major metabolic organs including heart, liver, muscle, pancreas as well as the nervous system (6, 75, 78, 79). Under normal physiological conditions the storage of triglycerides in non-adipose tissues (pancreas, liver and heart) is minimal and is strictly regulated (1, 6). However, excess fat accumulation in these vital organs occur during obese conditions (1, 6, 7). Therefore, any alterations in the normal function of adipose tissue may in turn impair metabolic functions of these vital organs that leads to the development of metabolic diseases such as insulin resistance and cardiovascular disease (7, 75, 78, 79) (See Appendix D 1 for more details).

1.1.3 Pathophysiology of adipose tissue

Leptin, resistin, visfatin, adiponectin, TNF-α, interleukin-1 (IL-1), IL-6, IL-8, IL-10, plasminogen activator inhibitor-1 (PAI-1), retinol binding protein-4 and monocyte-chemoattractant protein-1(MCP-1) are among the adipokines released from adipose tissue that play important roles in energy metabolism (72, 75, 79, 81) **(Figure 1-2).** Under the normal

healthy state, adipose tissue acts as a storage organ for deposition of excess triglycerides (7, 71-73). To accommodate excess fat, the fully differentiated adipocytes first enlarge to their maximum size. After that, further storage is done by increasing the number of adipocytes through the process of proliferation of progenitor cells. However, during obesity the normal physiological functions of adipose tissue are disrupted (7, 71-73) and pro-inflammatory cytokines such as TNF-α, IL-6, visfatin, resistin, angiotensin II and PAI-1 are produced mainly by adipose tissue (1, 5, 75, 77). In contrast, anti-inflammatory adipokines such as adiponectin, IL-10, IL-4, IL-13 and apelin are mainly produced by adipose tissue from non-obese individuals that facilitates physiological functions (72, 75, 79, 81). As a consequence, impaired adipokine production during obese conditions activates altered paracrine and endocrine immune responses that lead to the development of obesity related cardiovascular and metabolic disorders (72, 75, 79, 81). This indicates that depending upon the pathophysiological conditions of the body different kinds of adipokines regulate lipid and glucose metabolism in both a positive and negative manner.

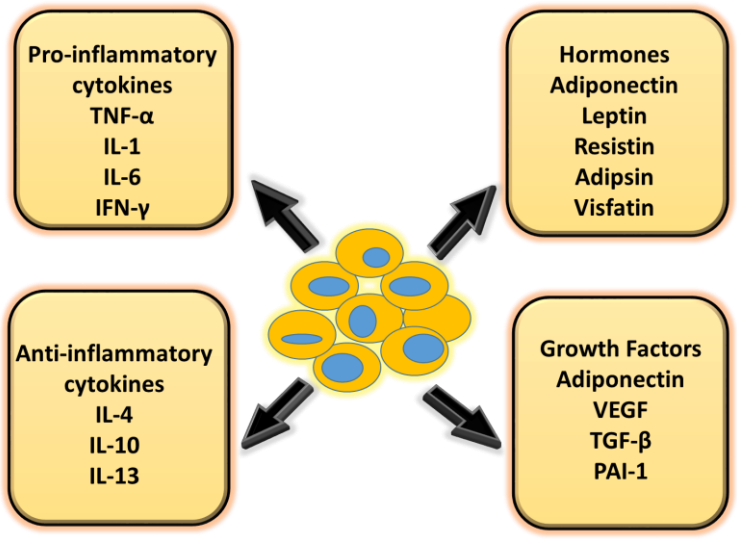

Figure 1-2: Adipokines produced by adipose tissue. Adapted and modified from *Pediatr Gastroenterol Hepatol Nutr 2013; 16: 143~152*

Abbreviations: Tumor necrosis factor (TNF-α); Interleukin (IL); Interferon-γ (IFN-γ); Vascular endothelial growth factor (VEGF), Transforming growth factor-β (TGF-β); Plasminogen activator inhibitor-1 (PAI-1).

1.1.4 Adipokines in obesity related metabolic disorders (See Appendix D2 for more details)

1.1.4.1 TNF-α

TNF-α is a pro-inflammatory cytokine that plays a central role in inflammatory and immune responses (3-5, 58, 82-84). It is a pleiotropic cytokine that mediates a wide range of biological activities. Depending upon cytokine levels, TNF-α exhibits dual roles in both physiology and pathology (85, 86). At normal physiological levels, TNF-α regulates apoptosis, host-defense interactions, cell proliferation, adipogenesis, lipid and glucose metabolism as well as participates in innate immune responses (58, 85, 87). However, elevated levels of TNF-α have been shown to mediate inflammatory responses and tissue injury (58, 85, 87) related to obesity and associated comorbidities (3-5, 58, 82-84, 88). The first molecular link between inflammation and obesity arise from the finding that in the rodent model of obesity, the pro-inflammatory cytokine TNF-α was overexpressed (76, 86, 89). Similarly, it was reported that both obese and type-2 diabetic animal models as well as obese individuals displayed overexpression of TNF-α (81, 83, 84, 90) in adipose and muscle tissue. In vitro and in vivo studies also revealed that administration of recombinant TNF-α impairs the insulin signaling pathway, while an improved insulin sensitivity is observed in knockout obese mice having dysfunctional TNF-α or TNF receptors, (91, 92). Studies showed that inhibition of TNF-α expression improved insulin sensitivity in obese rat and mice models (81, 93). Moreover, the long term inhibition of TNF-α improved glucose metabolism in patients with metabolic syndrome (94) and reduced insulin resistance in individuals with rheumatoid arthritis and psoriasis (95, 96). However, the beneficial

effect of TNF-α inhibition was not observed in a recent clinical study (97) and in the obese rat model (98), suggesting that blocking of TNF-α signaling may improve impaired insulin signaling under certain conditions of excessive inflammatory states (81).

Depending upon the stimuli, TNF-α is produced by different cell types including immune cells and adipocytes. The potent stimulator of TNF-α production from these cells includes bacteria, viruses, hypoxia, ischemia, complement factors and various pro-inflammatory cytokines (76, 86, 89). Interestingly, the production of TNF-α is governed by both positive and negative feedback loop mechanisms. The positive feedback loop mechanism includes TNF-α stimulated secretion of pro-inflammatory cytokines such as IL-1β, IFN-γ and IL-2 which, in turn governs TNF-α production. On the other hand, in the negative feedback loop mechanism, the production of TNF-α is inhibited by the TNF-α induced anti-inflammatory cytokine IL-10 (87). In hypertrophic adipose tissue, TNF-α is mainly secreted by activated macrophages and monocytes in response to inflammatory signals (87, 91). Increased levels of TNF-α in turn initiate the production of other pro-inflammatory cytokines and chemokines such as IL-6, MCP-1, resistin and PAI-1 (58, 88). As a consequence, more circulating monocytes are recruited at inflamed sites in response to chemokines such as MCP-1 (13). Thus, the interaction among macrophages, endothelial cells and adipocytes further amplifies inflammatory signals and promotes the secretion of pro-inflammatory cytokines and chemokines. This, leads to a state of local or systemic inflammation and subsequent insulin resistance (3-5, 58, 82-84). It is suggested that one of the possible mechanisms for TNF-α mediated impaired insulin signaling is that elevated levels of TNF-α inhibits tyrosine phosphorylation of the insulin receptor substrate 1 (IRS1) and thereby prevents binding of IRS1 to insulin receptors which in turn results in insulin resistance in muscle and adipose tissue (58, 81, 82, 99). In addition, it is reported that depending

upon stimuli, TNF-α activates different signaling pathways including, nuclear-factor κ B (NFκB), caspases and mitogen-activated protein kinases (MAP kinases) (82, 88) and the interactions among these pathways is responsible for a wide range of TNF-α mediated pathophysiological functions (13, 88). Thus, higher expression of TNF-α in adipose tissue is a characteristic feature of obesity and is a risk factor for insulin resistance and cardiovascular complications (3-5, 58, 82-84). Therefore, inhibition of TNF-α action might be a beneficial therapeutic tool against the pathogenesis of various diseases including obesity and cardiovascular disease.

1.1.4.2 IL-6

IL-6 is a multifunctional cytokine that plays an important role in the regulation of humoral and cellular immune responses, haematopoiesis, inflammation and tissue injury (16, 75, 100, 101). It is a pleiotropic cytokine with pro-inflammatory and anti-inflammatory action (75, 101). IL-6 belongs to a family of granulocyte colony stimulating factor proteins that includes IL-11, leukemia inhibitory factor, oncostatin M and ciliary neurotrophic factor as other cytokine members (101). It has been observed that these cytokines, including IL-6, signal through a ubiquitously expressed transmembrane glycoprotein 130 (gp130), a common β-receptor (102-105). However, each member of the IL-6 super family possesses a specific α-receptor. Under different conditions, the pleiotropic effects of IL-6 is initiated by formation of a heterocomplex through its binding to a receptor complex comprising a specific receptor subunit IL-6 receptor α (IL-6Rα) and gp130 (105-107). Binding of IL-6 to this heterocomplex, activates IL-6Rα, that initiates the activation of a signaling cascade namely, the janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway (108, 109). IL-6 is produced by various cell

types such as monocytes, fibroblasts, endothelial cells and also by the liver, skeletal muscle and adipose tissue (16, 75). However, secretion of IL-6 is cell specific and is mainly governed by immune, hormonal and metabolic stimuli (110). It is observed that under normal physiological conditions transient expression of IL-6 exerts anti-inflammatory function and regulates glucose metabolism (16, 75, 100). However, under the obesity-induced chronic inflammatory state, the elevated levels of systemic IL-6 induce dysfunctional insulin signaling (81, 111). IL-6 exhibits a dual functional state depending upon cell-type and metabolic state (16). For example, in skeletal muscles during exercise, IL-6 exerts its anti-inflammatory effect and facilitates glucose uptake that leads to muscle hypertrophy and myogenesis (16). In contrast, in liver and adipose tissue, increased levels of IL-6 are pro-inflammatory and promote insulin resistance (16, 75).

Under physiological conditions, nearly 15-35% of circulating IL-6 is produced from adipose tissue, therefore, IL-6 is also considered as an adipocytokine (112). However, compared to subcutaneous adipose tissue, IL-6 is secreted three times more from the stromal vascular fraction of vascular adipose tissue (16, 75). For this reason, IL-6 is considered as a marker for visceral adiposity (111). IL-6 has been identified as a key inflammatory molecule during the obese chronic inflammatory state (105), as approximately one-third of circulating IL-6 originates from adipose tissue (111). Elevated levels of circulating IL-6 have been reported in obese subjects (28, 31, 113). Moreover, high levels of IL-6 can be positively correlated with body mass and plasma FFA concentrations (101, 105, 114). However, in obese individuals who underwent bariatric surgery, subsequent weight loss resulted in low levels of serum IL-6 together with improved insulin sensitivity (28, 115). IL-6 has been implicated in the pathogenesis of various metabolic diseases including type-1 and type-2 diabetes, coronary heart disease and insulin resistance (105, 116). Increased IL-6 levels impair insulin signaling, elevate lipolysis and

augment FFA production (111). The exact mechanism for IL-6 induced impaired insulin signaling is not clear. However, it is suggested that IL-6 promotes the increased expression of the suppressor of cytokine signaling 3 (SOCS3) which binds and attenuates insulin receptor and IRS1 phosphorylation (31, 75). Although, increased plasma concentration of IL-6 is being regarded as a predictor for insulin resistance and cardiovascular disease (17, 101, 115, 116), the role of IL-6 in mediating insulin resistance is still ambiguous and depends upon metabolic state and tissue type. For example, in one IL-6 knockout mouse model, IL-6 was shown to develop insulin resistance and obesity, while the same results were not observed with different IL-6 knockout models (75). Interestingly, IL-6 has modulatory effects on adipokine production (75). For example, in human adipocytes, IL-6 was shown to suppress the expression and secretion of anti-inflammatory adiponectin and other markers of adipocyte differentiation (116). Thus, IL-6 is a pleiotropic cytokine that plays a key role in the pathogenesis of metabolic diseases (111, 116). Therefore, understanding its role in regulation of obesity-induced inflammatory states will help to counteract the adverse effects arising from obesity.

1.1.4.3 IL-1

The IL-1 family is a group of related cytokines that play important roles in metabolic inflammation and immune responses (115, 117). IL-1α, IL-1β, IL-18 are among the pro-inflammatory members, whereas, IL-1 receptor antagonist (IL-1Ra) and IL-37 are anti-inflammatory members of the IL-1 family (115, 117). The inflammatory mediators IL-1α and IL-1β are potent pyrogens and were among the first identified cytokines with similar structure and functional characteristics (118, 119). Both IL-1α and IL-1β are derived from precursor proteins (pro-IL-1α and pro-IL-1β) via enzymatic cleavage (119). It is reported that pro-IL-1α is the

biologically active form of the protein that is cleaved by calcium–dependent cysteine protease calpain to produce mature IL-1α. Both pro-IL-1α and IL-1α remain intracellular and are rarely detected in blood and other body fluids, till they are released by dying necrotic cells during disease conditions (119, 120). Under normal conditions, IL-1α was found to be a membrane bound protein, constitutively expressed at the surface of various cell types such as keratinocytes, platelets and epithelial cells as well as by the organs such as liver, kidney and lung (120). IL-1β is mainly secreted by blood monocytes, dendritic cells and tissue macrophages (120). Interestingly, cytokines such as TNF-α, IL-18, IL-1α and IL-1β itself acts as stimulators for IL-1β synthesis (120). In addition, compared to pro-IL-1α, IL-1β is first expressed in the form of a biologically inactive precursor (pro-IL-β). Pro-IL-1β is then cleaved by an intracellular cysteine protease caspase-1 to generate the active 17.5 KDa protein (120). In fact, caspase-1 itself needs to be activated via the formation of multimeric cytosolic molecular complex (inflammasome) in order to synthesize active and mature IL-β from its inactive precursor pro-IL-1β (119, 120). Once secreted, the pro-inflammatory effect of mature IL-1β is mediated with the help of IL-6 and C-reactive protein, that is known to be increased during inflammation and pathological conditions of hyperlipidemia and atherosclerosis (117). Both IL-1α and IL-1β exert their biological effects by binding to their cell surface receptor IL-1 receptor type 1 (IL-1R1), expressed by various cell types (119, 120). This in turn activates various downstream signaling cascades involving, c-Jun-N-terminal-kinase (JNK), extracellular signal-regulated kinases (ERKs), MAPK and NFκB (121). Furthermore, IL-1β has been implicated in the development of type-2 diabetes and insulin resistance in both in vitro and in vivo animal model as well as in humans (115, 122). In both IL-1α and IL-1β knockout mice, improvement in glucose metabolism and suppression of adipose tissue inflammation was observed (117). Thus, these findings

indicate that the blocking of IL-1β can be explored for the treatment of insulin resistance and obesity.

1.2 Obesity, inflammation and macrophages

1.2.1 Obesity as a pro-inflammatory state

Visceral obesity, in particular is a major risk factor for metabolic disorders including insulin resistance, diabetes and cardiometabolic complications (1-5, 15, 123-127). The common denominator that links obesity with other metabolic disorders is a chronic low grade inflammation (9, 10, 18, 24, 26, 27, 31, 71, 75, 77, 128-135). The identification of high levels of the pro-inflammatory cytokine TNF-α in adipose tissue of obese individual by Hotamisligil and colleagues (1993) provides the link between obesity, inflammation and insulin resistance. The subsequent studies demonstrated that an upregulated gene expression of inflammatory transcriptional factors and elevated production of various pro-inflammatory cytokines and chemokines occurs in hypertrophied adipose tissue (3, 5, 15, 124-127, 136). The exact molecular mechanism that leads to the initiation of the inflammatory response under obese conditions is largely unknown. However, one of the possible explanations is that due to the excessive deposition of fat, expansion of adipose tissue occurs in response to adipocyte hypertrophy and hyperplasia. As a consequence, large adipocytes became hypoxic that leads to the induction of various inflammatory pathways (67, 128). Obesity induced inflammation triggers overproduction of various pro-inflammatory cytokines (IL-1β, IL-6,TNF-α) and chemokines (MCP-1 and MIP1-α) from adipocytes, macrophages and other cells that play important roles in systemic inflammation (3, 5, 15, 124-127, 136) **(Figure 1-3).** Moreover, the increased macrophage infiltration during the inflammatory process was reported to be associated with a macrophage

polarization event where macrophages were polarized from an anti-inflammatory M2 to a pro-inflammatory M1 phenotype (19, 130, 137-140). In addition, during obesity, multiple signaling cascades including JNK, inhibitor of κ kinase (IKK), TLR4, NFκB and their downstream signaling molecules are activated that contribute to the stimulation of inflammatory processes in adipose tissue (69, 99, 141). The systemic inflammation upregulates SOCS proteins that suppress insulin receptor signaling and promote endothelial dysfunction (142). Thus, chronic low grade inflammation is an underlying mechanism that links obesity with insulin resistance and cardiometabolic complications (3, 5, 13-15, 24, 29, 68, 69, 91, 127, 135, 143-150).

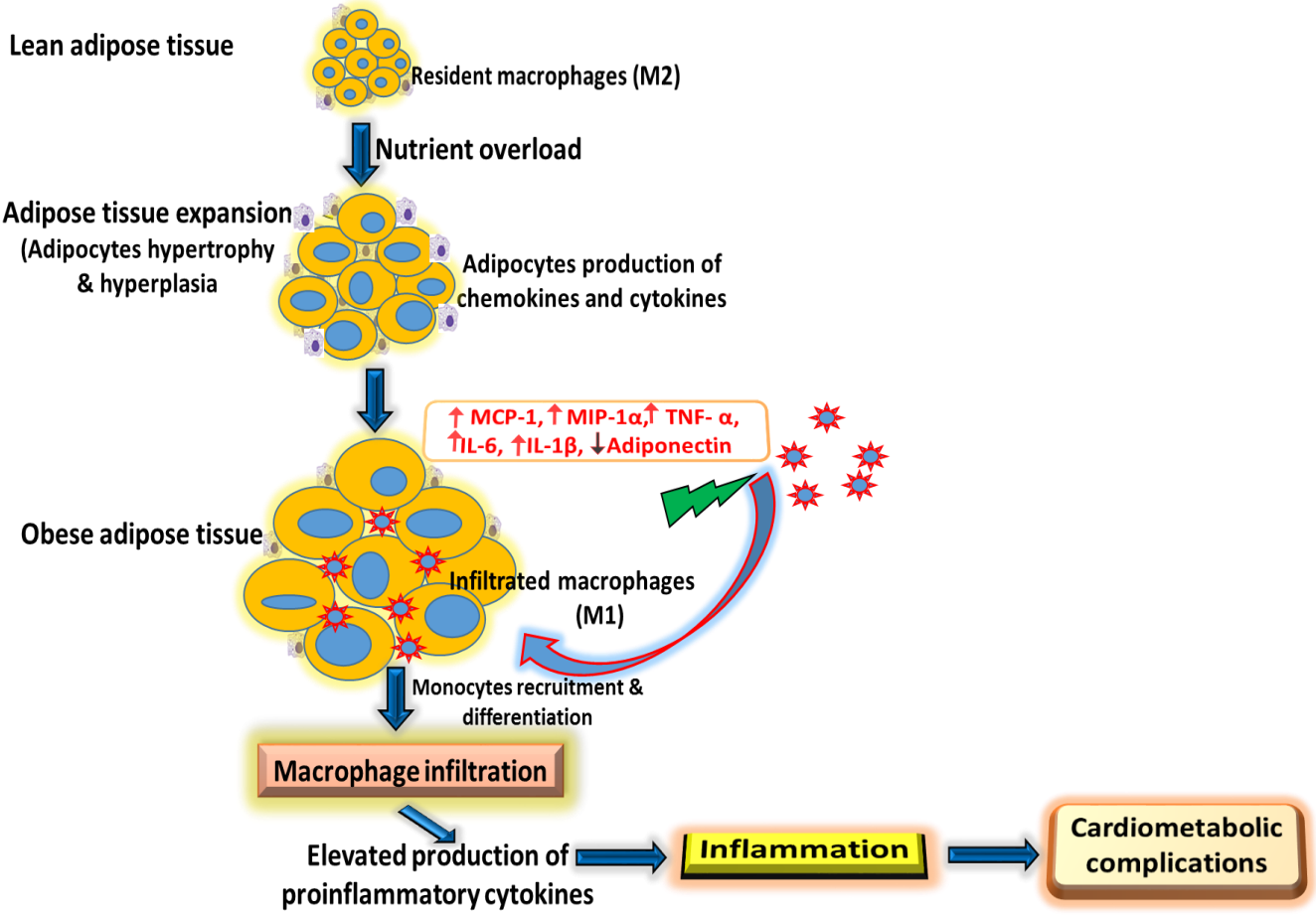

Figure 1-3: Obesity-induced macrophage infiltration and inflammation.

Abbreviations: Tumor necrosis factor (TNF-α); Interleukin (IL); Macrophage chemoattractant protein (MCP-1); Macrophage inflammatory protein 1 (MIP-1α).

1.2.2 Macrophages and inflammation

1.2.2.1 Origin, phenotype and function of macrophages

Macrophages are highly heterogeneous phagocytic cells that play an important role in metabolic homeostasis and immune response (137-139, 151-155). Under physiological conditions, macrophages contribute to host defense mechanisms, prevent inflammation by removing infected cells and pathogens via phagocytosis, contribute to wound healing, tissue repair and clearance of dead cells (153, 155) and acts as a link between adaptive and innate immunity. However, during the pathological condition of visceral obesity increased macrophage infiltration leads to inflammation and insulin resistance (19, 130, 137-140). Interestingly, increased numbers of both resident and infiltrated activated adipose tissue macrophages (ATMs) have been observed during obesity (137-140). For example, in lean animal models, macrophages comprise nearly 10-15% of the stromal cell population (135, 152). However, in obese animal models increased macrophage infiltration in adipose tissue increase their population to approximately 45-60% (135, 152). As a consequence, increased numbers of activated macrophages further elevates the levels of pro-inflammatory mediators including IL-6 and TNF-α that directly alters insulin signaling and exacerbates adipose tissue inflammation (135, 139, 154). In addition, ATMs are also involved in adipogenesis, angiogenesis and in hypoxia mediated responses in adipose tissue (154, 155). Thus, macrophages display heterogeneity in function.

How are macrophages able to mediate both physiological and pathological functions? One possible explanation for the diverse function of macrophages arises from their potential to exist in different activation states with specific properties (127, 135, 139, 151, 154, 156-160). On stimulation with different kinds of stimuli, macrophages gets activated and in turn express distinct surface receptors and produce distinct chemokines and cytokines that leads to diverse macrophage pro-inflammatory and anti-inflammatory functions (135, 139, 154). Moreover, ATMs exhibit a different pattern of surface markers and cytokine profiles and perform distinct functions in lean and obese animals (139). Depending upon site, stage and type of tissue insults, macrophages generally exhibit different phenotypes; M1 phenotype and M2 phenotype (135, 139, 154). It is reported that in lean animals, ATMs exhibit an alternative M2 phenotype characterized by anti-inflammatory gene expression (IL-10) and enhanced insulin sensitivity (139, 154). In contrast, visceral obese ATMs exhibit the classically activated M1 phenotype characterized by high expression of pro-inflammatory cytokines such as TNF-α and IL-6 and increased inducible nitric oxide synthase (iNOS) activity that inhibits insulin action directly and eventually develops adipose tissue insulin resistance (139, 154). Besides M1 and M2 phenotypes, a mixed M1/M2 phenotype has been observed in obese ATMs that also contribute to the chronic inflammatory process (139).

1.2.2.2 Tissue macrophage activation

In view of the diverse biological functions, activated macrophages possess the ability to acquire different phenotypes depending on the type of stimuli and site of insult in the local environment (127, 135, 139, 151, 154, 156-160). In response to various pathogens and cytokines macrophages may polarize towards the classical M1 phenotype or the alternative M2 phenotype

(151, 157, 160) **(Figure 1-4).** It is suggested that the phenotypic plasticity of macrophages resembles the Th1–Th2 polarization of T cells (151, 157).

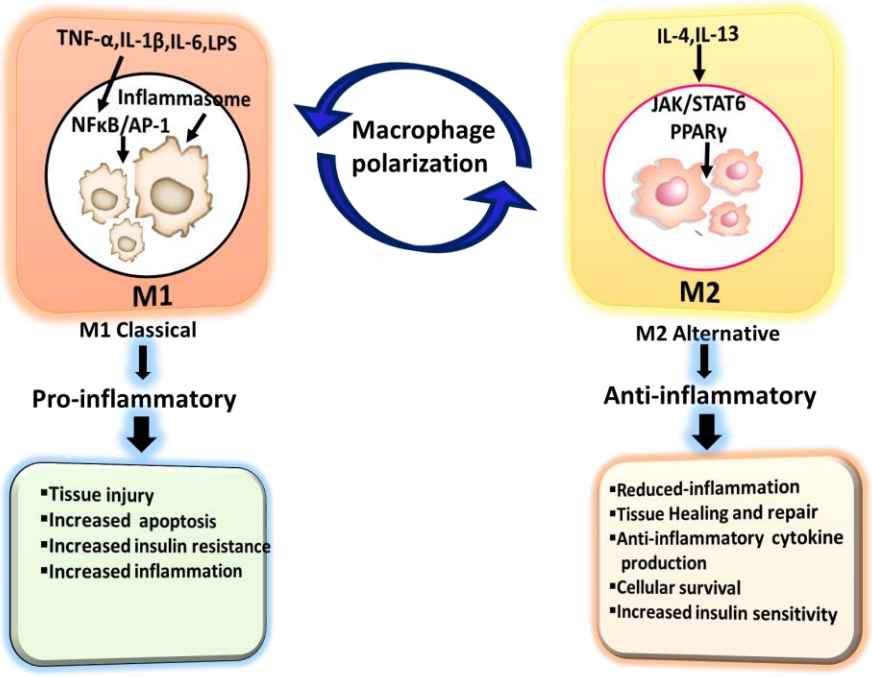

Figure 1-4: Schematic representation of macrophage polarization from classical M1 phenotype to alternative M2 phenotype.

Abbreviations: Tumor necrosis factor (TNF-α); Interleukin (IL); Interferon-γ (IFN-γ); Lipopolysaccharide (LPS), Nuclear factor kappa-B (NFκB); Activator protein-1 (AP-1); Signal transducer and activator of transcription 6 (STAT6); Peroxisome proliferator-activated receptor gamma (PPARγ).

1.2.2.2.1 Classically activated macrophage (M1 phenotype)

The M1 phenotype represents classically activated macrophages that participate in the immune response. Multiple factors such as interferon gamma (IFN-γ), toll-like receptor (TLR) ligands, lipopolysaccharide, granulocyte macrophage colony-stimulating factor (GMCSF)

activates the MI phenotype of macrophages. The M1 phenotype possesses microbicidal and tumoricidal properties and is characterized by elevated levels of pro-inflammatory cytokines such as TNF-α, IL-1β, Il-6, IL-12 and chemokines such as MCP-1 & MIP-1α, high levels of reactive oxygen species (ROS) generation and nitrogen intermediates which in turn leads to increased oxidative stress (19, 130, 137-140). Furthermore, M1 macrophages function as phagocytic cells for intracellular pathogens and can stimulate the Th1 response (156). Studies have demonstrated that in obese human and animal models, M1 macrophages mediated high levels of pro-inflammatory cytokines induce insulin resistance via the activation of an inhibitor of nuclear factor kappa-B kinase-β (IKKβ) and c-Jun N-terminal kinase (JNK)1 signaling pathways. These signaling pathways in turn promote inhibitory phosphorylation of IRS1 proteins at serine residues and activation of transcriptional factors including NFκB and activator protein 1 (AP-1) that play important roles in the inflammatory process (19, 132). Overall, classical activation of macrophages in response to various stress conditions promotes inflammatory responses and insulin resistance.

1.2.2.2.2 Alternative activated macrophage (M2 phenotype)

Alternatively activated macrophages, represent the M2 phenotype of macrophages that contribute to tissue function under physiological conditions (155, 160). M2 macrophages exhibit anti-inflammatory properties. M2 macrophages participate in the resolution of inflammation after infection or injury, in tissue repair and wound healing, and thus preserve tissue function in response to various conditions of stress (155, 160). Furthermore, M2 macrophages promote Th2 responses and participate in parasite clearance and immunoregulation (157). In general, the M2 phenotype is stimulated by the Th2 cytokines (IL-4, IL-13), TGF-β, glucocorticoids and immune

complexes (151, 160). M2 macrophages are characterized by low levels of pro-inflammatory cytokines, high production of anti-inflammatory cytokines (IL-10, IL1rα) and high expression of scavenger, mannose and galactose-type receptors (138, 157). Moreover, M2 phenotypic macrophages were found to be associated with a high production of the enzyme arginase that is known to block inducible nitric oxidase synthase activity which is a characteristic feature of M1 polarized macrophages (138). Interestingly, it is reported that activation of M2 macrophages in response to IL-4 cytokine is accompanied by the stimulation of transcription factors such as peroxisome proliferator-activated receptor gamma (PPARγ) and PPARγ coactivator 1β (PGC-1β) that play roles in lipid oxidative metabolism (138). Taken together, M2 polarized macrophages promote tissue repair, exhibit anti-inflammatory effects and exert cytoprotection against inflammation and insulin resistance (155, 160).

1.2.3 Mechanisms related to macrophage polarization and plasticity

Both heterogeneity and plasticity are the key features of macrophages (127, 135, 139, 151, 154, 156-160). ATMs from lean and obese animals display functional heterogeneity with relation to expression of surface markers and cytokine profiles (135, 139, 154). Lean ATMs display the anti-inflammatory M2 phenotype that plays important roles in insulin sensitivity, while excess nutrient driven obese ATMs are polarized towards the pro-inflammatory M1 phenotype that contribute significantly in systemic inflammation and associated insulin resistance (19, 137, 155). Interestingly, macrophage polarization is a phenomena that is known to occur during infection, pathological conditions of chronic inflammation, obesity, insulin resistance but also during homoeostatic conditions (19, 137, 155). One important question that still remains unanswered is that how does obesity switch the M2 activation state of macrophages

to the M1 activation state? At present, very little is understood regarding the mechanism that governs macrophage polarization. However, several possible explanations for obesity induced macrophage polarization have been postulated by different studies. It is predicted that a wide number of signaling molecules associated with different signaling pathways, various transcription factors and epigenetic processes are involved in the polarization of macrophages to distinct phenotypes (151). It is suggested that activation of the interferon regulatory transcription factor (IRF)/STAT molecular pathway by IFN-γ and TLR ligands via STAT1 favors the M1 phenotype of macrophage activation, whereas, stimulation by IL-4 and IL-13 via STAT6 activate the M2 phenotype of macrophages (151, 161). In addition, activation of macrophages by the STAT signaling pathway is governed by the proteins of the SOCS family. For example, IL-4 and IFN-γ in combination with TLR stimulation enhance the expression of SOCS1 and SOCS3 proteins that promote the inhibition of STAT1 and STAT3 action (151). In addition to these signaling pathways, various transcriptional factors play roles in macrophage polarization. The lipid sensing nuclear receptors such as PPARγ and PPARδ and their co-activator PGC-1β also mediate the expression of different genes associated with alternative macrophage activation (127, 151). STAT6 interacts with PPARγ and Kruppel-like factor 4 (proteins that participate in macrophage mediated function) to stimulate M2 gene expression and inhibit M1 genes via NFκB inhibition (27, 151, 159). In contrast, activation of TLR promote M1 macrophage activation via the induction of the NFκB signaling pathway and other inflammatory mediators (151). Recently, the important role of epigenetic regulation via histone methylation, acetylation and non-coding RNAs in macrophage polarization has been identified (151, 160). For example, in mice macrophages, IL-4 mediated induction of the histone demethylase was shown to promote M2 gene expression via the alterations of chromatin modifications (151). Thus, the physiological

and pathological functions of macrophages during normal and diseased states are mainly governed by phenotypic switching of macrophages between M1 and M2 activation states.

1.2.4 Macrophage infiltration during obesity

Macrophages infiltrate adipose and other tissues during the pathological condition of obesity and play direct roles in systemic inflammation and insulin resistance (19, 130, 137-140). The number and activation state of resident and infiltrated ATMs is reflective of diseased and healthy status of adipose tissue (151, 152). How does obesity promote increased macrophage infiltration? It is suggested that in response to excessive fat deposits adipose tissue expands. The continuous stress on adipocytes leads to the development of hypoxia, increased endoplasmic reticulum (ER) stress, high levels of FFA and ROS generation. This in turn promotes the increased production of pro-inflammatory mediators such as TNF-α, IL-6, IL-1β, MCP-1 and MIP-1α from adipocytes and other immune cells that result in the accumulation and activation of ATMs and various stress responsive signaling pathways (19, 130, 137-140). The activated ATMs further produce pro-inflammatory mediators that in turn exacerbate inflammatory responses and subsequently lead to the development of insulin resistance (19, 137, 155). The important chemoattractants for macrophage recruitment is MCP-1 and MIP-1α (130, 151, 158). It was shown that inhibition of MCP-1 and its receptor CCR2 in the obese animal model suppressed macrophage infiltration and improved insulin sensitivity (130). In addition, adipocyte necrosis observed during obesity might be another important factor that serves as a chemoattractant signal for macrophage recruitments (161). For example, in obese human and animal models, crown like structures formed by infiltrating macrophages has been observed around necrotic adipocytes (161). ATMs from obese humans and mice were reported to exhibit the M1 phenotype along

with high TNF-α and iNOS activity, while, ATMs from lean subjects were shown to polarize towards the M2 phenotype with high levels of IL-10 and arginase-1 production (138, 151). It is possible that with increasing obesity the anti-inflammatory M2 phenotype of macrophages switch to the pro-inflammatory M1 phenotype in adipose tissue (27). It is proposed that the pro-inflammatory cytokines such as TNF-α, IL-1β and TLR associated with M1 phenotype promote obesity induced insulin resistance by serine phosphorylation of IRS1 via the activation of IKK and JNK signaling pathways and downstream NFκB and AP-1 transcription factors, suggesting the pro-inflammatory role of M1 macrophages in obesity-induced inflammation and insulin resistance (19, 130, 137-140, 161).

1.3 Obesity and insulin resistance

Obesity is a major risk factor for insulin resistance (3, 5, 14, 15, 19, 24, 29, 68, 69, 127, 137, 145-149, 155). Insulin resistance is a state of reduced responsiveness of the metabolic tissues (muscle, liver, adipose tissue) to insulin action (135, 162-164). Insulin plays an important role in glucose homeostasis (135, 162, 165). In skeletal muscle, liver and adipose tissue, insulin increases glucose uptake in cells by activating the translocation of the intracellular glucose transporter, GLUT4 to the cell surface via the involvement of multiple signaling pathways (163-165) and thereby, helps in maintaining normal blood glucose levels. In addition, in adipose tissue insulin exerts an anti-lipolytic function by attenuating the release of FFA from adipocytes through the suppression of the activity of hormone-sensitive lipase and adipose triglyceride lipase (166). Furthermore, insulin decreases the release of hepatic glucose in the circulation through the inhibition of the expression of gluconeogenic enzymes and the glycogenolytic (breakdown of glycogen to glucose) process (164). Thus, any alteration in normal insulin action

and the corresponding insulin signaling pathways would in turn lead to subsequent development of insulin resistance in metabolic tissues.

1.3.1 Insulin signaling

Insulin initiates its metabolic action via its binding to and activation of the insulin receptor at the plasma membrane of metabolically active organs (162, 165). The insulin receptor is a member of the receptor tyrosine kinase subfamily that consists of insulin like growth factor-1 (IGF-1) receptor and the insulin receptor-related receptor (IRR) (167). The insulin receptor contains two α-subunits and two β-subunits that together form a heterotetrameric complex. Insulin binds to the extracellular α-subunits and thereby activates intracellular tyrosine kinase domain of β-subunits. This is accompanied by a series of trans-phosphorylation and conformational changes of the β-subunits that further leads to enhanced kinase activity (167). Insulin signaling involves complex and multiple signaling pathways downstream of the insulin receptor. The two important signaling pathways that participate in insulin signaling includes the phosphatidylinositol 3-kinase (PI3K) /protein kinase B (Akt or PKB) signaling pathway and MAPK (128). The PI3K/Akt pathway stimulate metabolic responses and governs insulin mediated glucose uptake and suppression of gluconeogenesis (128). The MAPK pathway activates growth factor-like responses and also governs cell growth and differentiation via interacting with the PI3K/Akt pathway (128, 168). The common connecting link between these two pathways is IRS proteins that consist of four different intracellular substrate members IRS 1-4. Out of the four members, IRS1 is the main protein that is susceptible to impaired insulin signaling (128). Insulin receptor activation via the binding of insulin at the cell surface promotes tyrosine phosphorylation of IRS1/2. Phosphorylated IRS1/2 proteins than activate PI3K that

converts phosphatidylinositol (PI) (4,5) bisphosphate (PIP2) to PI (3,4,5) triphosphate (PIP3)(162). PIP3 is a bioactive lipid that further activates 3-phosphoinositide dependent protein kinase-1/2 (PDK-1/2) which in turn leads to the phosphorylation and activation of downstream PI3k-dependent Akt and protein kinase B (PKB) signaling (162). PKB further activates the translocation of intracellular GLUT-4 to the plasma membrane to enhance glucose uptake in adipose, skeletal and myocardial tissue (128, 167, 168). The tyrosine phosphorylation of IRS-1 that involves the MAPK pathway activate downstream the extracellular signal-regulated kinases (ERK) signaling pathway via the activation of the GTP-binding protein RAS. The activation of these signaling pathways thereby, plays an important role in cell growth and remodeling, myocardial hypertrophy, cardiac fibrosis and dysfunctional endothelium (128, 167, 168). In contrast, the phosphorylation of IRS1 at the serine residue instead of the tyrosine residue attenuates insulin signaling via the suppression of insulin mediated tyrosine phosphorylation (128, 167, 168). Thus, this inhibitory action of serine phosphorylation acts as a negative feedback to insulin signaling and might be a mechanism that interconnects other signaling pathways that promote insulin resistance (128, 167, 168).

1.3.2 Inflammation, insulin resistance and obesity: A link

Both chronic low grade inflammation and insulin resistance are central to obesity related diseases such as type-2 diabetes, cardiovascular diseases, dyslipidemia, glucose intolerance and chronic kidney diseases (3, 5, 14, 15, 27, 29, 68, 69, 127, 145-149). How does obesity induce insulin resistance? The exact underlying mechanism for obesity-induced insulin resistance is poorly understood. However, obesity-induced insulin resistance is a complex and multifactorial process manifested by nutrient overload, ER stress, hypoxia, elevated levels of

cytokines/chemokines, increased ROS generation and chronic inflammation (164, 169). It is suggested that the stressful condition of obesity induces various stress-responsive molecules and their counter-regulatory signaling pathways such as JNK, ERK, and IKKβ that inhibit insulin signaling via the serine phosphorylation of IRS thus, suppressing insulin action (164, 169). In addition, abnormal secretion of pro-inflammatory cytokines (IL-6, IL1-β, TNF-α) and the chemokines (MCP-1, MIP-1α) from hypertrophied adipose tissue were reported to be the possible cause for the induction of systemic insulin resistance (156, 162, 164) **(Figure 1-5).** In particular, the pro-inflammatory cytokine TNF-α produced by activated macrophages is a major adipokine that links inflammation and insulin resistance (3-5, 58, 82-84, 86, 88, 164, 170). TNF-α acts in a paracrine manner and has a direct inhibitory effect on insulin-receptor signaling through the activation of JNK and IKK signaling pathways. In addition, activation of AP-1 and NFκB further exacerbates pro-inflammatory cytokine production (24, 99, 163, 171, 172).

Thus, elevated levels of pro-inflammatory mediators, in response to the inflammatory signal initiated by hypertrophied adipose tissue and infiltrated macrophages, leads to the abrogation of insulin sensitivity and acts as a link between obesity induced inflammation and insulin resistance **(Figure 1-5).**

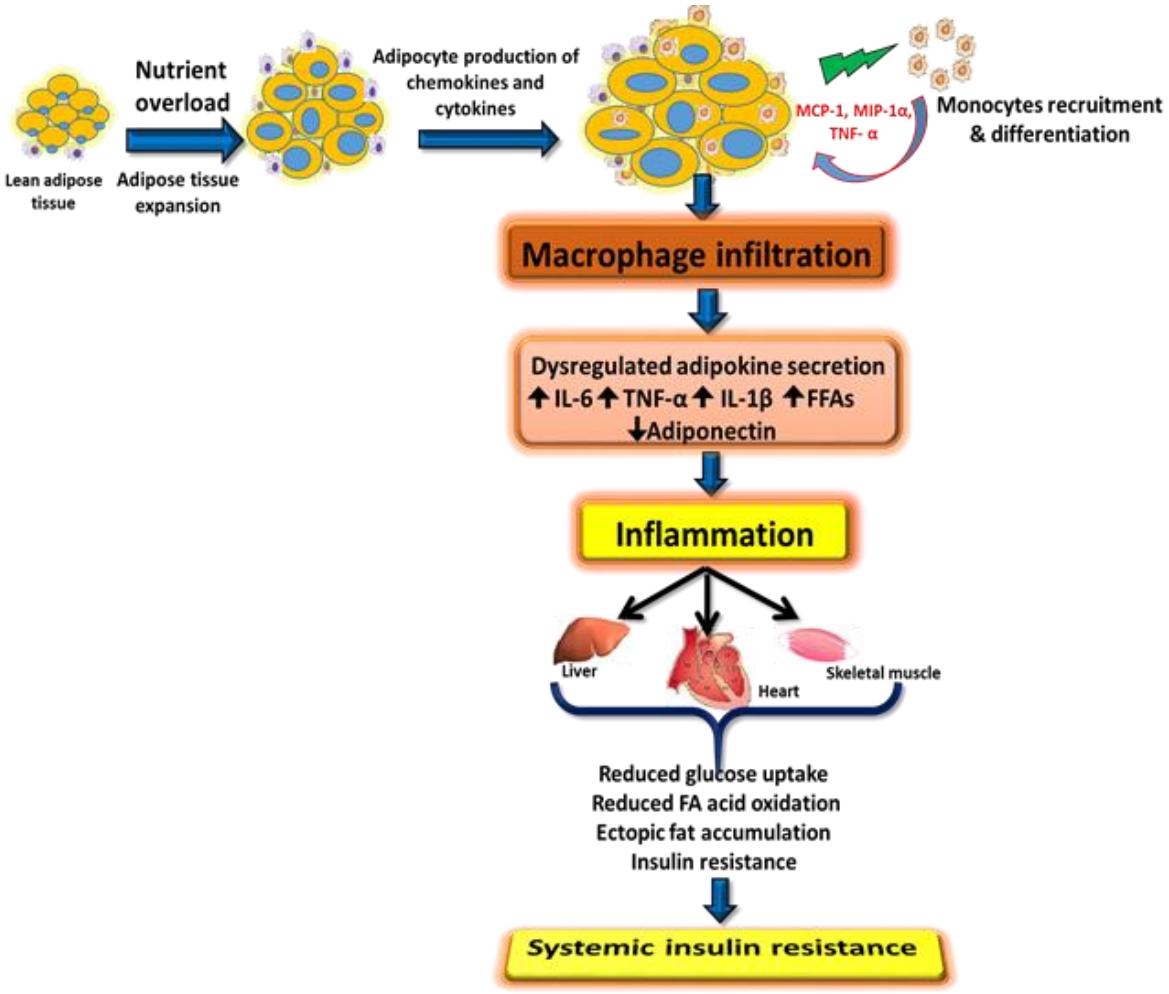

Figure 1-5 Obesity mediated alterations in adipokines secretion and the development of subsequent insulin resistance.

Abbreviations: Tumor necrosis factor (TNF-α); Interleukin (IL); Free fatty acids (FFAs).

1.3.3 Cardiac insulin resistance

Insulin resistance in the heart occurs independent of systemic insulin resistance (168), however, systemic insulin resistance participates in impaired cardiac insulin signaling (3, 5, 13-15, 24, 29, 68, 69, 127, 143-149, 168). The factors that contribute to dysfunctional insulin signaling in cardiac tissue include elevated levels of FFA, increased oxidative stress and aberrant production of pro-inflammatory cytokines/chemokines (128, 167, 168). Obesity leads to the

deposition of increased levels of FFA, diacylglycerol, ceramides and triglycerides in myocardial tissue that result in cardiac insulin resistance through the stimulation of kinases that promotes serine phosphorylation of the IRS1 protein. Moreover, it is proposed that increased oxidative stress activates ROS sensitive kinases, and angiotensin-II signaling that favors IRS1 phosphorylation at serine residues and contributes to insulin resistance (168, 173). Similarly, increased myocardial ROS production by mitochondria also contributes to impaired insulin signaling (168, 174). In addition, ER stress through the activation of MAPK/JNK has been shown to induce insulin resistance in cardiac tissue (168, 174). Furthermore, increased macrophage infiltration, elevated levels of IL-6, TNF-α, and decreased production of the insulin sensitizing hormone adiponectin, in response to pericardial adiposity contributes to the development of insulin resistance in cardiac tissue. Interestingly, TNF-α and IL-6 activates MAPK, PKC and SOCS3 that leads to the degradation of IRS1 protein (168, 175). The activation of the renin-angiotensin-aldosterone system (RAAS) might be another mechanism that contributes to insulin resistance in the heart during various pathological conditions of heart failure and myocardial infarction (168, 176). Besides angiotensin-II, increased secretion of IL-6 and TNF-α from expanded adipose tissue also stimulate aldosterone secretion from adrenal glands. Aldosterone and angiotensin-II in turn elevate ROS generation in cardiac vascular smooth muscle cells via the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzymes. As a consequence, ROS sensitive protein kinases are activated that phosphorylates serine residues in IRS1 and leads to the development of insulin resistance in the heart during various pathological conditions (168, 177).

1.4 Obesity and oxidative stress

1.4.1 Role of reactive oxygen species (ROS) in obesity

Oxidative stress is a condition of an imbalance between the levels of ROS and antioxidant defense systems (178, 179). ROS like the superoxide anion, hydrogen peroxide and hydroxyl radical are highly reactive molecules which are produced during normal biological processes or in response to different stress conditions (178, 179). Moreover, interaction between ROS and nitric oxide generate peroxynitrite that induce nitrosative stress and suppress nitric oxide bioavailability and thereby contribute to endothelial dysfunction (178-180). Increased oxidative stress has been implicated to play an important role in the pathogenesis of obesity, insulin resistance, diabetes and cardiovascular diseases (3, 5, 124, 178, 181-215). It is well documented that obesity is associated with increased ROS generation and reduced production of antioxidants (20, 178, 214, 216). It has been suggested that during obesity, adipose tissue is the main site for free radicals generation and contribute towards obesity related cardiometabolic complications (3, 5, 124, 178, 181-215). The exact mechanism responsible for dysfunctional adipose tissue and oxidative damage is not clear. However, increased fatty acid oxidation, impaired mitochondrial metabolism and decreased production of antioxidant enzymes might be possible mechanisms that lead to elevated ROS production during obesity (179, 215). It is suggested that increased accumulation of lipids in adipose tissue leads to adipocyte hypertrophy and hypoxia that contribute to oxidative stress (178, 215). Increased levels of FFA facilitate ROS generation via the stimulation of NADPH oxidase and attenuation of antioxidant enzymes (178, 215, 217). Elevated ROS in adipose tissue in turn contribute to enhanced inflammatory processes via the induction of redox sensitive NFκB (179, 215, 217), increased production of pro-inflammatory cytokines (215, 218) and attenuation of insulin sensitizing anti-inflammatory

adiponectin (219-223). Moreover, macrophage that play key roles in the chronic inflammatory processes also participate in ROS generation (135, 139, 154). In addition, local RAAS in adipocytes might be another possible mechanism that contributes to obesity mediated oxidative stress (168, 177, 215). Thus, oxidative stress in adipose tissue participates in the systemic inflammation process which in turn causes endothelial dysfunction and subsequent development of insulin resistance and cardiometabolic complications. The proposed mechanism by which elevated oxidative stress leads to obesity mediated insulin resistance involves the stimulation of JNK and NFκB signaling pathways that phosphorylates the insulin receptor and IRS proteins at serine residues (69, 99, 141, 178). Increased serine phosphorylation of IRS deactivates IRS-1 and impairs insulin signaling. This in turn, suppresses glucose uptake by GLUT-4 via the inactivation of IRS-1 and PI3k/Akt and eventually leads to the development of insulin resistance (168, 177, 178). Taken together, increased oxidative stress and systemic inflammation serves as a connecting link between obesity, insulin resistance and cardiovascular diseases.

1.4.2 8-isoprostane

Isoprostanes are a biologically active family of prostaglandin (PG)-like compounds that play important pathophysiological roles in the pathogenesis of several disorders associated with elevated oxidative stress (224-228). Importantly, isoprostanes are potent vasoconstrictors formed by a non–enzymatic process of peroxidation of phospholipid-bound arachidonic acid in response to increased superoxide radicals (224, 225). This is followed by a phospholipase mediated cleavage process and finally release to the circulation and excretion in the urine (224, 225). Since, isoprostanes are the most stable products of lipid peroxidation compared to other products, they are easily detectable in urine and plasma samples (224, 225). Therefore,

isoprostanes are now considered as authentic markers of oxidative stress under various pathological conditions (224-228). Among isoprostanes, 8-isoprostane is the stable downstream product of lipid peroxidation released during oxidative stress (225, 226). The levels of 8-isoprostanes are generally measured in plasma and urine samples (226). 8-isoprostane is a potent vasoconstrictor and a modulator of platelet activation. The basal level of 8-isoprostanes was found to be elevated in the pericardial fluid of patients with severe heart failure (227). Similarly, elevated levels of 8-isoprostane were found during various pathological conditions including obesity (228) reno-vascular hypertension (229) and diabetes (225). Thus, therapeutic tools that can reduce the levels of 8-isoprostane will be indicative of a strong antioxidant capacity and suppressors of oxidative stress. Thus, the measurement of 8-isoprostane in biological fluids might serve as a non-invasive marker for lipid peroxidation and will be helpful in determining the efficacy of therapeutic molecules employed to counteract the pathological condition of oxidative stress.

1.4.3 Endothelin-1

Endothelin (ET) is a vasoconstrictor peptide that plays a role in blood pressure and fluid homeostasis (230-232). Clinical studies demonstrated the pathophysiological role of the endothelin system in various disease conditions such as hypertension, diabetes, atherosclerosis, coronary artery disease, heart and renal failure (26, 230-235). The ET family comprises three structurally similar 21 amino acid peptides namely ET-1, ET-2 and ET-3. ET-1 is the most abundant and potent peptide of the ET family with respect to its biological effect (26, 230-235). ET-1 is produced by endothelial cells of the vascular system (232). In general, ET-1 functions in an autocrine or paracrine manner and the biological action of ET-1 is mediated by two distinct

G-coupled receptors ETA and ETB (179, 230). ET-1 via the stimulation of ETA receptors exhibits its vasoconstrictor, proliferative and hypertrophic effects (232, 235). It is reported that stimulation of the ETA receptor, predominant in vascular smooth muscle of arteries contributes to vasoconstriction via the increase in intracellular calcium levels (179, 231, 232), while, activation of the ETB receptor in the veins, pulmonary vessels and endothelial cells leads to vasodilation through the synthesis of NO and prostacyclin (179, 232). Various stimuli such as angiotensin-II, epinephrine, thrombin, insulin, vasopressin, cytokines and hypoxia acts as a trigger for ET-1 production (231-233). ET-1 is known to modulate other vasoactive molecules that play key roles in various biological functions (231-233). For example, ET-1 through its ETA receptor regulates angiotensin converting enzyme activity during obesity (231-233). ET-1 potentiates the action of noradrenaline and serotonin and thereby, contributes to increased vasoconstriction in hypertensive subjects (232, 236). High levels of ET-1 is associated with a parallel increase in ROS generation. In the DOCA-salt hypertensive rat model, increased generation of ET-1 leads to a high production of superoxide in vascular tissue that ultimately leads to endothelial dysfunction and reduced NO bioavailability (232, 234, 235). Likewise, in vascular smooth muscle of rat aorta as well as in humans, high secretion and expression of ET-1 was found to be associated with increased intracellular ROS production (232, 234). It is suggested that ET-1 potentiates ROS generation through the activation of signaling pathways including p38MAPK, JNK and ERK (232, 234, 237, 238). Overproduction of ROS in turn leads to an increased inflammatory response via the activation of the NFκB signaling pathway (69, 99, 179, 239, 240). Both increased ROS and inflammation acts as an underlying mechanism for the pathogenesis of cardiometabolic disorders and serves as a link between obesity and cardiovascular diseases (3, 5, 14, 15, 26, 27, 29, 68, 69, 127, 145-149, 161, 241).

ET-1 plays a major role in the pathogenesis of obesity and associated complications (26, 230-235). A positive correlation exists between elevated ET-1 plasma levels and obesity (26, 179, 232). For example, high levels of ET-1 in the circulation have been detected in normal obese and obese individuals with hypertension (179, 232). Moreover, in experiments done with mice fed on a high fat diet, increased expression of ET-1 at both molecular and cellular levels has been observed (179). One of the possible explanations for the obesity mediated increase in ET-1 levels is the interplay between ET-1 and the RAAS system (179, 232, 237). During obesity, increased production of angiotensin-II (a potent pro-inflammatory molecule), in response to the elevated RAAS system, stimulates high expression and production of ET-1 in the vascular system that leads to increased vasoconstriction (179, 232, 237). Moreover, increased production of ET-1 in response to leptin might be another mechanism for high ET-1 generation during obesity (242). The elevated ET-1 mediated vasoconstriction during obesity leads to the inhibition of endothelium dependent vasodilation and contributes to the pathological condition of hypertension, atherosclerosis, heart failure and related disorders (26, 179, 232).

High levels of ET-1 have been implicated in cardiac hypertrophy, fibrosis and vascular remodeling (231, 232, 235, 238). The ETA receptor plays a central role in the modulation of vascular activity (231). During the pathological conditions of heart failure, coronary artery disease, hypertension and endothelial dysfunction, high expression of ETA receptor has been observed (26, 230-235). Studies done with the hypertensive DOCA–salt animal model (235) and aldosterone-infused rats support the involvement of the ETA receptor in the pathogenesis of cardiac injury and remodeling. Blocking of the ETA receptor reduced oxidative stress, attenuated hypertrophic remodeling and fibrosis (231, 232, 235, 238). It is proposed that the mechanism for the ETA mediated inflammatory response in cardiac tissue involves NFκB activation,

stimulation of cell adhesion molecules and growth factors (231, 232, 235). NFκB is sensitive to increased oxidative stress and is stimulated in response to the NADPH oxidase-derived ROS (69, 99, 179, 239, 240). It is reported that ET-1 promotes cardiac hypertrophy through the stimulation of NADPH oxidase enzyme that produces ROS and activates the MAPK signaling pathway (26, 234, 238). The vasoconstrictor effect of ET-1 in cardiac tissue can be extended to renal tissue as well. ET-1 can modulate both vascular and tubular function in renal tissue and play a role in sodium homeostasis (230). Increased glomerular expression of ET-1 and prolonged stimulation of the ETA receptor may contribute to the progression of chronic kidney disease (230, 243). ET-1 via its ETA receptor has been reported to alter renal function by modulating the glomerular filtration rate and increasing albuminuria (230). Moreover, stimulation of the ETA receptor promotes phenotypic changes on the podocytes and contributes to hyperplasic lesion formation (230, 243). However, neutralization of ETA receptors by the ETA antagonist not only protected against diabetes induced renal injury, but also prevented fibrosis and atrophy of the kidney and improved renal function (230, 243). Collectively, ET-1 has inflammatory, hypertrophic and fibrotic effects in cardiac and renal tissue. Thus, blocking of ET-1 and its receptors would have therapeutic effects against obesity and cardiovascular complications.

1.5 Obesity and cardiomyopathy (See Appendix A for more details)

Visceral adiposity is an independent risk factor for progression and development of cardiovascular diseases (1, 3-5, 10, 14, 15, 23, 62, 71, 75, 123, 127, 150, 244-254). Obesity itself is associated with multiple pathological conditions which themselves serve as important risk factors for various metabolic disorders including insulin resistance, type-2 diabetes, dyslipidemia, cardiovascular and renal diseases (3, 5, 14, 15, 24, 27, 29, 58, 68, 69, 127, 149,

255-257). The dysfunctional adipose tissue is a key feature that links obesity and cardiometabolic complications (1-5, 15, 18, 72, 75, 124-127). Initially, under the normal physiological state, deposition of excessive fat takes place in non-ectopic tissue such as subcutaneous adipose tissue (1, 6, 72, 258). However, during conditions of obesity, once the maximal limit of subcutaneous adipose tissue is achieved, the later deposition of excess fat occurs in ectopic tissue sites including heart, liver, kidney and the vasculature (1, 6, 72, 75, 79, 258). Thus, this ectopic fat (perivascular, epicardial and myocardial fat) exerts adverse effects on cardiac tissue and vasculature in both an endocrine and paracrine manner (1, 6, 7, 72, 253, 258). In general, pericardial fat depots were found along the large coronary arteries and on the surface of cardiac tissue. Furthermore, the ectopic fat deposition in the heart is the spread of epicardial fat into the atria and ventricles (1, 6, 72, 253, 258). This in turn causes structural and hemodynamic alterations of cardiac tissue as well as endothelial dysfunction, eventually leading to the development of obesity induced cardiomyopathy (1, 6, 7, 72, 75, 79, 150, 253, 258).

1.5.1 Mechanisms responsible for obesity associated cardiomyopathy

How does obesity induce cardiomyopathy? The exact mechanism responsible for obesity associated cardiovascular disorders is not completely understood. However, different mechanisms such as increased FFA levels, increased hemodynamic preload and afterload, insulin resistance, cardiac hypertrophy and fibrosis that correlate obesity and cardiovascular disease have been proposed (1, 6, 7, 72, 150, 253, 258, 259). It is proposed that excessive hypertrophy of adipocytes in response to chronic nutrient overload alters normal metabolic functions of adipose tissue and generates various stress responsive molecules and signals to neighboring cells. This in turn produce adipocytes apoptosis, local hypoxia, as well as ER stress

and leads to the abnormal secretion of adipokines and increased macrophage infiltration to the inflamed site (1, 3, 5, 15, 67, 76). As a consequence chronic low grade inflammation, increased oxidative stress and adipose tissue fibrosis alters insulin signaling and eventually leads to the development of insulin resistance (72, 150, 175, 260, 261). Thus, defective insulin signaling and subsequent insulin resistance is an important mechanism that links visceral obesity to cardiomyopathy (3, 5, 14, 15, 24, 29, 68, 69, 127, 145-150). Insulin plays a regulatory role in substrate metabolism in the heart (259, 262). Excess accumulation of lipids in cardiac tissue elicits adverse endocrine, paracrine and immune responses (259, 262). This results in increased production of inflammatory/ROS mediators, increased fatty acid oxidation, altered response to insulin and decreased glucose uptake that altogether contribute to an impaired IRS1/PI3K/Akt insulin signaling and subsequent development of insulin resistance in cardiomyocytes (259, 263, 264). As a consequence, the development of left-ventricular hypertrophy, cardiac tissue remodeling, diastolic and systolic dysfunction eventually contribute to obesity induced cardiomyopathy and predispose to heart failure (129, 248, 265) **(Figure 1-6).** Moreover, obesity is also a contributing factor for hyperinsulinemia and hyperglycemia that leads to altered cardiac structure and function. It is proposed that hyperglycemia participates in cardiac injury via the enhanced production of ROS that leads to apoptosis of myocardial cells (129, 248, 265, 266). In addition, hyperglycemia-mediated generation of advanced glycation end-products and excessive remodeling of ECM proteins contribute to compromised cardiac function (129, 248, 265, 266).

Another possible mechanism that contributes to obesity related cardiomyopathy is an altered RAAS system in response to dysfunctional adipose tissue metabolism (1, 13, 66, 91, 129, 132, 248, 265, 267-277). It is reported that adipose tissue has been implicated in the production of components of the RAAS system including angiotensinogen, angiotensin-converting-enzyme

(ACE) and AT-1 (1, 254, 271, 276-279). Angiotensinogen is an important component of the RAAS system that is catalytically cleaved by renin to AT-I and subsequently to AT-II by the action of ACE. AT-11 acts as a growth factor for myocardial cells and contributes to different cellular process including hypertrophy, apoptosis, cell proliferation, fibrosis and cardiac dysfunction (129, 248, 265). In addition, during obesity abnormal activation of the RAAS system accompanied by upregulated renin activity and elevated aldosterone levels contribute to increase volume overload, vasoconstriction and insulin resistance and the subsequent development of hypertension, myocardial and renal dysfunction (1, 66, 254, 271, 276-280).

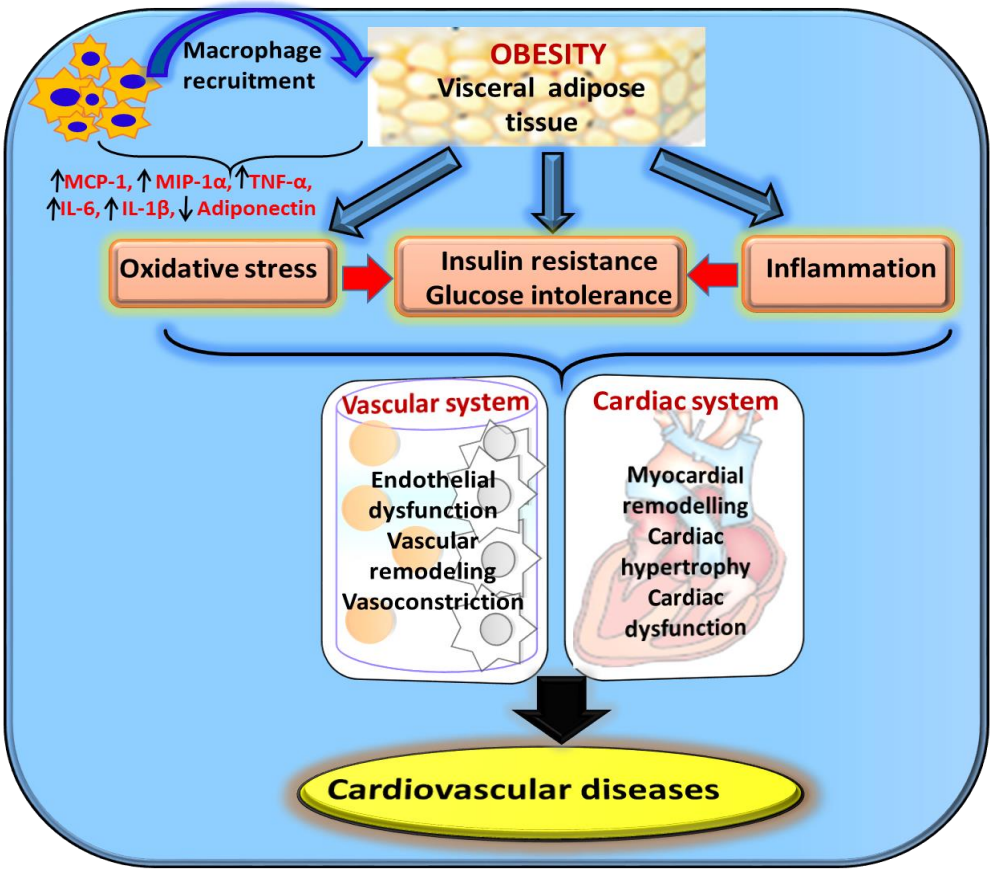

Figure 1-6: Schematic representation of the link between increased visceral adiposity, inflammation, insulin resistance, oxidative stress and their effects on cardiovascular diseases.

Abbreviations: Tumor necrosis factor (TNF-α); Interleukin (IL); Macrophage chemoattractant protein-1 (MCP-1); Macrophage inflammatory protein -1α (MIP-1α).

1.5.2 Altered cardiac structure and function in obesity (See appendix D 3 for more details)

Obesity has various pathophysiological consequences affecting the cardiovascular system (1, 14, 23, 71, 129, 244-252, 265). Deposition of excess fat in ectopic and non-ectopic tissue adversely effects metabolic homeostasis and induces various structural adaptations and alterations in cardiac structure and function which may altogether contribute to cardiovascular diseases (1, 6, 72, 253, 258, 263).

A recent study demonstrated that excessive fat accumulation in the body induces cardiac structural and functional changes as a beneficial adaptive response in order to maintain body homeostasis (60). This adaptive response during the initial phase of obesity is characterized by increased cardiac output and stroke volume in response to elevated plasma volume along with reduced peripheral resistance (60). However, with increasing duration and stage of obesity this beneficial adaptive response switches to a harmful maladaptive pathological response of obesity and associated cardiometabolic complications (1, 14, 23, 71, 244-252). With increasing duration of obesity, elevated plasma and blood volume shifts Frank-Starling curves to the left due to increased cardiac load (60, 129, 254). As a consequence, cardiac overload induces left-ventricular remodeling characterized by increased left-ventricular wall thickness and left-ventricular cavity enlargement. This ultimately leads to the development of left-ventricular hypertrophy which is an important predictor of heart failure (60, 129, 254, 281).

Left-ventricular hypertrophy, myocardial fibrosis as well as increased cross linking of collagen are risk factors that contribute to altered diastolic and systolic function (60, 129, 248,

254, 265, 266, 281). The altered cardiac function in turn elevates myocardial oxygen demand (281-283) accompanied by increased apoptosis and necrosis of myocardial cells as well as accumulation of advanced glycation end-products in the myocardium. These pathological changes result in elevated myocardial inflammation and endothelial dysfunction that eventually predisposes to heart failure (60, 129, 254, 281, 283). The exact mechanism responsible for myocardial dysfunction needs further explanations. However, both volume and pressure load dependent alterations were suggested as the underlying mechanism for obesity induced cardiac dysfunction (10, 284). In addition, it was demonstrated that during the condition of excess accumulation of FFA in the cardiac tissue, there is a parallel increase in β-oxidation of FFA (254). When the deposition of FFA exceeds the capacity of the myocardium to further oxidize FFA, lipotoxicity occurs leading to cardiac dysfunction (10, 285), indicating that impaired myocardial metabolism plays an important role in obesity induced cardiac dysfunction (60, 129, 254, 281, 283).

1.5.3 Myocardial remodeling and fibrosis

Myocardial remodeling and fibrosis is an important pathophysiological process that leads to structural and functional changes in the heart in response to increased pressure and volume load and cardiac injury (129, 248, 265, 281, 283, 286). This process has been implicated in the development of left-ventricular hypertrophy and is associated with a chronic inflammatory process (129, 287-290). The heart is composed of various kinds of cells including endothelial cells, vascular smooth muscle cells, mast cells and cardiomyocytes surrounded by extracellular matrix (ECM) (6, 286, 287). Cardiomyocytes nearly comprise 70-80% of the heart mass and is composed of bundles of myofibrils that contain sarcomeres as repeating micro-anatomical units

(6, 286, 287). Under normal conditions, cardiomyocytes are surrounded by a network of collagen fibers that provide mechanical support. The ECM also contributes to maintain the elliptical shape of the heart and thickness and thereby, provides tensile strength to myocytes and blood vessels (291, 292). During the physiological condition of increased hemodynamic load, cardiomyocytes exhibit a homogenous hypertrophic response and undergo expansion by the synthesis of new contractile proteins (286, 287, 293). However, during pathological conditions of chronic overload, the fine network of collagen fibers is disrupted by the inhomogeneous abnormal deposition of interstitial collagen and overexpression of ECM proteins such as TGF-β and fibronectin leading to mechanical stiffness of cardiac tissue with subsequent diastolic dysfunction that can progress to impaired systolic function (286, 292).

Various molecular, mechanical and biochemical stimuli including mechanical stress, neurohormonal activation, endothelin, inflammation and increased ROS generation has been implicated in the excessive synthesis of collagen from fibroblasts and vascular smooth muscle cells that leads to the remodeling and fibrosis of cardiac tissue (1, 66, 129, 254, 271, 276-280, 286, 292). This in turn cause either apoptosis or necrosis of cardiomyocytes that induces the release of growth factors in the connective tissue to form new fibroblasts and ECM proteins. The abnormal increase in fibrosis, ECM synthesis and apoptosis of cardiomyocytes contribute to decreased contractile force, myocardial stiffness and compromised cardiac function (129, 248, 265, 281, 283, 286, 292). In addition, chronic activation of the RAAS system via aldosterone and the sympathetic nervous system also contribute to cardiac tissue fibrosis and the development of left-ventricular hypertrophy during obesity (1, 66, 129, 254, 271, 276-280, 292). Thus, increased myocardial stiffness and reduced contractility in response to pathological cardiac remodelling serves as an important predictor of heart failure (286).

1.5.4 Markers of heart failure

1.5.4.1 Osteopontin

Osteopontin (OPN) is a glycosylated phosphoprotein first isolated in 1986 from mineralized bone matrix (294). As the name implies, OPN is the main component of bone, however, it is produced by several cell types and tissues including osteocytes, chondrocytes, fibroblasts, macrophages, arterial smooth muscle cells, skeletal muscle, endothelial cells, brain and kidney (295-297). OPN exists as both an extracellular and intracellular protein and can be found as soluble protein in body fluids such as plasma, urine and milk (295-297). OPN has been implicated to play a pathophysiological role in various biological processes such as inflammation, fibrosis, bone mineralization, tissue modeling and obesity (294, 295, 297-308).

1.5.4.1.1 Osteopontin and inflammation

Mounting evidence suggests that OPN has been implicated in the pathogenesis of diseases related to chronic inflammation including obesity, diabetes, insulin resistance and cardiovascular disease (294, 295, 297-308). OPN is expressed by inflammatory macrophages and is highly activated in response to various inflammatory molecules such as LPS,TNF-α, IFN-γ, TGF-β, angiotensin-II, nitic oxide, IL-1β (295, 297, 299). The exact regulatory mechanism that governs OPN secretion from inflammatory macrophages is still not clear. It is proposed that stimulation of PI3K, ERK and JNK signaling cascade activate OPN expression in LPS induced macrophages (295, 299, 300, 303, 306). OPN plays a role in the macrophage infiltration process to the site of inflammation (295, 299, 300, 303, 306). Studies conducted using genetic approaches or through the use of neutralizing antibodies has shown that blocking of OPN

attenuates the recruitment of monocytes, macrophages and leukocytes to the inflamed tissue (295, 300). Moreover, the OPN promoter possesses the binding sites for pro-inflammatory transcriptional factors including AP-1 and NFκB and hence can modulate the expression of various inflammatory proteins (295, 301). OPN also plays an important role in tissue remodeling by stimulating the expression of matrix metalloproteinase (MMP) such as MMP-2 and MMP-3 that degrades matrix and facilitates cell migration (295, 302). Interestingly, OPN favors inflammatory cytokine IL-12 induction and attenuates anti-inflammatory IL-10 cytokine production by macrophages during inflammation (295, 300). Taken together, OPN plays important roles in the expression of different cytokines and transcriptional factors and is a key player in the inflammatory process.

1.5.4.1.2 Osteopontin and obesity

OPN plays a key role in the pathogenesis of adipose tissue inflammation and insulin resistance (295, 300, 303-305). OPN expression is strongly elevated in adipose tissue of genetically obese mice or in the high fat diet induced obese mouse model (295, 298, 303). Similarly, in obese individuals as well as in obese diabetic and insulin resistant patients, the OPN levels in circulation and its expression in adipose tissue is highly upregulated (295, 298, 303). In contrast, blunting of the OPN expression by antibodies and deficiency of the OPN gene in mice improved insulin sensitivity, glucose tolerance and suppressed inflammation via the reduction of adipose tissue mediated IL-6, TNF-α, MCP-1 and iNOS expression (295, 303, 304). The exact mechanism for upregulation of OPN expression in inflammatory adipose tissue is not clear. It is proposed that the primary source for high OPN expression under obese conditions are adipose tissue macrophages in response to high glucose, TLR4 activation, IL-6 and IL-18 factors (295,

303). A recent study demonstrated that the glucose dependent insulinotropic peptide (GIP) an incretin hormone that plays an important role in the process of adipogenesis also induce OPN expression in adipocytes (295, 305). OPN in turn, activates several inflammatory signaling cascades such as Akt, p38 MAPK, and ERK, increases the expression of TNF-α and MCP-1 in macrophages that leads to the subsequent development of adipose tissue mediated insulin resistance and diabetes (295, 306). Thus, OPN is implicated as an important component in the development of obesity associated inflammation and insulin resistance.

1.5.4.1.3 Osteopontin and myocardial disorder

Under physiological conditions, the expression of OPN in the myocytes, fibroblasts and endothelial cells of cardiac tissue is low at a basal level. However, pathophysiological conditions such as hypertrophy and factors such as endothelin-1, angiotensin-II, IFN-γ and cytokine like IL-1β triggers a strong expression of OPN in cardiac tissue (294, 298, 307). Angiotensin-II is regarded as a potent inducer of OPN in the vasculature during the atherosclerotic process (297). It is reported that in myocytes, high expression of OPN is stimulated in response to increased myocyte apoptosis and impaired myocardial function (294, 298, 307). In contrast, in various models of cardiac hypertrophy and heart failure, deficiency of the OPN gene is related to decreased myocardial fibrosis and myocyte apoptosis (294, 298, 307). In patients with heart failure, high levels of OPN in plasma is found compared to their normal counterparts (308). The elevated OPN expression in cardiac tissue and in the circulation can be related to myocyte hypertrophy, myocardial dysfunction and the severity of heart failure (294, 308). Recently, it was demonstrated that increased OPN activity in heart failure patients was associated with a parallel increase in the activity of lysyl oxidase (an enzyme involved in extra-cellular protein

remodeling) and collagen deposition (308). Furthermore, increased plasma levels of OPN during pulmonary hypertension was associated with altered right ventricle function and tissue remodeling (294, 298, 307). These findings suggest that elevated expression of OPN in circulation and in cardiac tissue is a predictor of impaired cardiac function and remodeling and subsequent heart failure.

1.5.4.2 Osteoprotegerin

Osteoprotegerin (OPG) is a secreted glycoprotein of the tumour necrosis factor receptor (TNFR) super family (309, 310). It plays an important regulatory role in bone turnover and inhibits osteoclast differentiation. OPG exerts its effect via binding to two ligands, RANKL (receptor activator of NFκB ligand) and TRAIL (TNF related apoptosis inducing ligand). RANKL is a cytokine produced by osteoblasts and bone marrow stromal cells that with the help of its receptor RANK induces osteoclast differentiation (311, 312). OPG blocks RANKL-RANK ligation, thereby inhibiting osteoclastogenesis (310). OPG is reported to be constitutively expressed by smooth muscle and endothelial cells in the vasculature (309, 310). In addition, various growth factors and pro-inflammatory cytokines such as TNF-α, IL-1β, platelet derived growth factor (PDGF) and angiotensin-II can stimulate the OPG expression during inflammatory processes in both vascular smooth muscle cells and endothelial cells (309, 310). Activation of endothelial cells by TNF-α and IL-1β, might be one of the mechanisms for high plasma levels of OPG in patients with cardiovascular complications and is a contributing factor for endothelial cell dysfunction (309, 311). OPG induces migration and adhesion of immune cells (leukocyte, macrophages, lymphocytes) (310), a primary step of endothelial dysfunction through the activation of various endothelial adhesion molecules including ICAM-1 (intercellular adhesion

molecule-1), VCAM-1 (vascular cell adhesion molecule-1), and E-selectin as well as stimulation of metalloproteinase activity in the vasculature (309). OPG promotes remodeling of ECM by potentiating the induction of expression of profibrotic proteins such as TGF-β, fibronectin and collagen type I, III and IV (309, 310). A recent study proposed that a vicious cycle prevails between TGF-β and OPG, as TGF-β mediated activation of smooth muscle cells results in the high production of OPG in response to PDGF and angiotensin II (310).

The role of bone regulatory protein OPG in cardiovascular physiology is a topic of much interest. OPG is known to play key pathophysiological roles in cardiovascular disease and exhibit inflammatory and proliferative effects (309-312). OPG is a critical regulator of arterial calcification during the atherosclerotic process that leads to the development of cardiovascular disease (309-312). Infact, the RANKL/RANK/OPG system participates in the inflammation related atherosclerotic process (309). Despite these findings, the actual mechanism that interrelates inflammation, OPG and cardiovascular events still needs to be clarified. However, OPG can serve as an important biological marker for cardiovascular diseases and heart failure.

1.6 Atrial natriuretic peptide (ANP) (See Appendix D 4 for more details)

ANP is a peptide hormone mainly secreted by the heart atria (313-316). It belongs to a structurally related hormone family composed of brain natriuretic peptide (BNP) and the C-type natriuretic peptide (CNP) (313-316). Under physiological and pathophysiological conditions, ANP can function as an autocrine and paracrine factor in different organs including, heart, kidney, lung, thymus and liver (315-317). ANP plays an important role in the immune response and contributes to innate immunity (315-324). ANP plays important roles in blood pressure homeostasis and exhibits anti-inflammatory, natriuretic and diuretic effects (315-324).

Moreover, ANP attenuates cardiac hypertrophy (314, 316, 324), promotes peripheral vasodilation and has a modulatory role in cardiac and vascular remodeling (315, 317). It suppresses total peripheral resistance via its inhibitory effect on the release of rennin, vasopressin and aldosterone (315). ANP also possess anti-proliferative properties (315, 317). It is suggested that in vascular and cardiac cells, ANP promotes anti-proliferative effects via the reduction of intracellular adenosine 3', 5'-cyclic monophosphate (cAMP) levels and inhibition of MAPK and protein kinase G signaling pathways (315, 317).

1.6.1 Obesity and ANP

Mounting evidence confers that an inverse relationship exists between obesity and natriuretic peptide levels in the circulation (319, 320, 325). Interestingly, increased ANP levels due to genetic polymorphism in the ANP promoter region was found to be associated with reduced blood pressure, BMI and lower risk for obesity and metabolic syndrome (319-321, 326, 327). Moreover, increased levels of natriuretic peptide are accompanied by reduced VAT and ectopic fat deposition in metabolic organs (319). In obese individuals, the circulating levels of natriuretic peptides is highly reduced compared to lean subjects that can be restored by physical exercise done for long duration (319). In addition, in obese individuals an altered secretion of myocardial natriuretic peptide has been observed (321). One of the possible reasons for decreased natriuretic peptide levels might be the degradation of natriuretic peptide by neutral endopeptidase neprilysin and NPRC receptor in obese individuals (319). It is demonstrated that in obese individual with hypertension, higher expression of NPRC is observed in adipose tissue compared to their normal counterparts (319). In human adipocytes and monocytes, NPRC expression was increased in response to hyperinsulinemia (319).

ANP plays a major role in lipid metabolism. ANP acts as a stimulator for lipolysis in adipose tissue (319, 321, 328). ANP-induced lipolysis in turn promotes FFA release which serves as a substrate for metabolic tissues and results in increased lipid oxidation via the β-oxidation pathway in adipose tissue (319). It is observed that short term administration of intravenous ANP for a enhanced lipid oxidation in normal individuals (328). More investigation to delineate the initial steps of the mechanism responsible for ANP-induced enhanced lipid oxidation and mitochondrial biogenesis may help to design novel targets for the treatment of obesity related metabolic diseases.

1.7 Adiponectin (See Appendix D 5 for more details)

1.7.1 Adiponectin and obesity

Adiponectin is an anti-inflammatory adipokine that plays a major role in the regulation of tissue inflammation, lipid metabolism and cardiovascular homeostasis (219, 220, 222, 329-339). It serves as a key molecule that links obesity to insulin resistance and cardiovascular disease (220, 329, 334, 336, 337). Compared to other adipokines, adiponectin exhibits diverse biological functions owing to its anti-diabetic, antioxidant, anti-atherogenic and insulin sensitizing effects (219, 220, 222, 329-338). Adiponectin is vasoprotective and demonstrated to be beneficial against pathological conditions of obesity, inflammation, insulin resistance, type-2 diabetes and atherosclerosis (219, 220, 222, 329-338). Studies conducted on a knockout mice model revealed that compared to wild type, adiponectin-knockout (Adipo-KO) mice fed a high fat diet exhibited dysfunctional insulin signaling, high levels of TNF-α in both plasma and adipose tissue and were highly insulin resistant (219, 332). However, administration of adiponectin via adenovirus transfer improved insulin sensitivity in these Adipo-KO mice (219). Adiponectin is mainly

secreted from adipocytes; however, it is produced by cardiomyocytes as well (329, 340). It is interesting to know that although, adipose tissue is the main site for adiponectin production, reduced levels of adiponectin is associated with obesity (219-223). The exact mechanism responsible for this is not clear. It is suggested that obesity-induced hyperinsulinemia and overproduction of pro-inflammatory cytokines such as TNF-α and IL-6 exert reciprocal effects on adiponectin expression that results in reduced systemic levels of adiponectin during obesity (222). Moreover, ER stress-induced impaired processing of adiponectin protein might be another reason for reduced adiponectin levels during obesity (222, 335). In addition, the size of adipocytes and insulin sensitivity are the driving force for adiponectin production. For example, adipocytes that are large and are insulin resistant secrete less adiponectin (222, 335).

In general, the physiological levels of circulating adiponectin is found to be nearly, 0.5-30 μg/ml, which is about 0.01% of total plasma proteins (333). An inverse relationship has been observed between circulating levels of adiponectin and various pathological conditions of obesity and cardiovascular disease (219, 221, 222, 331, 332, 334). It is reported that low plasma levels of adiponectin are found in obese humans and in obese animal models with insulin resistance (219-221). Moreover, reduced circulating levels of adiponectin were reported in patients with type-2 diabetes, coronary artery disease, hypertension, dyslipidemia and during excessive oxidative stress conditions (219-221). In contrast, during chronic heart or renal failure, elevated levels of adiponectin were reported (219). However, the underlying mechanism responsible for high levels of adiponectin during these disease conditions is still not clear.

Taken together, these studies support that adiponectin plays a pivotal regulatory role in cardiometabolic functions and exerts protection against obesity and related cardiovascular diseases.

1.8 Heme oxygenase (HO) system (Refer Appendix B and Appendix C for more details)

Section 1.8 (Shuchita et al., *Curr Pharm Des. 2014; 20(9):1354-69*) has been reformatted from the original version of the manuscript, co-authored with my supervisor Joseph Fomusi Ndisang for inclusion in the thesis. In this review article, as primary author, I was responsible for the first draft of the manuscript and revised the manuscript as suggested by the supervisor.

1.8.1 Overview of HO system

HO is an evolutionarily conserved stress-responsive protein that protects from several immune-mediated inflammatory diseases and restores cellular homeostasis upon exposure to various stimuli. HO is the rate-limiting enzyme that catalyzes the degradation of the heme molecule generating an equimolar ratio of carbon monoxide (CO), biliverdin (IXa) isomer and free iron. Biliverdin is subsequently converted to bilirubin, another cytoprotective compound, via the action of biliverdin reductase and free iron is promptly sequestered into ferritin, an antioxidant (34, 35, 341, 342). The downstream products of HO possess important antioxidant, anti-inflammatory properties through which HO exhibits its cytoprotective function depicted in **Figure 1-8** (3-5, 32-54). Thus the HO system generates a tetrad of cytoprotective molecules, suggesting that compounds like HO inducers that enhance HO activity could have a pharmacological role against various disease conditions.

The Heme oxygenase system

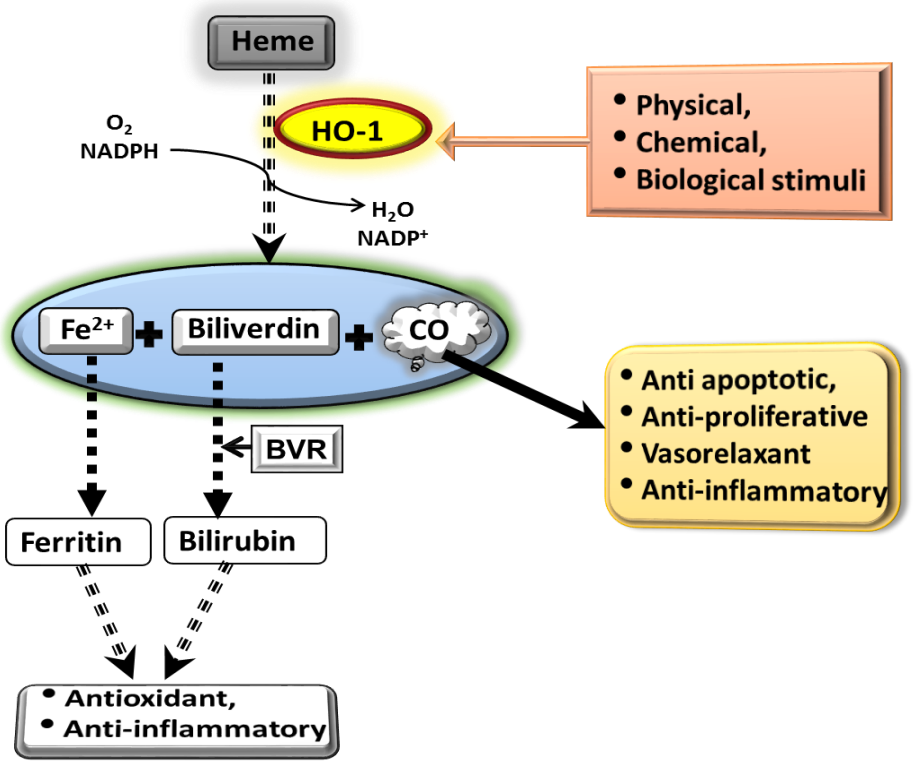

Figure 1-8: Schematic representation of cytoprotective role of the heme oxygenase system. Adapted and reformatted from Shuchita et al., *Curr Pharm Des. 2014; 20(9):1354-69.* Abbreviations used; CO (carbon monoxide), Biliverdin Reductase (BVR); Fe^{2+} (Iron); HO-1(heme oxygenase-1); NADPH (Nicotinamide adenine dinucleotide phosphate)

1.8.2 Distribution and isoforms of HO

The HO enzyme consists of three isoforms, the inducible HO-1, also known as heat shock protein (hsp 32), the constitutive HO-2 and HO-3, a pseudo-derivative of HO-2 (41, 341, 342). HO-1 (~32 KDa), encoded by the HMOX1 gene, is induced by a broad spectrum of pharmaceutical agents (35) and stimuli with widespread tissue distribution, including liver, kidney and lung. The constitutively synthesized HO-2 (~36 KDa) encoded by the HMOX2 gene

is localized primarily in the brain, testis, and the vascular endothelium (37, 44, 343). Still poorly understood HO-3 (33 KDa) is a constitutively expressed pseudogene derived from HO-2 transcripts, with very little demonstrable activity and unknown function (344, 345). Both HO-1 and HO-2 catalyzes the heme degradation pathway and are similar in respect to mechanism of heme oxidation, cofactors and substrate specificities (5, 35, 52).

1.8.3 Cytoprotective role of HO and its by-products

Upregulation of HO-1 by a number of agonistic stress stimuli plays beneficial roles and is associated with potential clinical applications in various pathological states including atherosclerosis, transplant rejection, hypertension, acute renal injury hypoxia, ischemia and inflammation (3-5, 32-54). HO-1 is induced by both heme and non-heme products including hydrogen peroxide, ultraviolet (UV) radiation, heavy metals, hypoxia, nitic oxide, shear stress, endotoxin, cytokines, growth factors and oxidized low-density lipoprotein (LDL)(36, 43, 343, 346). A plethora of studies have demonstrated that the induction of HO-1 and the subsequent metabolites of heme catabolism play vital roles in regulating important biological responses including inflammation, oxidative stress, cell survival, and cell proliferation (3-5, 32-54). The proposed mechanisms by which HO-1 exerts its biological effects include its ability to degrade the pro-oxidative heme (derived from various ubiquitously distributed heme proteins), the release of biliverdin and subsequent conversion to bilirubin, both of which have antioxidant properties and the generation of carbon monoxide, which has vasodilatory, anti-proliferative, and anti-inflammatory properties (38, 39, 44, 205, 347-352). HO-1-derived bilirubin is an efficient scavenger of reactive oxygen and nitrogen species (RONS) (38-40, 205, 347-351). The beneficial role of induction of HO-1 expression resulting in anti-hypertension, anti-diabetes and

reno-protection has been reported in various animal models (3-5, 32-54). Although, mechanisms underlying HO-1 induction is still not unveiled completely, it is demonstrated that the regulation of HO-1 is complex and essentially regulated at the transcriptional level which is cell and tissue specific (352, 353).

Taken together, **t**hese findings have led to the notion that the HO and its catabolic products could be used as a promising therapeutic target against vascular dysfunction, inflammatory disease states and cardiovascular diseases.

1.9. Summary

Collectively, the introductory material above gives a detailed picture of the current state of knowledge related to obesity and cardio-metabolic complications (see appendices for more detailed description). In general, obesity is associated with various health consequences including insulin resistance, type-2 diabetes, cardiomyopathy, nephropathy and hypertension (1, 11, 14, 15, 27, 57-60, 62, 64, 65, 68, 69). Visceral obesity, like pericardial adiposity, is an established risk factor for cardiovascular diseases (6-8, 12, 354). Moreover, elevated inflammation, increased oxidative stress, altered lipid metabolism, insulin resistance, impaired glucose metabolism and excessive ECM deposition are among the possible mechanisms which are implicated in altered cardiac structure and function (13-15, 27, 29, 30, 58, 68, 69, 149, 150) and links obesity with cardiomyopathy. Since the cytoprotective role of HO is well acknowledged (3-5, 32-54) in the present thesis the effects of upregulating HO with hemin on cardiomyopathy in obese ZF rats was investigated and the mechanism by which HO improves insulin signaling, glucose metabolism and cardiac function was evaluated.

1.10 Rationale

Visceral obesity is an established cardiovascular risk factor and a serious health issue worldwide (1-12, 14, 15, 80, 123, 129, 248). In obesity, high levels of pro-inflammatory cytokines/chemokines, increased oxidative stress and excessive infiltration of macrophages (1, 3, 5, 15, 16, 20, 67, 76, 150, 175) constitute a major damaging force for tissue homeostasis. In obese individuals, vital organs including the heart are exposed to incessant inflammatory and oxidative insults that ultimately leads to cardiac dysfunction and impaired insulin signaling. If this conditions persist, heart failure and other cardiovascular complications may arise (6, 12, 26, 135, 150, 265, 355, 356).

Generally, heart failure is characterized by elevated levels of osteopontin (294, 297, 298, 308), osteoprotegerin (311, 357, 358), endothelin-1 (230-232, 234), 8-isoprostane (224, 227) and ECM/profibrotic factors such as TGF-β, collagen, and fibronectin (21, 286, 292, 293), pro-inflammatory cytokines (TNF-α, IL-6, IL-1β) (1, 3, 5, 15, 24, 67, 76, 115) and chemokines (MCP-1 and MIP-1α) (16, 19, 132). Interestingly, these inflammatory/oxidative mediators are elevated during obesity (2, 16, 26, 30, 75, 83, 131, 132, 179, 217, 232, 257, 298) that would act in concert to further exacerbate cardiac injury and compromising cardiac function. Thus, there is need for new therapeutic strategies with novel mechanisms of action that can be used clinically to counteract the adverse effects associated with obesity and cardiomyopathy. An upregulated HO can be used as an alternative approach to combat obesity and associated cardiovascular comorbidities. Our lab and other clinical studies have acknowledged the beneficial role of the HO system in response to various pathophysiological stress conditions (3-5, 32-54). However, the activation of HO by different stress stimuli under pathophysiological conditions only results in the transient increase in HO activity that falls below the optimum level required for the

stimulation of downstream signaling components through which the HO system confers cytoprotection. Hence, to activate the HO system above the threshold levels, HO inducers like hemin may be needed for cardioprotection (4, 53, 54, 195, 196, 359).

Our lab recently reported the anti-diabetic and insulin sensitizing effects of the HO inducer hemin in different animal models such as the spontaneously hypertensive rat model, deoxycorticosterone acetate-salt hypertensive rat model and Goto-Kakizaki rat model (53, 192, 195, 201, 202). However, so far no systemic study has been carried out to demonstrate the effects of an upregulated HO system by hemin on macrophage polarization and its effects on insulin signaling and cardiomyopathy in ZF rats, an obese rat model characterized by insulin resistance, hyperinsulinemia, glucose intolerance, hypertriglyceridemia, hypercholesterolemia, and cardiomyopathy. Similarly, the effect of the HO system on pericardial adiposity in ZF rats remains to be elucidated. Furthermore, no study has been done on the effects of hemin therapy on the markers of heart failure such as osteopontin and osteoprotegerin in cardiac tissue of ZF rats. Moreover, the effects of an upregulated HO system on cardiac ANP or adiponectin in obese ZF rats need to be elucidated. It is important to investigate, if an upregulated HO system by hemin would alleviate the aberrant insulin signaling and impaired glucose metabolism in obese ZF rats and attenuates cardiomyopathy.

1.11 General hypothesis

Based on the above background knowledge, it is hypothesized that upregulation of the HO system by hemin would improve insulin signaling and cardiac function in obese ZF rats.

1.12 Thesis objectives: The main objectives of my thesis work are

- to determine the effect of hemin on HO-1 concentration in the left ventricle tissue of ZF rats.
- to investigate the effects of hemin on pro-inflammatory cytokines and chemokines in the left ventricle tissue of ZF rats.
- to investigate whether hemin treatment attenuates macrophage infiltration and to analyze its effects on pro-inflammatory M1-phenotype and anti-inflammatory M2-phenotype markers in cardiac tissue of ZF rats.
- to assess whether hemin treatment suppresses oxidative stress in ZF rats.
- to determine if hemin treatment exhibits protection against excessive deposition of extracellular matrix and profibrotic proteins in the left ventricle tissue of ZF rats.
- to investigate whether an upregulated HO system potentiates important proteins of the insulin signal transduction pathway in obese and insulin resistant ZF rats.
- to study the effects of hemin on glucose intolerance and insulin resistance in ZF rats.
- to investigate whether hemin treatment enhances ANP and adiponectin in ZF rats.
- to assess whether hemin treatment attenuates cardiac lesions in ZF rats.
- to investigate whether treatment with hemin improves cardiac function by measuring various echocardiographic and hemodynamic parameters and suppresses the markers of heart failure such as osteopontin and osteoprotegerin in cardiac tissue of ZF rats.

The results of this thesis has been published as two manuscripts

CHAPTER 2

The risk of heart failure and cardiometabolic complications in obesity may be masked by an apparent healthy status of normal blood glucose

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This chapter has been published as a research paper in

Oxidative Medicine and Cellular Longevity Volume 2013, Article ID 253657, 16 pages

My contribution to this paper: (Shuchita et al., *Oxid Med Cell Longev*. 2013; 2013:253657) was co-authored with Manish Mishra, Ashok Jadhav, Courtney Gerger, Paul Lee, Lynn Weber, and my supervisor Joseph Fomusi Ndisang. This chapter describes the role of an upregulated HO system by hemin on cardiomyopathy in ZF rats and the multifaceted mechanisms by which hemin therapy improves insulin signaling, glucose metabolism and cardiac function. Moreover, this chapter suggests that perturbations in insulin signaling and cardiac function may be forerunners to overt hyperglycemia and heart failure in obesity. The experimental approach was designed in accordance with objectives as described in chapter 1. In this study, I was responsible for performing all experiments, collection of all data, statistical analyses, making figures and

tables. As primary author, I drafted the first version of the manuscript, given all major interpretations, synthesized information, and revised the manuscript with input from all co-authors. My supervisor Joseph Fomusi Ndisang conceived the idea for the work and provided critical input towards my experimental protocol and manuscript writing.

2.1 Abstract

Although, many obese individuals are normoglycemic and asymptomatic of cardiometabolic complications, this apparent healthy state may be a misnomer. Since heart failure is a major cause of mortality in obesity, we investigated the effects of heme-oxygenase (HO) on heart failure and cardiometabolic complications in obese normoglycemic Zucker-fatty-rats (ZF rats).Treatment with the HO-inducer, hemin reduced markers of heart failure such as osteopontin and osteoprotegerin, and abated left-ventricular hypertrophy/fibrosis, extracellular matrix/pro-fibrotic proteins including collagen-IV, fibronectin, TGF-β1, and reduced cardiac lesions. Furthermore, hemin suppressed inflammation by abating macrophage chemoattractant protein-1, macrophage-inflammatory protein-1 alpha, TNF-α, IL-6 and IL-1β but enhanced adiponectin, atrial-natriuretic-peptide (ANP), HO-activity, insulin sensitivity and glucose metabolism. Correspondingly, hemin improved several hemodynamic/echocardiographic parameters including left-ventricular -diastolic wall-thickness, left-ventricular -systolic wall-thickness, mean-arterial pressure, arterial-systolic pressure, arterial-diastolic pressure, left-ventricular -developed pressure, +dP/dt and cardiac output. Contrarily, the HO-inhibitor, stannous-mesoporphyrin nullified the hemin-effect, exacerbating inflammatory/oxidative insults and aggravated insulin resistance (HOMA-index).

We conclude that perturbations in insulin-signaling and cardiac function may be forerunners to overt hyperglycemia and heart failure in obesity. Importantly, hemin improves

cardiac function by suppressing markers of heart failure, left-ventricular hypertrophy, cardiac lesions, extracellular matrix/pro-fibrotic proteins, inflammatory/oxidative mediators, while concomitantly enhancing the HO-adiponectin-ANP axis.

2.2 Introduction

The recent escalation of obesity in every segment of the population including children, adolescences and adults poses a great health challenge of considerable socioeconomic burden (61, 360). The impact on healthcare systems may become unsustainable given the numerous chronic diseases such as type-2 diabetes, dyslipidemia, hypertension and other related cardiometabolic complications associated with obesity (61, 360, 361). Cardiac complications including heart failure is among the major causes of mortality in obese individuals. Obesity causes lipotoxicity and adipose tissue dysfunction with excessive production of adipokines like tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), IL-1β, all of which are implicated in heart failure and related cardiometabolic complications (206, 254). However, obesity may not always translate into increased risk for these comorbidities (362). Some obese individuals dubbed 'metabolically healthy' are protected against obesity-related metabolic diseases. These 'metabolically healthy' obese individuals are insulin-sensitive with normal lipid metabolism and cardiac function similar to healthy lean individuals, which is in stark contrast to 'metabolically unhealthy' obese individuals with high risk of developing cardiometabolic complications (80, 362). However, the apparent state of good health in 'metabolically healthy' obese sub-phenotype may be a misnomer because the development of several characteristics of metabolic syndrome is now being observed in many adults who previously manifested the healthy obese phenotype (363), suggesting that individuals with a healthy obese phenotype may not remain healthy for

their entire lives. Several parameters including environmental and behavioral factors may modify obesity sub-phenotypes, and the transition from healthy to unhealthy. Whether healthy obese individuals can maintain insulin sensitivity during the entire life or whether healthy obesity simply represents delayed onset of obesity related cardiometabolic complications has to be clarified.

In obesity, excessive oxidative stress, intense inflammatory activity, insulin resistance, deregulated lipid metabolism, altered glucose metabolism and impaired mitochondrial biogenesis are among the pathophysiological driving forces that precede the early stages of cardiac dysfunction. Many cardiac complications have the common denominator of elevated inflammation due to the infiltration of macrophage M1-phenotype (364). Generally, macrophages exhibit two different forms dubbed "classical" or M1-phenotype and "alternative" or M2-phenotype (364), and each phenotype express distinct patterns of surface receptors when responding to different stimuli. The M1-phenotype stimulates inflammation while the M2-phenotype blunts inflammation (364). During macrophage infiltration, the M1-phenotype is stimulated by different chemokines including macrophage inflammatory protein-1 alpha [(MIP-1α), chemokine (C-C motif) ligand-3 (CCL3)] and macrophage chemoattractant protein-1 [(MCP-1) (158), chemokine (C-C motif ligand-2 (CCL2)] (158). The activation of the macrophage M1-phenotype is generally associated with elevated levels of pro-inflammatory cytokines including TNF-α, IL-6 and IL-1β (5, 365, 366). Moreover, the levels of macrophage M1-phenotype, MCP-1, TNF-α, IL-6 and IL-1β are elevated in obesity and insulin resistance (3, 158, 365), and these factors play a major pathophysiological role in heart failure (254). In obesity, markers of heart failure such as osteopontin (367) and osteoprotegerin (357) are elevated (306, 358). Similarly, the levels of extracellular matrix/profibrotic proteins like transforming

growth factor beta (TGF-β), collagen and fibronectin are elevated in obesity (368). Therefore, in obesity elevated chemokines, cytokines and increased macrophage-M1 infiltration would act in concert with elevated extracellular matrix/profibrotic and heart-failure proteins to exacerbate cardiac tissue destruction and compromise heart function. Thus, novel strategies capable of selectively suppressing macrophage M1-phenotype, pro-inflammatory cytokines/chemokines and extracellular matrix/profibrotic proteins are needed.

In many pathophysiological conditions, various stress-response immune-regulatory proteins, including heme oxygenase (HO-1) are activated as an innate defense mechanism (369-372). However, the pathophysiological activation of HO-1 may only result in a transient or marginal increase of HO-activity that falls below the threshold necessary to activate the downstream signaling components through which the HO system elicits its cytoprotective effects, so a robust and surmountable increase of HO-activity with HO-inducers like hemin may be needed for cardio-protection (193, 201, 202, 207, 373). Generally, HO is composed of two main isoforms (HO-1 or inducible) and (HO-2 or constitutive), which are largely responsible for the anti-oxidant and anti-inflammatory effects of HO (205). We recently reported the cardioprotective effects of the HO system in Zucker diabetic fatty rats (ZDFs) (3), a model characterized by obesity, insulin resistance and overt hyperglycemia. However, because ZDFs are hyperglycemic, their pathophysiological profile is not reflective of individuals dubbed ‘metabolically healthy’, a subtype of obesity characterized by normoglycemia (363). Given that the incidence of cardiometabolic complications is increasing in many adults who previously manifested the metabolically healthy obese phenotype (363), novel studies with animal models that closely mimic the pathophysiological profile of metabolically healthy obese subtype are needed. Therefore, this study will investigate the effects of the HO system on cardiometabolic

complications in Zucker fatty rats (ZF rats), an obese model with normoglycemia and cardiometabolic complications (374) that closely mimic the pathophysiological profile of metabolically healthy obese individuals with normoglycemia and an apparent state of good health. Although the HO system is cytoprotective, its effects on cardiomyopathy in ZF rats remain to be elucidated.

Since, dysfunctional insulin signaling, obesity, elevated inflammation and cardiac hypertrophy are forerunners to heart failure, this study will also investigate the multifaceted mechanisms by which the HO system preserves cardiac function in ZF rats. Whether, an upregulated HO system by hemin is capable of modulating macrophage polarization towards the M2-phenotype that blunts inflammation, while suppressing the pro-inflammatory M1-phenotype will be assessed. As macrophage infiltration is stimulated by chemokines like MIP-1α and MCP-1 (158), and the effects of the HO system on these chemokines in ZF rats have not been reported, this study will also determine left-ventricular MIP-1α and MCP-1 and correlate changes of these chemokines to the expression of the pro-inflammatory macrophage-M1-phenotype in the left ventricle of ZF rats. Similarly, the effect of hemin therapy on important markers of heart failure such as osteopontin (357) and osteoprotegerin (306) will be investigated. Importantly, no study has reported the levels of expression of osteopontin and osteoprotegerin in myocardial tissue of ZF rats. Therefore this study will unveil the multifaceted mechanisms by which hemin therapy improves cardiac function and insulin signaling in obesity.

2.3 Materials and Methods

2.3.1 Animals, treatment groups and biochemical parameters

Our experimental protocol was in conformity with the Guide for Care and Use of Laboratory Animals stipulated by the Canadian Council on Animal Care and the National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was approved by University of Saskatchewan Animal Ethics Committee. Male ZF rats (12 weeks old) and sex/age-matched Zucker lean (ZL) rats were purchased from Charles River Laboratories (Willington, MA). The animals were housed at 21°C with 12-hour light/dark cycles, fed with standard laboratory chow and had access to drinking water *ad libitum*.

The HO-inducer, hemin (30 mg/kg i.p., Sigma, St Louis, MO), and HO-blocker stannous-mesoporphyrin [(SnMP) 2 mg/100 g body weight i.p.] were purchased from Porphyrin Products (Logan, UT), and prepared as we previously reported and administered biweekly for 8 weeks (3, 124, 200). At 16 weeks of age, the animals were randomly assigned to the following experimental groups (*n=6* per group): **(A)** controls (ZF and ZL), **(B)** hemin-treated ZF and ZL, **(C)** ZF+Hemin+SnMP and **(D)** ZF+vehicle dissolving hemin and SnMP.

During the treatment period body-weight and glucose were monitored on a weekly routine. Body-weight was measured using a digital balance (Model Mettler PE1600**,** Mettler Instruments Corporation, Greifensee, Zurich, Switzerland). At the end of the 8-week treatment period, the animals were 24 weeks of age. A day prior to killing, the animals were fasted in metabolic cages for 24-hr urine collection and weighed. Systolic blood pressure was determined by non-invasive tail-cuff method, while a Millar Mikro-Tip ultra-miniature tip sensor pressure transducer catheter (Model SPR-407, Harvard Apparatus**,** Montreal, Canada) for invasive hemodynamic parameters. In addition, a Vevo 660 high frequency ultrasound machine (Visual

Sonics, Markham, ON, Canada) equipped with B-mode imaging was used for echocardiographic measurements as we previously reported (3). After anaesthetizing the animals with pentobarbital sodium (50 mg/kg i.p.), blood was collected by cardiac puncture, and the pericardial fat pad and the heart isolated, cleaned and weighed with an analytical balance as previously reported (187). The atria were removed from the heart and the right ventricle free wall separated from the left ventricle including the septum as we previously reported (187).

Left-ventricular HO activity was evaluated spectrophotometrically as we previously reported (187, 200). ELISA kits were used for HO-1 (Stressgen-Assay Design, Ann Arbor, MI, USA), adiponectin (Phenix Pharmaceuticals, Inc, Burlingame, CA, USA), TNF-α, IL-6 and IL-1β (Immuno-Biological Laboratories Co Ltd, Gunma, Japan), MIP-1α and MCP-1 (OmniKine™, Assay Biotechnology Company Inc, Sunnyvale, CA, USA) (203, 375), while EIA kits for 8-isoprostane, ANP, ET-1, cGMP and kits for cholesterol and triglyceride were purchased from Cayman following the manufacturers' instructions as we reported (3, 187, 200). Intraperitoneal glucose tolerance test (IPGTT) and homeostasis model assessment of insulin resistance (HOMA-IR) were done as we previously reported (200).

2.3.2 Histological, morphological and immunohistochemical analyses of left ventricle

Histological and morphometric analyses were done as we previously described (186). Sections of 5 μm were cut and stained with hematoxylin and eosin for histological analysis. Masson's Trichrome staining detected left-ventricular collagen deposition. Morphometrical evaluation of left-ventricular longitudinal myocytes thickness was done by randomly measuring 30 cardiac muscle fibers from each experimental group by a blinded researcher using a microscope (Aperio Scan Scope Model CS, Aperio Technologies Inc, Vista, CA, USA) and

analyzed using Aperio Image Scope V11.2.0.780 software (Aperio, e-Pathology Solution, Vista, CA, USA). Morphologic assessment of collagen deposition in left-ventricular sections was accessed using Aperio Image Scope (Aperio Technologies Inc, Vista, CA, USA). Each left-ventricular section was magnified at 200X, and 20 random snaps were taken per slide (20 x 6 = 120 images per group) subsequently scored semi-quantitatively by a blinded researcher as we previously reported (3, 186).

Immunohistochemistry was done as we previously reported (188). Sections of 5 μm of left-ventricular tissue were treated with bovine serum albumin in phosphate buffered saline to block non-specific staining, and incubated overnight with ED1 (1:500 dilution sc-59103, Santa Cruz Biotechnology, CA). The sections were later treated with with goat anti-mouse IgG for 30 min (1:200 dilution; Jackson Immuno-Research Laboratories, Inc., ME, USA). Immunohistochemical staining was done using the standard avidin-biotin complex method with the chromogen 3, 3'-diaminobenzidine (DAB) at the final detection step. Sections of heart tissue were scanned using virtual microscope (Aperio Scan Scope Model CS, Aperio Technology Inc, Vista, CA, USA). Quantitative assessment of ED1 was done by a blinded researcher who randomly examined 20-22 fields of each left-ventricular section magnified at 200X. Macrophages which were positively stained with ED1 (brown from immune-stained sections) were quantified by manually counting the ED1-stained cells around the blood vessels and interstitial spaces of myocardium.

2.3.3 Western Immunoblotting

Pericardial fat and left-ventricular tissues were homogenized as previously reported (3, 124, 186, 187, 200). Primary antibodies [(Santa Cruz Biotechnology, Santa Cruz, CA, USA),

ED-1 (CD68), sc-59103), ED-2 (CD163) (sc-58956), CD-14 (sc-9150), CD-206 (sc-48758), CD-36 (sc-9154), osteopontin (sc-21742), osteoprotegerin (sc-11383), PI3K (sc67306), IRS-1 (sc-559), collagen-IV (sc-11360), fibronectin (sc-18825), TGF-β1/2/3 (sc7892)] and GLUT4 (ab 654, Abcam Inc, Cambridge, MA, USA) were used. Densitometric analysis was done with UN-SCAN-IT software (Silk Scientific Inc, Orem, Utah, USA). G6PDH antibody (A9521, Sigma St Louis, MO, USA) was used as a control to ascertain equivalent loading.

2.3.4 Statistical Analysis

All data are expressed as means ± SEM from at least four independent experiments unless otherwise stated. Statistical analyses were done using two-way ANOVA, using Statistical Analysis System (SAS), software Version 9.3 (SAS Institute Inc., Cary, NC, USA) and Student's *t*-test. Group differences at the level of $p<0.05$ were considered statistically significant.

2.4 Results

2.4.1 Hemin therapy upregulates the HO system to improve cardiac function

To investigate the mechanisms underlying the improvement of cardiac function in obese insulin-resistant ZF rats, we measured the concentration of HO-1 and HO activity. In ZF-control rats, the basal level of HO-1 concentration and HO-activity were significantly lower than in ZL-control (**Figs. 2-1A and 2-1B**). However, hemin administration increased HO-1 and HO-activity in ZF by 8.4- and 11.3-fold respectively. The enhanced HO activity would increase endogenous carbon monoxide that would in turn stimulate cGMP (187, 200). Both cGMP and carbon monoxide are known to enhance insulin signaling and glucose metabolism (376). Accordingly,

we detected a 3.4-fold increase of cGMP in hemin-treated animals (**Fig.2-1C**). In contrast, the co-administration of the HO-blocker, SnMP and the HO-inducer, hemin abolished the hemin-induced increase of HO-1 and HO-activity, with corresponding reduction of cGMP levels (**Fig.2-1C**). Hemin therapy also enhanced HO-1, HO-activity and cGMP levels in ZL-control rats (**Fig.2-1A, 2-1B and 2-1C**). In hemin-treated ZLs, HO-1, HO-activity and cGMP were enhanced by 3.1-, 2.8-, 2.4-fold respectively as compared to 8.4-, 11.3- and 3.4-fold respectively in hemin-treated ZF rats, suggesting greater selectivity of hemin to the HO system in unhealthy ZF rats characterized obesity, insulin resistance and cardiomyopathy (374).

Since cardiac hypertrophy is amongst the forerunners to heart failure, we investigated the effects of hemin on cardiac hypertrophy. Our results indicate that hemin therapy significantly reduced cardiac hypertrophy in ZF, whereas the co-administration of hemin and SnMP nullified the effect (**Table 2-1**). Echocardiography was used to further assess left-ventricular hypertrophy. Our hemodynamic data obtained during catheterization of the left side of hearts from ZF rats showed association between elevated myocardial hypertrophic response and obesity. A significant 2-fold increase in left-ventricular free wall thickness, an important index of cardiac hypertrophy (377) was observed during diastole and systole, and interestingly these were abated by hemin by 33.3 % and 15.6 % respectively **(Figure 2-9 & 2-10)** (**Table 2-2**). Other hemodynamic deficiencies in ZF rats including abnormalities in left-ventricular end-diastolic volume, left-ventricular end-systolic volume, stroke volume and cardiac output which were reduced by 17.5%, 16%, 8.3%, 7.7% respectively (**Table 2-2**), were increased by hemin therapy by 15.2%, 27.3%, 13.6% and 12.4% respectively. Hemin therapy also improved cardiac hemodynamics by lowering arterial-systolic pressure, arterial-diastolic pressure, mean-arterial pressure and total-peripheral resistance by 12.4%, 11.4% and 12.2%, 17.6% respectively, with

corresponding reduction of +dp/dt (the maximal-rate of increase in left-ventricular pressure), left-ventricular developed pressure and heart rate.

Treatment with hemin and SnMP caused loss of body-weight in ZL-controls and ZF rats, which however did not exceed 9% **(Table 2-1)**. The loss of weight may not be due to toxicity as we recently showed that several indices of toxicity including plasma gamma-glutamyltransferase, aspartate aminotransferase and alanine aminotransferase were within normal range (200). Although ZF rats had normoglycemia, hemin and SnMP affected blood glucose. In hemin-treated animals, there was a slight but significant reduction of glycemia, whereas in SnMP-treated animals a slight increase was observed (**Table 2-1**). Similarly, hemin therapy slightly reduced glycemia in ZL-controls. The vehicle dissolving hemin and SnMP had no effect on the measured parameters.

2.4.2 Hemin therapy abates MCP-1, MIP-1α, TNF-α, endothelin-1, 8-isoprostane but enhanced ANP in ZF rats

Since 8-isoprostane stimulates ET-1 (378), and both ET-1 and 8-isoprostane are involved in the oxidative destruction of tissue, we measured ET-1 and 8-isoprostane. ET-1 in untreated ZF rats were markedly elevated as compared to ZL-controls (**Fig.2-2A**), but were significantly abated by hemin. In contrast, the co-administration of hemin and the HO-blocker, SnMP annulled the effect of hemin (**Fig.2-2A**). Because elevated oxidative stress is linked to impaired insulin-signaling and cardiac dysfunction, we measured urinary 8-isoprostane, an important marker of oxidative stress (379). In ZF rats, the basal levels of 8-isoprostane were significantly elevated (**Fig.2-2B**), but were reduced by hemin, whereas co-treatment of hemin with SnMP nullified the effects. Given that ET-1 and ANP are known to interact reciprocally (380), we

investigated whether the hemin-dependent suppression of ET-1 (**Fig.2-2A**) would be associated with a parallel potentiation of ANP. In ZF rats, the basal ANP levels were markedly depressed by 1.7-fold (**Fig.2-2C**), but interestingly were robustly enhanced by hemin by 3.3-fold. In contrast, the co-administration of hemin with SnMP abolished the effects of hemin.

We also investigated the effects of hemin on MIP-1α and MCP-1 since these chemokines trigger macrophage infiltration (158). In ZF rats, the basal MCP-1 levels were significantly increased by 4.6-fold (**Fig.2-2D**), but were attenuated by hemin by 2.8-fold, whereas the co-administration with SnMP nullified the effects of hemin (**Fig.2-2D**). Although hemin therapy greatly attenuated MCP-1 by 64% in ZF, however comparable levels as observed in the ZL-controls were not reinstated. Hemin therapy was also effective in suppressing MIP-1α (**Fig.2-2E**). In ZF rats, the basal MIP-1α levels were significantly elevated by 4.9-fold, but were reduced by hemin by 3.5-fold, whereas the co-treatment of hemin with SnMP nullified the effects (**Fig.2-2E**). Since TNF-α is implicated in macrophage infiltration (158), we also assessed the effects of hemin on TNF-α. In ZF rats, the basal levels of TNF-α were elevated by 3.5-fold, but were significantly attenuated by hemin by 2.6-fold (**Fig.2-2F**), whereas co-treatment with SnMP abolished the effect of hemin.

Hemin therapy also affected ET-1, 8-isoprostane, ANP, MCP-1, MIP-1α in ZL-controls, although the magnitude of effect was smaller than in ZF rats (**Fig.2-2**).

2.4.3 Hemin selectively abated the pro-inflammatory macrophage M1-phenotype, but enhanced the anti-inflammatory M2-phenotype in the left ventricle of ZF rats

After having observed the hemin-dependent reduction of cytokines/chemokines implicated in macrophage infiltration such as MIP-1α, MCP-1 and TNF-α, we investigated

whether the suppression of these chemokines/cytokines in the left ventricle of ZF rats would be accompanied by the selective attenuation of the pro-inflammatory macrophage M1-phenotype using a specific marker such as ED1 (381) to quantify the expression of the pro-inflammatory M1-phenotype in left-ventricular tissue, and other markers for the assessment of anti-inflammatory M2-phenotype including ED2 (381), CD14 (382, 383), CD206 (364), CD36 (384, 385).

Our Western immunoblotting and relative densitometry revealed that the basal expression of the pro-inflammatory macrophage M1-phenotype marker, ED1, in ZF rats was markedly elevated by 4.8-fold as compared to ZL-controls (**Fig.2-3A**), but was significantly reduced by hemin by 3.5-fold, although control levels were not attained. On the other hand, the basal expression of several markers of the anti-inflammatory macrophage M2-phenotype including ED2, CD206, CD36 and CD14 were significantly depressed in ZF rats by 2.1- , 5.7-, 3.6- and 2.9-fold respectively (**Figs.2-3B, 2-3C, 2-3D and 2-3E**). Interestingly, hemin therapy greatly enhanced the depressed ED2, CD206, CD36 and CD14 in ZF rats by 3.8-, 4.1-, 2.3- and 2.6-fold respectively, suggesting that a novel mechanism by which hemin therapy blunts inflammation is by selectively modulating the polarization of macrophage toward the M2-phenotype that dampens inflammation.

2.4.4 Hemin therapy suppressed macrophage infiltration in the left ventricle of ZF rats

Following the observation from our Western blot experiment that hemin therapy reduced left-ventricular ED-1, a marker of macrophage infiltration, we use the ED-1 antibody to further confirm macrophage infiltration in the left ventricle by immunohistochemistry (**Fig.2-4A**). Our results reveal that left-ventricular sections from ZL-control rats were almost devoid of the dark

brown ED1 positive staining. However, in untreated ZF-control rats, a greater number of ED1-positively stained dark brown cells were observed, indicating increased macrophage infiltration. Interestingly, in hemin-treated ZF rats, there was a marked reduction in the number of dark brown stained macrophages, suggesting reduction of macrophage infiltration. Correspondingly, hemin therapy significantly reduced the quantitative ED1 score (**Fig.2-4B**).

2.4.5 Hemin therapy enhanced insulin signaling but suppressed extracellular matrix and profibrotic proteins implicated in cardiac injury

Since visceral adiposity and elevated inflammation impairs insulin signaling (8), we investigated the effects of hemin therapy on the expression of important components of the insulin signal transduction pathway including IRS-1, PI3K and GLUT4. In ZF rats, the basal expression of IRS-1, PI3K and GLUT4 were significantly reduced by 11.2-, 2.5- and 2.3-fold as compared to the ZL-control (**Figs.2-5A, 2-5B and 2-5C**), but were enhanced by hemin by 5.7-, 4.01- and 1.9-fold respectively. To further confirm the anti-hypertrophic effect of hemin therapy, we measured collagen-IV an important protein implicated in cardiac hypertrophy and fibrosis (186). In ZF rats, the basal expression of left-ventricular collagen-IV was significantly elevated by 6.9-fold, but was abated by hemin by 2.8-fold (**Fig.2-5D**). Given that excessive deposition of extracellular matrix/profibrotic proteins and inflammation due to macrophage infiltration are cardinal pathophysiological events implicated in cardiac insult (8, 386), while atrial natriuretic peptide (ANP) and adiponectin are known to suppress fibrosis caused by the deposition of extracellular matrix (387, 388), we investigated whether the concomitant potentiation of ANP, adiponectin and the HO-system by hemin would abate TGF-β. In ZF rats, the basal expression of TGF-β was significantly elevated by 4.6-fold, but was markedly attenuated by hemin by 3.4-fold

(**Fig 2-5E**). Since TGF-β mobilizes the extracellular matrix by stimulating fibronectin and collagen to cause fibrosis and cardiac injury (21, 387), we also measured the expression of fibronectin. In ZF rats, the basal expression of fibronectin was increased by 7.5-fold, but was markedly attenuated by hemin therapy by 4.5-fold (**Fig.2-5F**).

2.4.6 Hemin improved glucose tolerance, enhanced the insulin-sensitizing protein, adiponectin but abated insulin resistance

After having observed the hemin-induced potentiation of insulin signaling, to further confirm the role of hemin therapy on glucose metabolism, we assessed the effects of hemin on glucose tolerance, insulin resistance and the insulin-sensitizing protein, adiponectin in ZF rats, an obese model with elevated inflammation. Since inflammation due to macrophage infiltration is implicated in insulin resistance and cardiomyopathy (8, 386), and ZF rats are characterized by insulin resistance (374), we investigated whether the hemin-dependent suppression of macrophage infiltration would be accompanied by improved glucose metabolism. In untreated ZF rats, IPGTT analysis showed marked increase in glycemia as compared to ZL-controls and hemin-treated ZF rats at all time-points tested (**Fig.2-6A**), suggesting improved glucose tolerance in hemin-treated ZF rats. Although ZF rats were hyperinsulinemic with elevated basal glycemia, when challenged with a bolus injection of glucose, only to a meagre glucose-stimulated insulin release was observed (**Fig.2-6B**), suggesting glucose intolerance. On the other hand, glucose challenge to ZL-controls and hemin-treated ZF rats greatly stimulated insulin release (**Fig.2-6B**), suggesting improved glucose tolerance. Hemin also reduced the elevated insulin resistance HOMA-IR in ZF rats (**Fig.2-6C**), whereas co-administration with SnMP reversed the effects of hemin (**Fig.2-6C**).

We also investigated the effects of hemin therapy on adiponectin, an anti-inflammatory, insulin sensitizing and cardioprotective protein (389, 390). Interestingly, hemin therapy significantly enhanced the depressed basal adiponectin levels in ZF rats, whereas treatment with SnMP abolished and further reduced the depressed levels of adiponectin (**Fig.2-6D**). Hemin therapy also reduced HOMA-IR index in ZL-controls and enhanced adiponectin, although the effect was less-intense as compared to ZF rats.

2.4.7 Hemin therapy suppressed left-ventricular fibrosis, cardiomyocyte hypertrophy and longitudinal cardiac myofibril thickness in ZF rats

Histological and morphometric analyses using Masson's trichrome and hematoxylin and eosin staining were done to further confirm the cardio-protection by hemin. Cardiomyocytes appeared as dark reddish while extracellular matrix, such as collagen, stained blue (**Fig.2-7A**). Left-ventricular sections from ZL-controls appeared morphologically normal, with scanty interstitial collagen deposition. In contrast left-ventricular images from ZF rats showed moderate to severe fibrosis, with scarring of cardiomyocytes, and interstitial and perivascular collagen depositions (**Fig.2-7A**). Interestingly, hemin therapy attenuated the severity of scarring, and intestinal and perivascular collagen deposition, evidenced by reduced extracellular and perivascular blue staining (**Fig.2-7A**). Correspondingly, semi-quantitative analysis showed that hemin therapy significantly abated the elevated collagen deposition and perivascular fibrosis in ZF rats, reinstating comparable levels to ZL-control (**Fig.2-7B**).

Hemin therapy was also effective against cardiomyocyte hypertrophy (**Fig.2-7C**). In ZF rats, cardiomyocytes were enlarged with increscent nuclei and the inner myofibril spaces were decreased, as compared to normal cardiomyocytes in ZL-controls (**Fig.2-7C**). In ZF rats, the

longitudinal cardiac myofibril thickness was 37% higher than ZL-controls (**Fig.2-7D**), but was reduced by 27% in hemin-treated ZF rats. Although ZL-control values were not reinstated, hemin increased inter-myofibril spaces in ZF rats close to the levels observed in ZL-controls (**Fig.2-7D**).

2.4.8 Hemin therapy suppressed the elevated expression of markers of heart failure in the left ventricle of ZF rats

To further confirm the cardioprotective effects of an upregulated HO-system, we investigated the effects of hemin therapy on important markers of heart failure such as osteopontin (367) and osteoprotegerin (357). Since left-ventricular hypertrophy is associated with heart failure (391), we determined whether the hemin-dependent suppression of left-ventricular hypertrophy in ZF rats would be accompanied by the reduction of markers of heart failure. Our results indicate that, in ZF rats, the basal expression levels of osteopontin and osteoprotegerin were significantly elevated by 4.6- and 7.1-fold respectively as compared to ZL-controls (**Fig.2-8A and 2-8B**). Interestingly, treatment with hemin attenuated the expressions of osteopontin and osteoprotegerin by 3.5- and 3.3-fold respectively (**Figs.2-8A and 2-8B**).

2.5 Discussion

The present study indicates that the multifaceted mechanisms by which hemin therapy improves cardiomyopathy in obesity includes: **(i)** the suppression of visceral adiposity, **(ii)** the reduction of macrophage M1-phenotye, **(iii)** the attenuation of markers of heart failure, **(iv)** the reduction of extracellular matrix/profibrotic proteins and **(v)** the amelioration of insulin resistance, with corresponding enhancement of glucose metabolism. In ZF rats, excessive

visceral adiposity, increased macrophage infiltration and the elevated levels of 8-isoprostane, MIP-1α, MCP-1, TNF-α, IL-6, IL-1β, ET-1, proteins of heart failure and extracellular-matrix deposition are among the complex molecular processes that characterize the intricate relationship between inflammation, oxidative stress, cardiac fibrosis, and the progressive development of insulin resistance and cardiomyopathy (8, 206, 386, 387, 392-394). Importantly, the present study unveils that hemin therapy selectively enhance the anti-inflammatory macrophage M2-phenotype in pericardial fat and left-ventricular tissue of ZF rats while concomitantly abating the pro-inflammatory M1-phenotype, suggesting that a novel mechanism by which hemin therapy suppress cardiac inflammation in obesity is by selectively favoring the polarization of the macrophage towards the M2-phenotype that ablate inflammation. Correspondingly, hemin therapy abated several chemokine and cytokines that promotes macrophage infiltration including MIP-1α, MCP-1, TNF-α, IL-6 and IL-1β (158, 365, 366). Interestingly, the suppression of visceral adiposity and inflammation in hemin-treated ZF rats was accompanied by reduced insulin resistance and improved glucose intolerance, and the potentiation of important components of the insulin signal transduction pathway including IRS-1, PI3K and GLUT4, which in addition to the hemin-dependent enhancement of adiponectin, an anti-inflammatory, insulin sensitizing and cardioprotective protein (389, 390) may account for improved glucose metabolism in obese conditions.

Hemin therapy also reduced left-ventricular hypertrophy, cardiac fibrosis, cardiomyocyte longitudinal muscle-fiber thickness, a pathophysiological feature of cardiomyocyte hypertrophy (186), with corresponding suppression of markers of heart failure such as osteopontin and osteoprotegerin (357, 367), as well as the reduction of extracellular matrix protein like TGF-β, fibronectin and collagen which are implicated in cardiac hypertrophy and fibrosis (387, 394).

Since TGF-β mobilizes the extracellular matrix by stimulating fibronectin and collagen causing tissue damage and hypertrophy (387, 394), the concomitant reduction of TGF-β, fibronectin and collagen-IV in ZF rats may account for reduced cardiac lesions. Another mechanism by which the HO system suppress extracellular matrix and pro-fibrotic agents like TGF-β and ET-1 may be due to the HO-dependent potentiation of ANP, a substance known to suppress extracellular matrix (380, 395) . Generally, ANP and ET-1 have opposing effects (322). For example, ANP reduces fibrosis by inhibiting TGF-β and fibronectin (380), while ET-1 acts in conjunction with TGF-β to stimulate the synthesis of fibronectin (395). Similarly, ANP suppress inflammation to reduce insulin resistance (322), while ET-1 stimulates inflammatory/oxidative insults causing insulin resistance (396). On the other hand, ANP stimulates the production of adiponectin (397), a protein with insulin sensitizing and anti-inflammatory effects (389). The effects of ANP are largely mediated by cGMP (398), and adiponectin is also known to enhance cGMP (399). Moreover ANP and the HO system have mutual stimulatory effects. Accordingly, ANP enhance HO (400, 401), and similarly, the HO system has been shown to upregulate ANP and adiponectin (196, 202). Therefore, the synergistic potentiation of the HO-adiponectin-ANP axis and insulin signaling with corresponding ablation of extracellular matrix/heart failure proteins, the reduction of oxidative stress and inflammation mediators such as macrophage M1-phenotype, MIP-1α and MCP-1, TNF-α, IL-6, IL-1β, ET-1 and 8-isoprostane are among the multifaceted mechanisms by which hemin therapy improved cardiac function. Thus, novel strategies capable of potentiating the HO-adiponectin-ANP axis would improve cardiomyopathy and insulin signaling in obesity.

Cardiomyocyte hypertrophy and myocardial fibrosis are early microscopic changes in heart failure. Subsequently, macroscopic alterations including increased left-ventricular wall thickness, diastolic/systolic dysfunction and impaired cardiac hemodynamics become evident.

Interestingly, hemin therapy modulated several hemodynamic and echocardiographic parameters to improve cardiac function. These include the reduction of left-ventricular diastolic wall-thickness, left-ventricular systolic wall-thickness, mean-arterial pressure, arterial-systolic pressure, arterial-diastolic pressure, left-ventricular developed pressure, +dP/dt and total-peripheral resistance, with corresponding enhancement of stroke volume and cardiac output, and thus improved cardiac function in hemin-treated ZF rats. The improved myocardial function in hemin-treated ZF rats was also associated with the reduction of total-cholesterol, triglycerides and pericardial fat. Moreover, pericardial adiposity is associated with left-ventricular diastolic dysfunction (12). Hemin therapy also enhanced the HO system, cGMP, adiponectin and ANP, and abated 8-isoprostane, MCP-1, MIP-1α, TNF-α, IL-6, IL-1β, ET-1 and lowered HOMA-IR index in ZL-control rats, although the magnitude was smaller as compared to ZF rats with depressed HO-activity. The reasons for this selective effect of HO are not fully understood. However, it is possible that as ZL-controls are healthy animals with normal/functional insulin-signaling, the HO system may be more stable as compared to ZF rats which have deregulated HO system with depressed HO-1 and HO-activity. Importantly, the selectivity of the HO system in diseased conditions could be explored against the co-morbidity of insulin-resistant diabetes and obesity

Although we recently reported the cardioprotective effects of the HO system in ZDFs (3), a model characterized by obesity, insulin resistance and overt hyperglycemia, pathophysiological profile of ZDF is not reflective of the metabolically healthy individuals who are characterized by obesity and normoglycemia (363). In contrast, ZF rats closely mimic the pathophysiological profile of metabolically healthy obese individuals with normoglycemia, so our findings may be applicable to this subtype of obese individuals. Moreover, with the rising incidence of

cardiometabolic complications in many adults who previously manifested the metabolically healthy obese phenotype (363), novel studies with animal models that closely mimic the pathophysiological profile of metabolically healthy obese subtype are needed. Therefore, studying the effects of the HO system on ZF rats may offer new perspective in the pathophysiology of cardiometabolic complications, and especially the progressive deterioration of cardiac function which may eventually lead to heart failure given the elevated levels of proteins of heart failure detected in untreated ZF rats.

Collectively, our study unveils the beneficial effect of upregulating the HO system in the co-morbidity of obesity and insulin resistance, and suggest that the suppression of oxidative mediators, macrophage-M1-phenotye infiltration and extracellular matrix/remodeling proteins are among the multifaceted mechanisms by which the HO system maintains enhance insulin signaling and counteract diabetic cardiomyopathy. These data suggest that although ZF rats are normoglycemic, perturbations of insulin signaling and cardiac function may be forerunners to overt hyperglycemia and heart failure in obesity.

2.6 Conclusion

The novelty of our study includes: **(i)** the hemin-induced selective enhancement of the anti-inflammatory M2-phenotype in pericardial fat and left-ventricular tissue of ZF rats, and parallel reduction of the pro-inflammatory macrophage M1-phenotype and MIP-1α, a chemokine implicated in macrophage infiltration; **(ii)** the hemin-dependent suppression of heart failure proteins such as osteopontin and osteoprotegerin; **(iii)** the suppression of pericardial adiposity and reduction of inflammatory cytokines in ZF rats; and **(iii)** the hemin-induced reduction of insulin resistance and improvement of cardiac function in ZF rats. This study also suggests a

possible paracrine and inter-organ cross-talk between the heart and pericardial fat. Importantly, the concomitant modulation of macrophage polarization in pericardial adipose tissue and left ventricles towards the anti-inflammatory M2-phenotype alongside the parallel reduction of pro-inflammatory cytokines and chemokines implicated in macrophage infiltration and tissue destruction may be indicative of a putative inter-organ cross-talk and the movement of inflammatory mediators from the pericardial fat to the myocardium or vice versa, and this may be particularly important in the progressive development of cardiomyopathy, insulin resistance and related cardiometabolic complications. Although this linkage has to be established, this study would set the stage for further exploration of the putative inter-organ communication between pericardial fat and the heart.

Acknowledgements

This work was supported by a grant from the Heart & Stroke Foundation of Saskatchewan, Canada to Dr. Joseph Fomusi Ndisang.

Declaration of interest

The authors have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

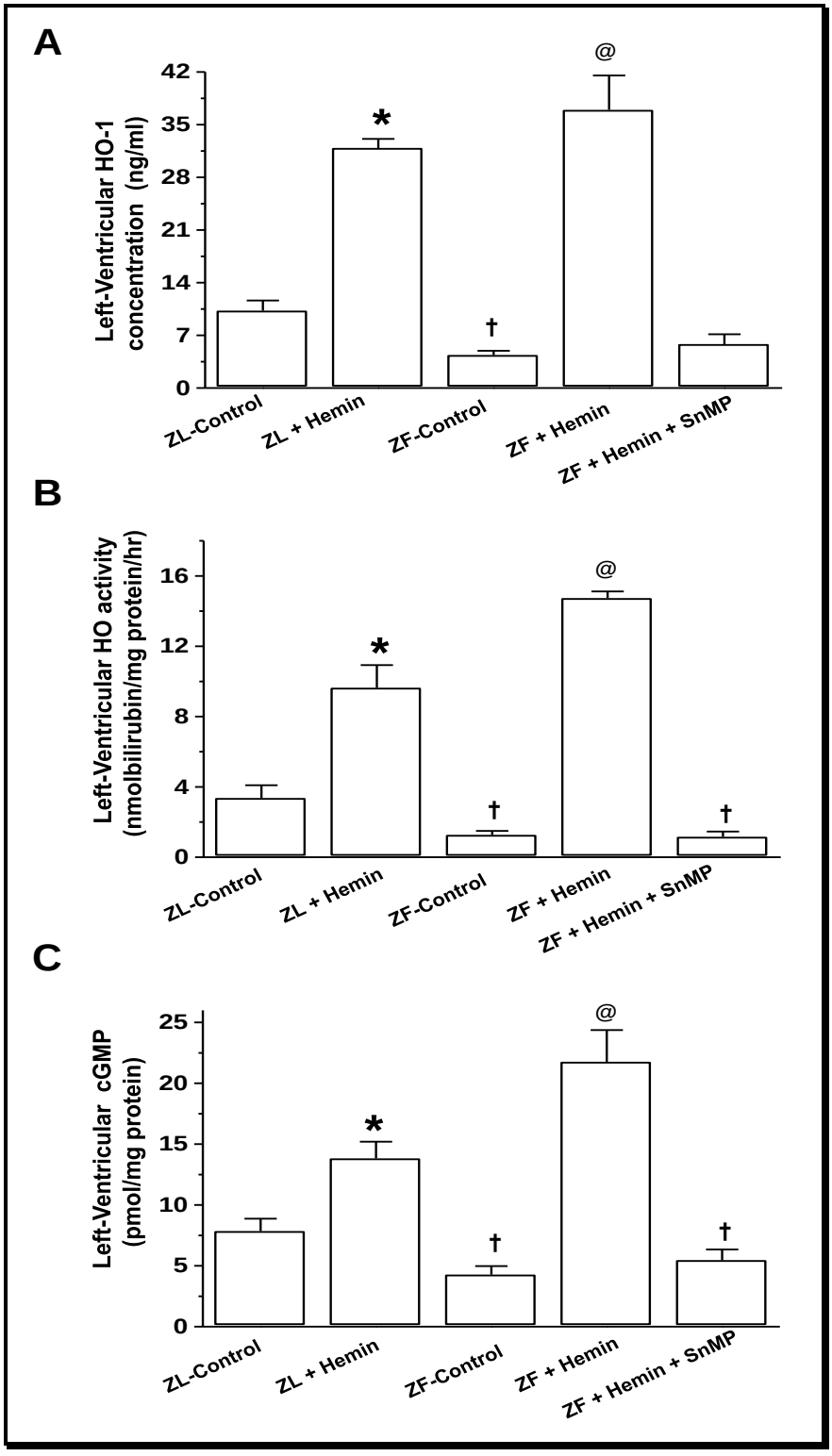

Figure 2-1: Effects of the HO inducer, hemin and the HO inhibitor, SnMP on HO-1, HO-activity and cGMP in the left ventricle of ZLs and ZF rats. (**A**) Hemin enhanced HO-1, whereas SnMP

nullified the effects of hemin. (**B**) Hemin increased HO-activity, while SnMP abolished the hemin effect. (**C**) Hemin enhanced cGMP, which however was abolished by SnMP. Bars represent means ± SEM; *n=6* rats per group (*p<0.01 *vs* ZL-Control; †p<0.05 *vs* ZL-Control; @p<0.01 *vs* ZF+Hemin+SnMP or ZF-control).

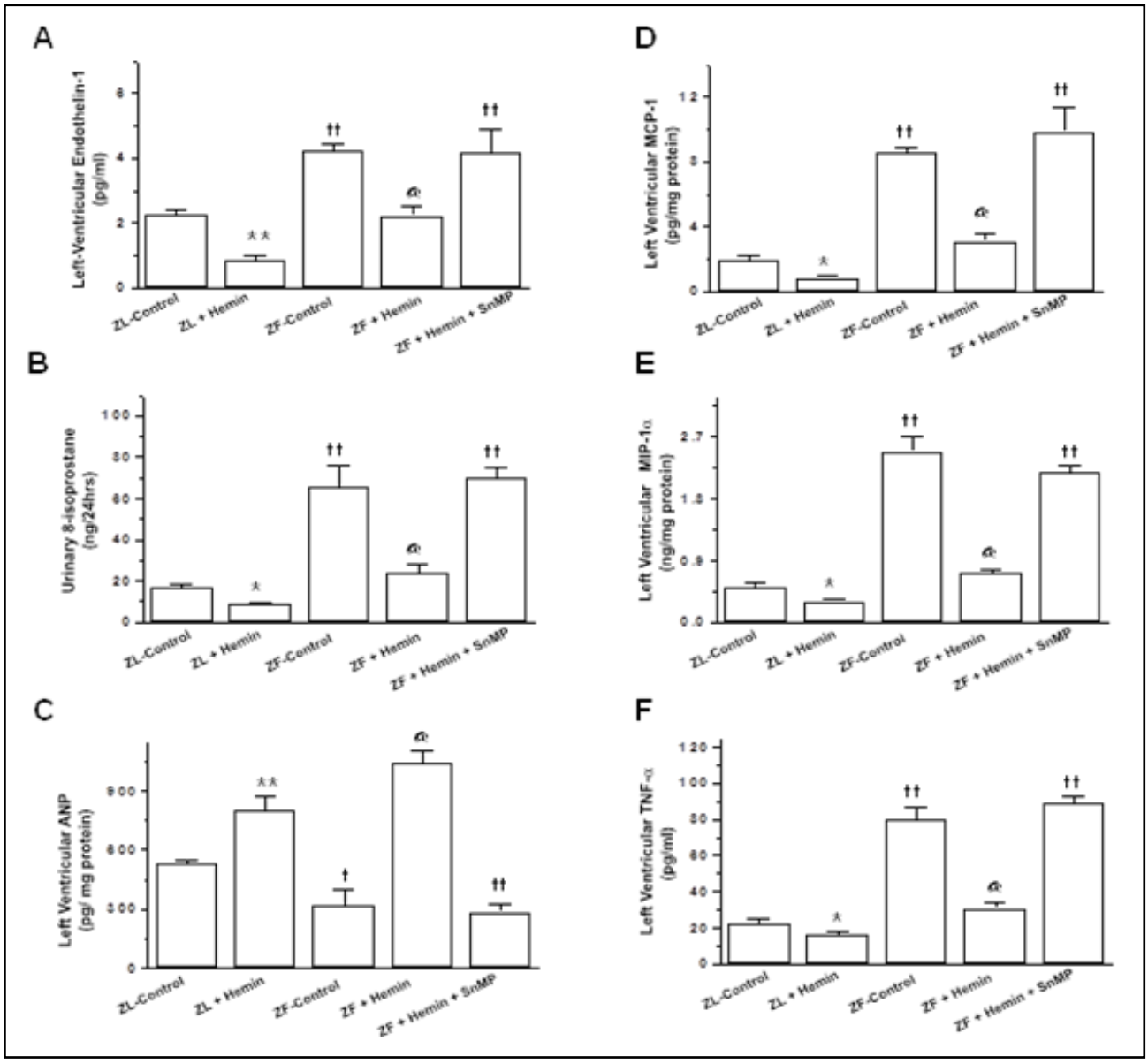

Figure 2-2: Effects of the HO inducer, hemin and the HO inhibitor, SnMP on endothelin-1, ANP, MCP-1, MIP1-α and TNF-α in left-ventricular tissue from ZLs and ZF rats. Hemin therapy (**A**) reduced endothelin-1, (**B**) attenuated 8-isoprostane, (**C**) increased ANP, (**D**) suppressed

MCP-1, (**E**) abated MIP-1α and (**F**) reduced TNF-α, whereas SnMP abolished the hemin effects. Bars represent means ± SEM; *n=6* rats per group (*p<0.05, **p<0.01 *vs* ZL-Control; †p<0.05, ††p<0.01 *vs* ZL-Control; @p<0.01 vs ZF+Hemin+SnMP or ZF-control).

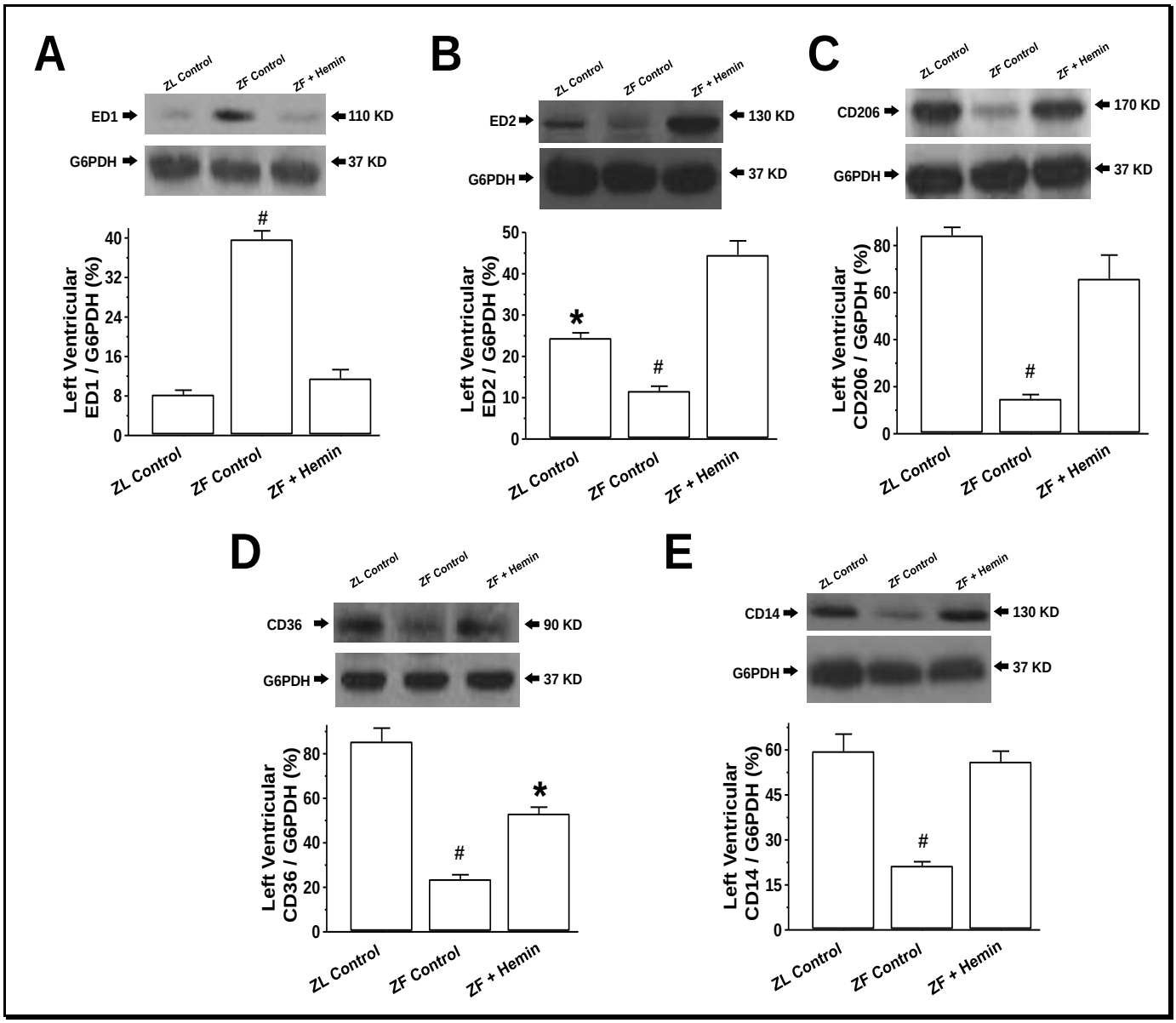

Figure 2-3: Effects of hemin on ED1, ED2, CD206, CD36 and CD14 in left-ventricular tissue from ZLs and ZF rats. Hemin therapy (**A**) abated ED1, but (**B**) enhanced ED2, (**C**) increased CD206, (**D**) enhanced CD36, and (**E**) increased CD14 in ZF rats. Bars represent means ± SEM; *n=4* rats per group (*p<0.01 *vs* all groups; #p<0.01 *vs* all groups).

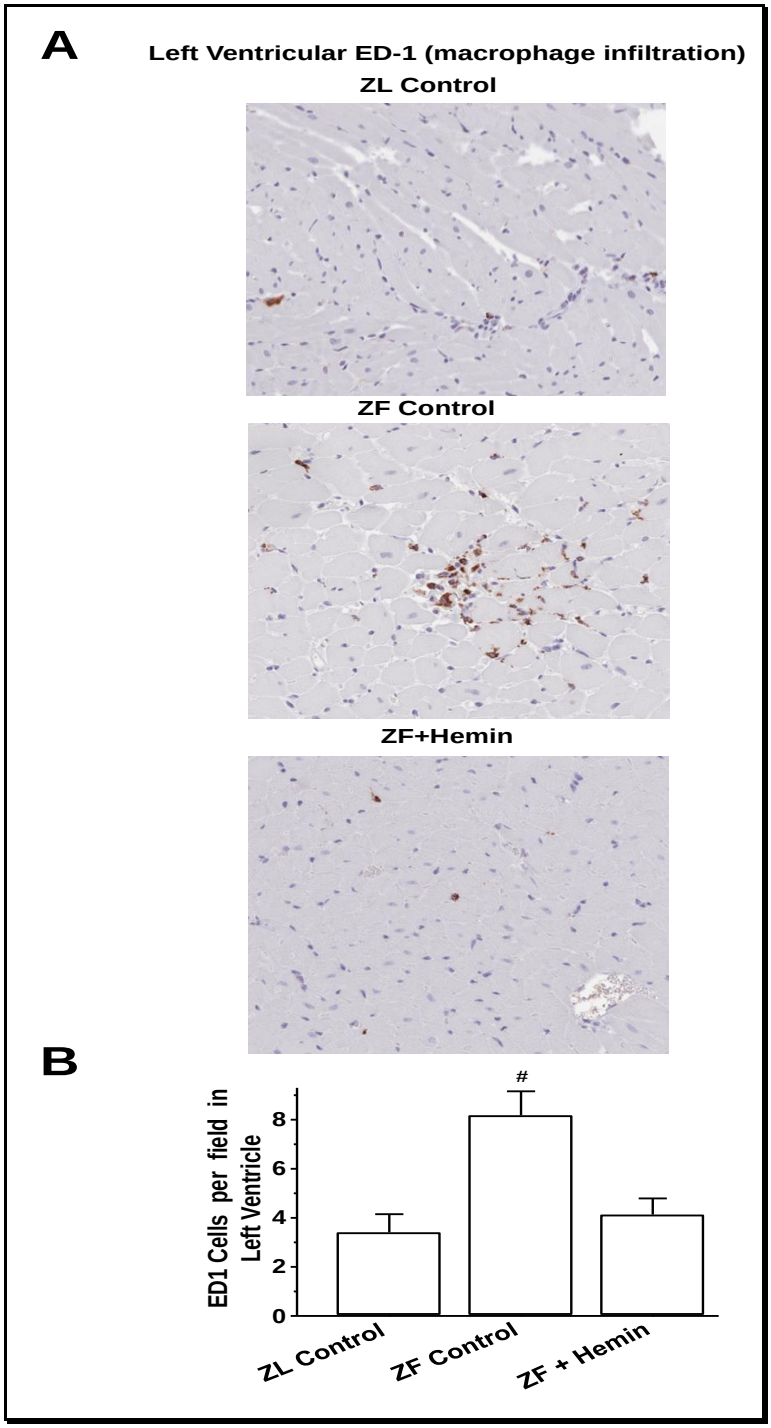

Figure 2-4: Representative photomicrographs of cross-sections of the left ventricle showing macrophage infiltration (ED1-positive cells stained dark brown) (magnification×200). (**B)** Quantitative analyses of macrophage infiltration per field indicating that in ZF rats the number of ED1-positive dark-brown cells (macrophage infiltration) was markedly elevated as compared to

ZL-control, but interestingly were significantly attenuated by hemin therapy. Bars represent means ± SEM; *n=6* rats per group ($^{\#}p<0.01$ *vs* all groups).

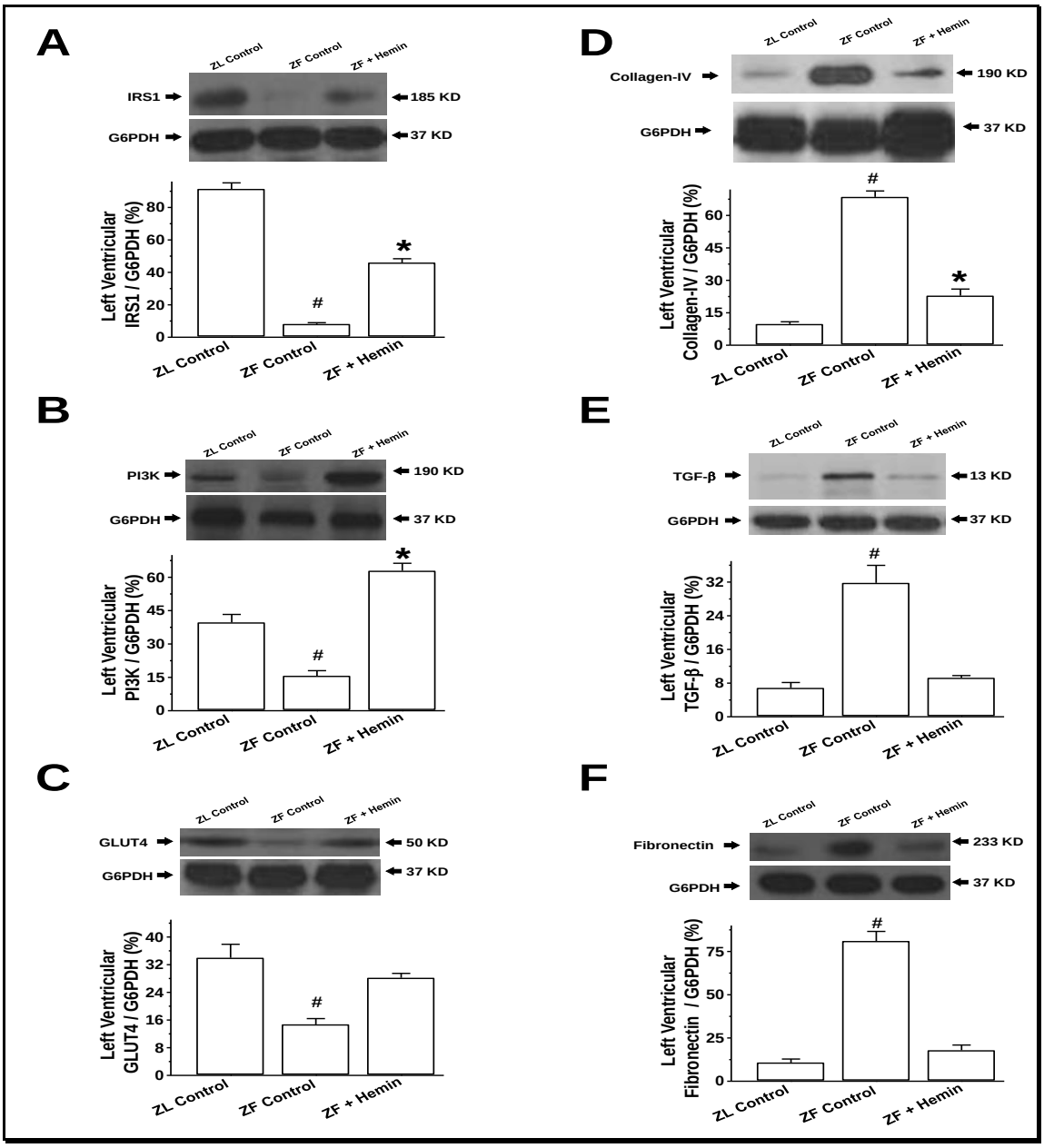

Figure 2-5: Effects of hemin on the expression of important proteins of the insulin signal transduction pathway such as IRS-1, PI3K, GLUT4 and the expression of profibrotic/extracellular matrix proteins including, collagen-IV, TGF-α and fibronectin in left-ventricular tissue from ZLs and ZF rats. Representative Western immunoblotting and relative

densitometry of the expressed proteins normalized by G6PHD indicates that hemin therapy significantly (**A**) enhanced IRS-1, (**B**) increased PI3K, (**C**) upregulated GLUT4, but (**D**) abated Collagen-IV, (**E**) reduced TGF-β, and (**F**) suppressed fibronectin in ZF rats. Bars represent means ± SEM; *n=4* rats per group (*p<0.01 *vs* all groups; #p<0.01 *vs* all groups).

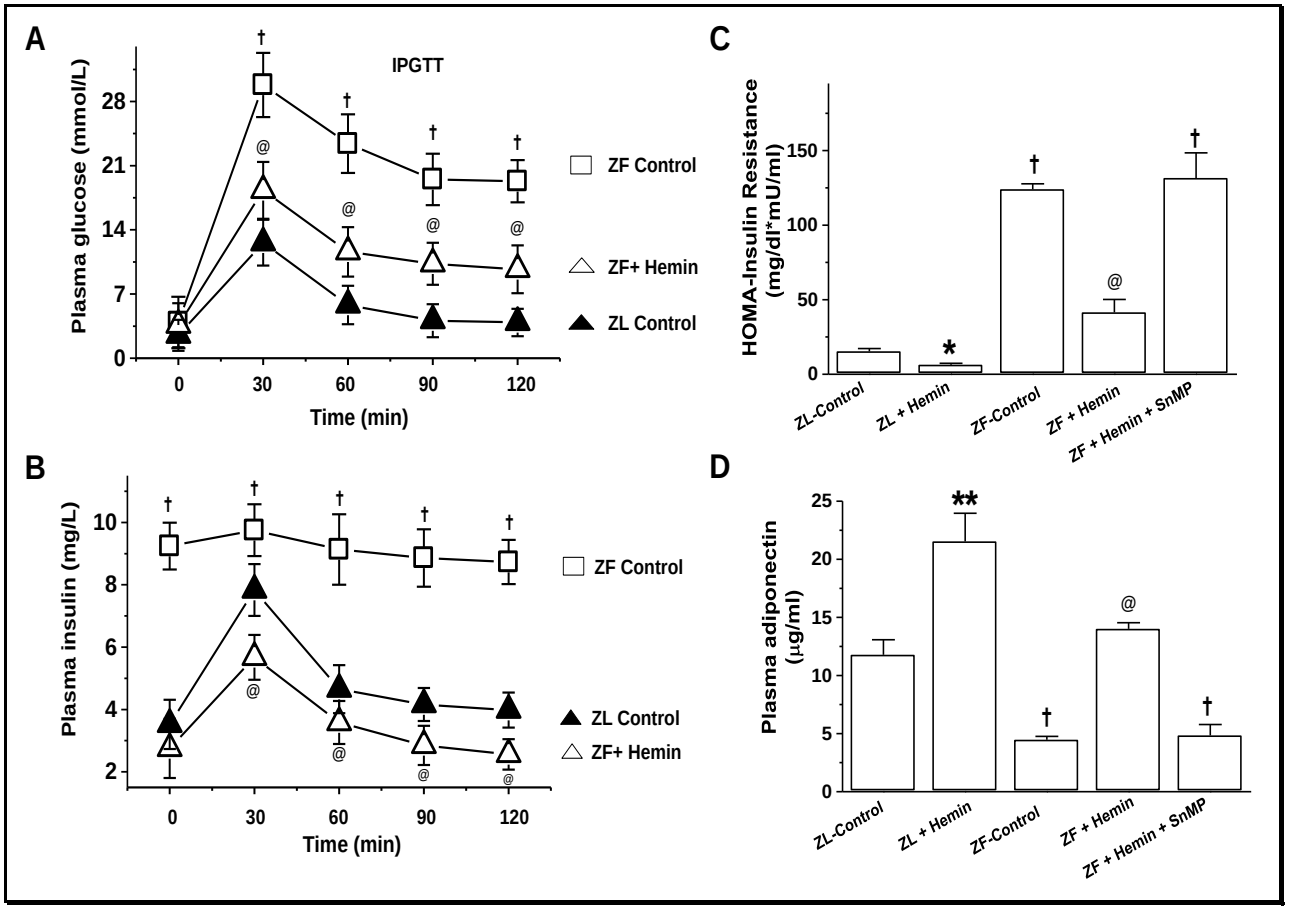

Figure 2-6: Effects of hemin on glucose tolerance, insulin resistance (HOMA-IR index) and adiponectin. Hemin therapy (**A**) improved glucose tolerance (IPGTT), (**B**) increased glucose-stimulated insulin release, (**C**) reduced insulin resistance and (**D**) increased adiponectin. Bars represent means ± SEM; *n=6* rats per group (*p<0.05, **p<0.01 *vs* ZL-Control; †p<0.01 vs. ZL-Control; @p<0.01 *vs* ZF+Hemin+SnMP or ZF-control).

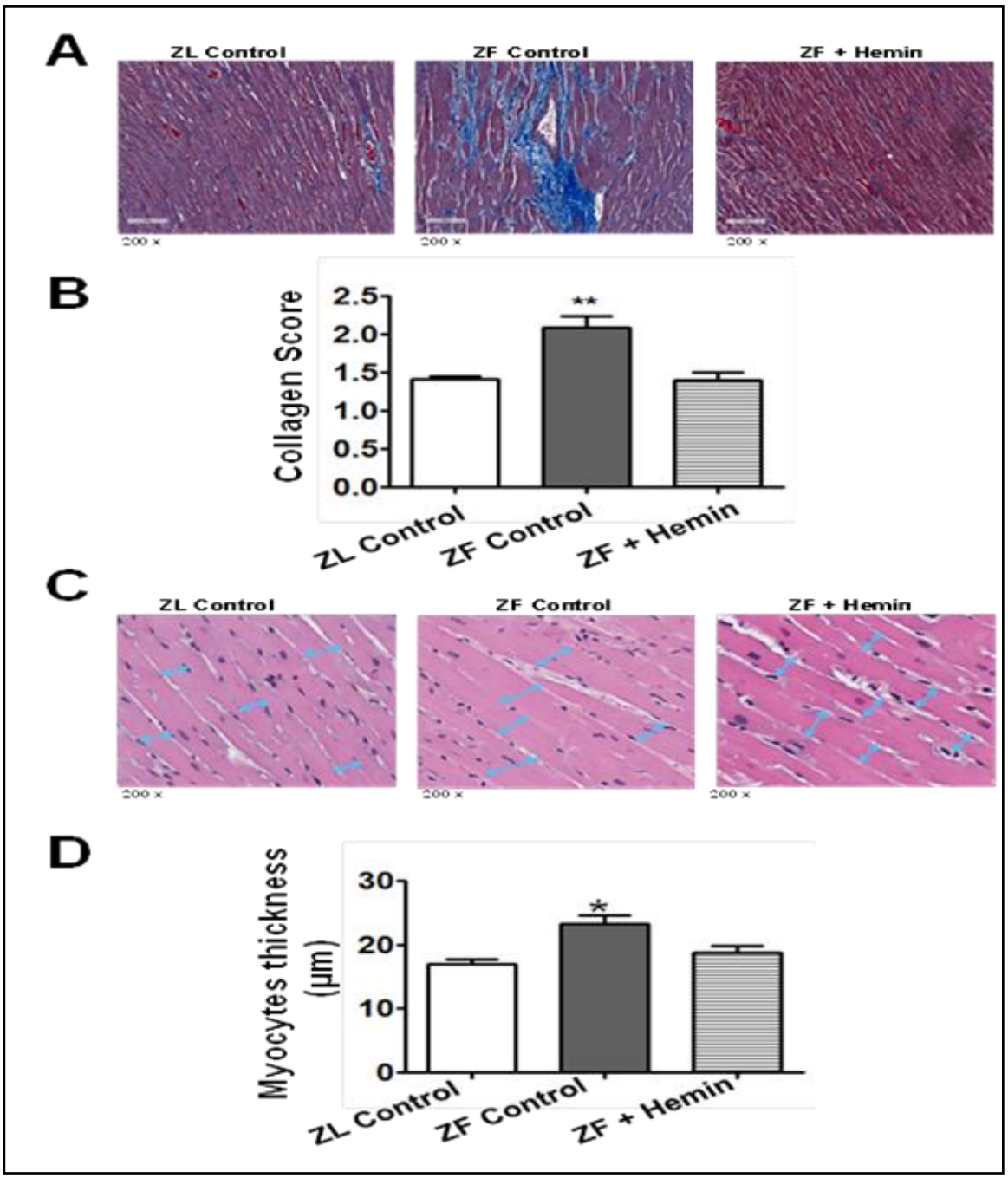

Figure 2-7: Effect of hemin on histological lesions in the left ventricle of ZLs and ZF rats. (**A**) Representative Mason's trichrome-stained images revealing severe cardiac muscle scaring and collagen deposition in ZF rats. (**B**) Semi-quantitative evaluation showed that hemin therapy reduced collagen deposition in ZF rats. (**C**) Representative hematoxylin and eosin-stained images revealing severe longitudinal muscle-fiber thickness in ZF rats. (**D**) Quantitative evaluation

showed that hemin reduced longitudinal muscle-fiber thickness. ± SEM; *n=6* rats per group (*p<0.05, **p<0.01 *vs* all groups).

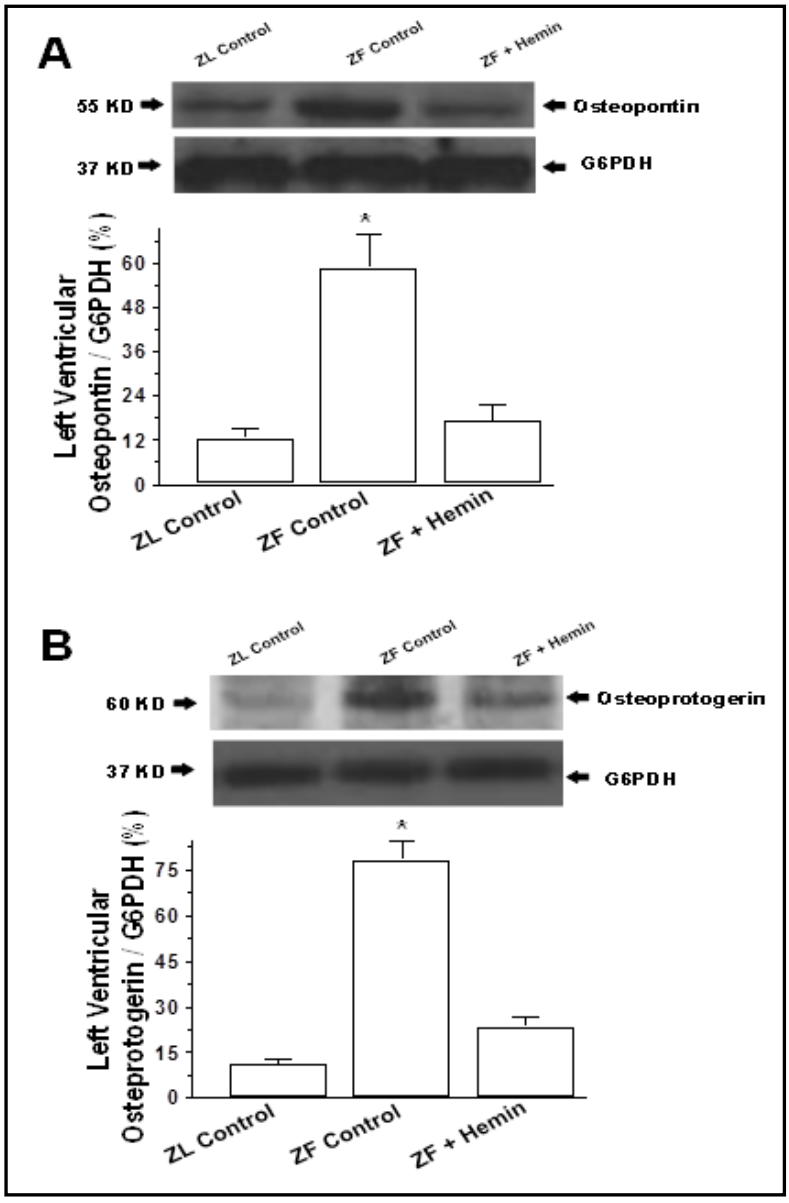

Figure 2-8: Effect of hemin on markers of heart failure such as osteopontin and osteoprotegerin in the left ventricle of ZLs and ZF rats. (**A**). Representative Western immunoblotting and relative densitometry of the expressed proteins normalized by G6PHD indicates that hemin therapy significantly (**E**) abated osteopontin and (**F**) reduced osteoprotegerin in ZF rats. Bars represent means ± SEM; *n=4* rats per group (*p<0.01 *vs* all groups).

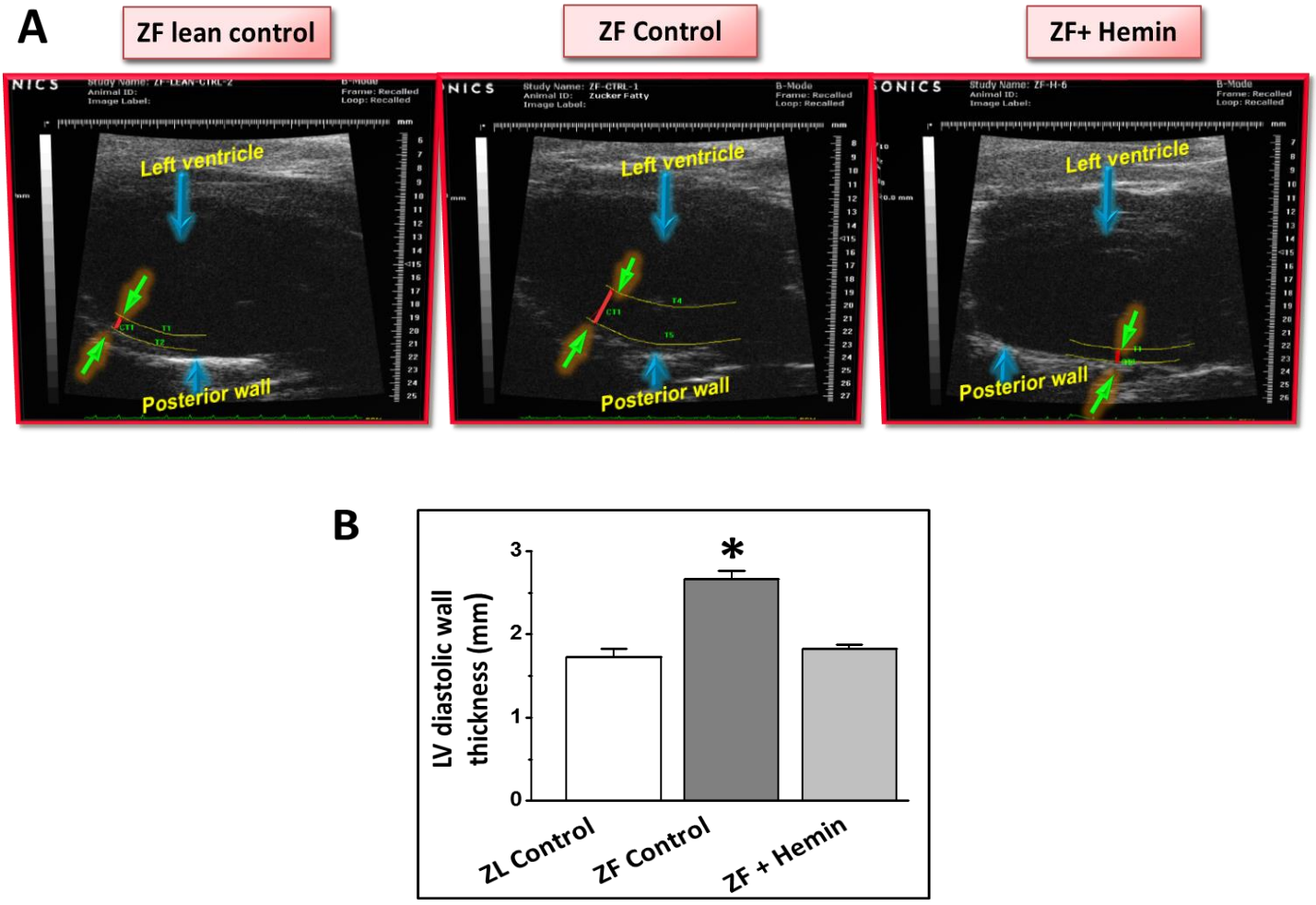

Figure 2-9: Hemin therapy suppressed left-ventricular free wall thickness in ZF rats (**A**). B-Mode image of left ventricle in the long axis view - Diastolic (**B**) Quantitative analyses indicates that hemin therapy suppressed left ventricle free wall thickness in ZF rats. Bars represent means ± SEM; *n=6* rats per group (*$P < 0.05$ vs all groups).

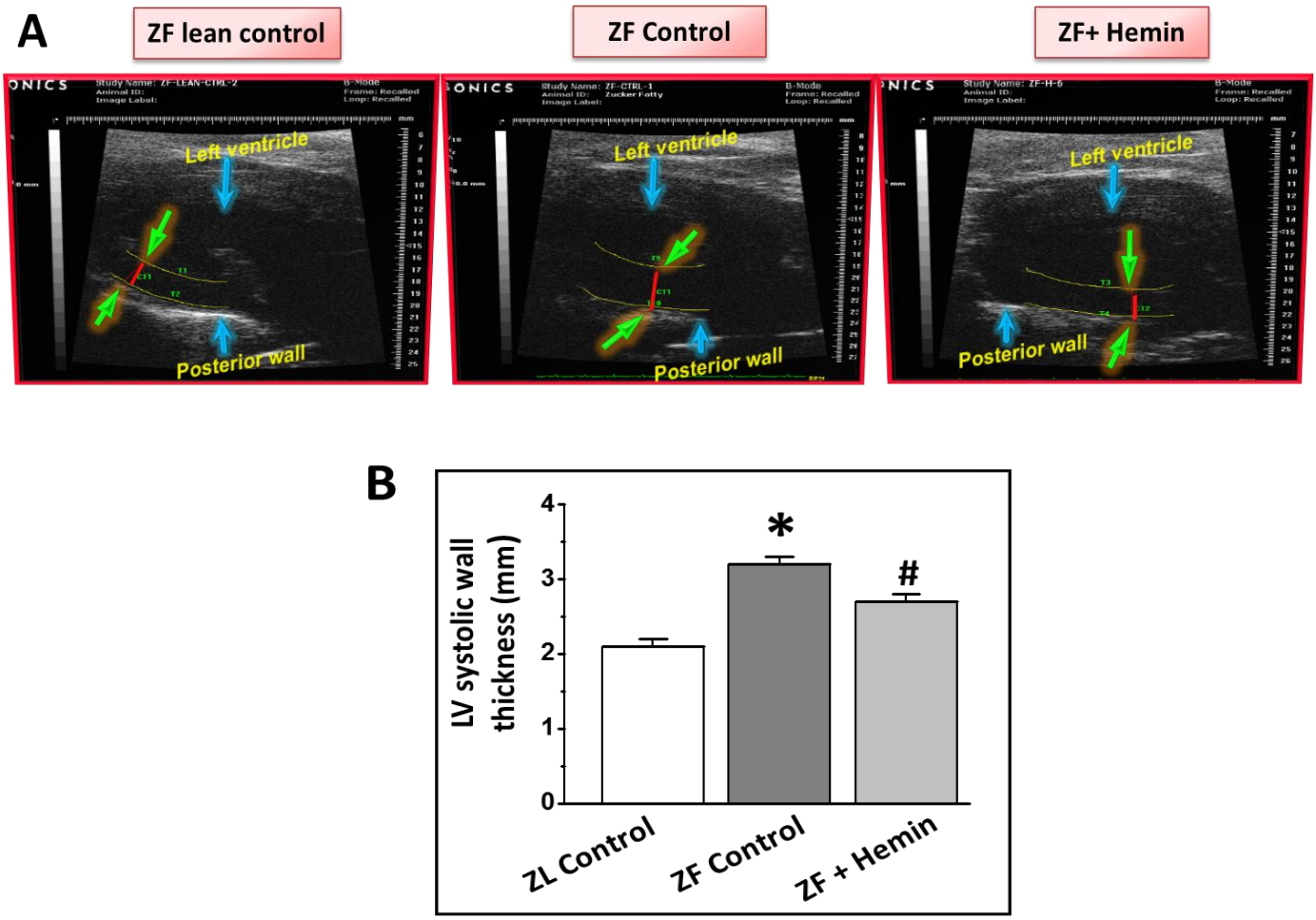

Figure 2-10: Hemin therapy suppressed left-ventricular free wall thickness in ZF rats (**A**). B-Mode image of left ventricle in the long axis view - Systolic (**B**) Quantitative analyses indicates that hemin therapy suppressed left ventricle free wall thickness in ZF rats. Bars represent means ± SEM; *n=6* rats per group (*$P < 0.05$ vs all groups, $^{\#}P < 0.05$ versus all groups).

Parameters	Animal groups					
	ZL Control	ZL + Hemin	ZF Control	ZF + Hemin	ZF + Hemin + SnMP	ZF + Vehicle
Body weight (g)	472.9 ± 9.7	445.5 ± 11.6†	746.8 ± 21.5§	685.9 ± 14.7$^{\$}$	691.4 ± 15.2$^{\$}$	702.8 ± 24.6
Fasting glucose (mmo/L)	7.2 ± 0.6	6.5 ± 0.3*	8.2 ± 0.5§	6.9 ± 0.4*	8.5 ± 0.4 *	8.1 ± 0.4
Heart weight (g)	1.5 ± 0.03	1.1 ± 0.02†	3.4 ± 0.06§	2.0 ± 0.04$^{\$}$	3.0 ± 0.03$^{\$}$	3.1 ± 0.04
Cardiac hypertrophy (g/Kg body weight)	3.1 ± 0.07	2.5 ± 0.06*	4.6 ± 0.16§	2.9 ± 0.08*	4.4 ± 0.17*	4.4 ± 0.09

$^{\dagger}p<0.05$ vs ZL; $^{\$}p<0.05$ vs ZF; $^{\S}p<0.05$ vs ZL Control; $^{*}p<0.05$, **$p<0.01$ vs ZF-control or ZL-Control, n=6 per group

Table 2-1: Effect of hemin and stannous mesoporphyrin (SnMP) on physiological and biochemical variables in Zucker fatty (ZF) and Zucker lean (ZL) rats.

Parameters	Experimental groups			P-value		
	ZF	ZL	ZF + Hemin	ZF *vs* ZL	ZF *vs* ZF+ Hemin	Effect of hemin on ZF
Arterial systolic pressure (mmHg)	153 ± 5.2	124 ± 3.4 #	134 ± 6.3 *	0.001	0.018	Reduced by 12.4 %
Arterial diastolic pressure (mmHg)	109 ± 3.2	92 ± 2.5 #	96 ± 4.7 *	0.003	0.024	Reduced by 11.9 %
Mean arterial pressure (mmHg)	123 ± 3.8	102 ± 2.7 #	109 ± 5.2 *	0.002	0.022	Reduced by 11.4 %
Total peripheral resistances (mmHg.min/mL)	1.7 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	0.060	0.080	Reduced by 17.6%
LV developed pressure (mmHg)	161 ± 4.1	136 ± 8.0 #	140 ± 5.8 *	0.012	0.030	Reduced by 13.0 %
+ $dp/dt_{(max)}$ (mmHg/sec)	3634 ± 127	3050 ± 200 #	3050 ± 169 *	0.027	0.027	Reduced by 16.1 %
Heart rate (beats/min)	328 ± 9.2	331 ± 7.2	289 ± 14.2 *	0.814	0.020	Reduced by 11.9 %
LV diastolic wall thickness(mm)	2.7 ± 0.1	1.7 ± 0.1 #	1.8 ± 0.1 *	0.0001	0.0001	Reduced by 33.3%
LV systolic wall thickness (mm)	3.2 ± 0.1	2.1 ± 0.1 #	2.7 ± 0.1 *	0.0001	0.007	Reduced by 15.6 %
LV end diastolic volume (ml)	0.33 ± 0.01	0.40 ± 0.04	0.38 ± 0.02	0.075	0.169	Increased by 15.2%
LV end systolic volume (ml)	0.11 ± 0.01	0.16 ± 0.01 #	0.14 ± 0.01	0.001	0.089	Increased by 27.3 %
Stroke volume (ml)	0.22 ± 0.01	0.24 ± 0.02	0.25 ± 0.02	0.564	0.311	Increased by 13.6 %
Cardiac output (ml/min)	72.8 ± 3.6	78.9 ± 9.0	81.8 ± 9.8	0.519	0.412	Increased by 12.4 %

Table 2-2: Effect of hemin therapy on Hemodynamic and Echocardiographic Parameters.

Values for each echocardiography and hemodynamic end-point were averaged for each rat and the mean values used in statistical analyses, with *n* = number of rats. Differences among treatment groups were compared using 1-way ANOVA followed by Fisher's Least Square Difference (LSD) posteriori tests. Differences of $p<0.05$ were considered statistically significant. Values are means ± SE; *n* = 6 per group. $^{*}P < 0.05$ vs. control ZF rats, $^{\#}P < 0.05$ vs. control ZL rats. n=6 per group

LV, left ventricle; + dp/dt (max), maximal rate of increase in left-ventricular pressure.

Chapter 3

The heme oxygenase system selectively enhances anti-inflammatory M2 macrophage phenotype, reduces pericardial adiposity and improves diabetic cardiomyopathy in Zucker diabetic fatty rats

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This chapter has been published as a paper in

***The Journal of Pharmacology and Experimental Therapeutics*, May 2013; 345:239–249**

My contribution to this paper: (Jadhav et al., JPET.2013 May; 345(2):239-49) was co-authored with Ashok Jadhav, Paul Lee and my supervisor Joseph Fomusi Ndisang. In this manuscript, I and Ashok Jadhav were equal contributors. This chapter unveils the beneficial effects of hemin therapy against pericardial adiposity, dysfunctional insulin signaling and diabetic cardiomyopathy. The experimental approach was designed in accordance with objectives as described in chapter 1. In this study, I was responsible for performing experiments, collection of all data, statistical analyses, making figures and tables. As first co-author, I contributed to the writing of the manuscript, synthesized information and revised the manuscript with input from all co-authors. Ashok Jadhav (post-doctoral fellow) participated in performing experiments, data analysis and manuscript writing. Dr. Paul Lee (Assistant Professor) was

responsible for teaching me some of the techniques, performed experiments, analyzed the data and provided critical input towards my experimental work. My supervisor Joseph Fomusi Ndisang participated in research design, performed data analysis and provided critical input in manuscript writing.

3.1 Abstract

Cardiac function is adversely affected by pericardial adiposity. We investigated the effects of the heme-oxygenase (HO) inducer, hemin on pericardial adiposity, macrophage polarization and diabetic-cardiomyopathy in Zucker-diabetic-fatty rats (ZDF). Echocardiographic, quantitative-RT-PCR, Western-blot, EIA and spectrophotometric analysis were used. In ZDF-rats, hemin administration increased HO-activity, normalized glycaemia, potentiated insulin-signaling by enhancing insulin-receptor-substrate-1 (IRS-1), phosphatidylinositol-3-kinase (PI3K) and protein-kinase-B (PKB), suppressed pericardial adiposity and reduced several pro-inflammatory/oxidative mediators including, NF-κB, c-Jun-N-terminal-kinase (c-JNK), endothelin (ET-1), TNF-α interleukin (IL)-6, IL-1β, activating-protein (AP-1) and 8-isoprostane, whereas the HO-blocker, stannous-mesoporphyrin (SnMP) nullified the hemin-effects. Furthermore, hemin reduced the pro-inflammatory macrophage-M1-phenotype, but enhanced M2-phenotype that dampens inflammation. These were associated with improved ejection-fraction and cardiac-output, but reduced total peripheral resistance. Interestingly, the hemin effects were less pronounced in Zucker-lean rats with healthy status, suggesting greater selectivity of HO in unhealthy ZDF-rats. Since NF-κB activates TNF-α, IL6 and IL-1β, while TNF-α, JNK and AP-1 impair insulin-signaling, and the high levels of these cytokines in obesity/diabetes would create a vicious cycle that together with 8-isoprostane and

ET-1 exacerbates cardiac injury, compromising its function. Therefore, the concomitant reduction of pro-inflammatory cytokines and macrophage infiltration coupled to increased expression of IRS-1, PI3K, PKB and cardiac hemodynamics may account for enhanced glucose metabolism and improved cardiac function. The preferential polarization of macrophages towards anti-inflammatory M2-phenotype in cardiac tissue, suppression of pericardial adiposity and hemin-dependent improved cardiac function in ZDF-rats are novel findings. Collectively, our study unveils the beneficial effects of hemin on pericardial-adiposity, impaired insulin-signaling and diabetic-cardiomyopathy, and suggests that the multifaceted mechanisms include the suppression of inflammatory/oxidative mediators.

3.2 Introduction

The inflammatory and metabolic systems have been evolutionarily well-conserved in species, and are fundamental for survival (402). However, these systems can be offset by obesity or nutrition-overload leading to inflammation in metabolic sites like the adipose tissue and skeletal muscles. Generally, obesity and insulin resistance are closely associated with a state of low-grade inflammation due to incessant activation of a wide variety of inflammatory mediators including nuclear-factor kappa B (NF-κB), tumor-necrosis factor-alpha (TNF-α and c-Jun-N-terminal kinase (JNK) (5, 76, 92, 136, 403-410). Moreover, NF-κB stimulates TNF-α, interleukin (IL)-6 and IL-1β, which in turn may activate JNK to create a vicious cycle that may aggravate insulin resistance and tissue damage (5). These destructive processes may be further exacerbated by macrophage infiltration, an event characterized by elevated levels of ED-1 (ED-1 is the primary antibody for activated macrophages (411). Generally, macrophages express distinct patterns of surface receptors when responding to different stimuli. Currently, two distinct

polarization states of macrophages, “classical” or M1 and “alternative” or M2 have been characterized (5, 412). While the M1 phenotype promotes inflammation, the M2 phenotype dampens inflammatory events. Therefore, the concomitant reduction of M1 phenotype, NF-κB, TNF-α, JNK, IL6 and IL-1β would limit tissue insults and decrease the oxidative destruction of important metabolic regulators like adiponectin and insulin in type-2 diabetes (T2D) (413, 414).

Emerging evidence indicates that adipocytes from different body compartments have distinct inflammatory phenotypes based on their anatomical location (415). Generally, pericardial or ectopic adiposity is more malignant than subcutaneous adiposity, although they are both implicated in the pathogenesis of obesity-related cardio-metabolic complications like insulin resistant T2D and coronary artery disease in lean and obese individuals (8, 415). Although we recently reported the insulin sensitizing effects of the heme oxygenase (HO) inducer, hemin, in Zucker diabetic Fatty rats (ZDF) (200), the effects of the HO system on pericardial adiposity remains largely unclear. Similarly, the effect of upregulating the HO system with hemin on macrophage polarization in cardiac tissue has not been reported. Whether hemin therapy will improve cardiac function in ZDF after suppressing the M1 phenotype, NF-κB, TNF-α, JNK, IL6 and IL-1β in the left ventricle will be investigated. Therefore this study was designed to investigate the role of hemin on pericardial adiposity and the mechanisms by which hemin ameliorates diabetic cardiomyopathy in ZDF, an insulin-resistant T2D model with diabetic cardiomyopathy (374, 416).

3.3 Materials and methods

3.3.1 Animal treatment and biochemical assays

Our experimental protocol was approved by the University of Saskatchewan Standing Committee on Animal Care and Research Ethics, which is in conformity with the Guide for Care

and Use of Laboratory Animals stipulated by the Canadian Council on Animal Care and the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Twelve-week-old male ZDF rats, a genetically obese leptin receptor-deficient (*fa/fa*) animal model of T2D and their corresponding age/sex-matched Zucker-lean (ZL) littermates were purchased from Charles River (Willington, MA, USA). The animals were housed at 21°C with 12-hour light/dark cycles, fed with standard laboratory chow and had access to drinking water *ad libitum*. The drugs used for this study were hemin, an inducer of HO (30 mg/kg i.p., Sigma, St Louis, MO) and stannous-mesoporphyrin (SnMP), a blocker of HO (2 mg/100 g body weight i.p, Porphyrin Products, Logan, UT). The doses of SnMP and hemin used in this study have been shown to be effective in previous studies (48, 192, 200, 202, 417). Hemin and SnMP were prepared as we previously reported and were given biweekly for eight weeks (48, 187, 192, 200, 202, 417). Hemin has been approved by the FDA against porphyria (56), while SnMP has successfully completed phase-III clinical trials (418). At 14 weeks of age, the animals were randomly assigned to five experimental groups (*n=6-14* per group): **(A)** controls (ZDF and ZL), **(B)** hemin-treated ZDF and ZL, **(C)** ZF+Hemin+SnMP, **(D)** ZDF+SnMP and (**E**) ZF+vehicle dissolving hemin and SnMP.

During the treatment period glucose was monitored weekly after 6 hrs of fasting in metabolic cages with a glucose-meter (BD, Franklin Lakes, NJ, USA). Body weight was also measured on a weekly basis. At the end of the 8-week treatment period, the animals were 22 weeks of age. A day prior to sacrifice, the animals were fasted in metabolic cages for 24-hr urine collection and weighed. Systolic blood pressure was determined by non-invasive tail-cuff method (Model 29-SSP, Harvard Apparatus, Montreal, Canada). Plasma was collected by intra-cardiac puncture and the pericardial fat pad and the heart were isolated, cleaned and weighed

using an analytical balance (Precisa Instruments Ltd, Switzerland). The atria were removed from the heart and the right ventricle free wall separated from the left ventricle including the septum as we previously reported (187).

Left-ventricular HO activity was evaluated spectrophotometrically as bilirubin production using our established method (187, 196, 200, 202), while HO-1 (Stressgen-Assay Design, Ann Arbor, MI, USA), TNF-α, IL-6, IL1-β (Immuno-Biological Laboratories Co Ltd, Takasaki-shi, Gunma, Japan) were measured by commercially available ELISA kits. Other biochemical parameters such as left-ventricular 8-isoprostane and endothelin-1 were measured by EIA (Cayman Chemical, Ann Arbor, MI) as we previously reported (187, 196).

3.3.2 Measurement of cardiac hemodynamic parameters (invasive hemodynamic measurements)

The animals were anesthetized with isoflurane through inhalation using a vaporizer. The vaporizer was set at 5.0% isoflurane/L O_2 (g) to induce, and 2.0%/L O_2 (g) for maintenance of anesthesia. To measure the hemodynamic parameters, a Millar Mikro-Tip ultra-miniature tip sensor pressure transducer catheter (SPR-407) with a catheter size of 1.5 French was inserted through the right carotid artery and advanced into the left-ventricular chamber to measure the left-ventricular hemodynamic parameters. After positioning of the pressure transducer into the left ventricle, the rat was allowed to stabilize for 10 minutes before the left-ventricular hemodynamic measurements were recorded. Arterial blood pressure was subsequently recorded by pulling the catheter out of the ventricular chamber into the aorta. Central venous pressure was measured by inserting the miniature tip sensor pressure transducer catheter into the superior vena cava through the right jugular vein. Data was acquired on a Biopac Data Acquisition system and assessed on AcqKnowledge software as previously reported (187, 419).

3.3.3 Echocardiography

Echocardiographic evaluation was done in rats using a Vevo 660 high frequency ultrasound machine (Visual Sonics, Markham, ON) equipped with B-mode imaging. For consistency, all measurements were done by the same investigator and all ultrasound procedures did not exceed 30 minutes for each rat. Prior to ultrasound experiments, anesthesia was induced with 5% isoflurane, maintained at 0.5-1% isoflurane (Abbott Laboratories, Saint-Laurent, QC), and the rats placed with the ventral side up on an electrocardiogram (ECG) plate (Visual Sonics, Markham, ON) with each paw covered with electrode cream (Sigma Crème, Parker Laboratories, Fairfield, NJ) and secured to ECG contacts with surgical tape in order to monitor heart rate throughout each experiment. A temperature probe was inserted rectally to maintain internal body temperature at 37°C. To prevent artifact with this high resolution ultrasound system, the animal was depilated by wiping from the chest area with depilatory cream (Nair, New York, NY). Thereafter, Eco Gel 200 (Eco-Med Pharmaceuticals, ON) was then applied to the thorax for ultrasound.

A RMV 710B scan head was used to gather all parasternal short and long-axis views of the rat ventricle in B-mode. The areas of three different short axis views along the ventricle were measured and designated as A_1, A_2, A_3 while the ventricular length of one long axis view was measured and divided by four to give ventricular height (h). Using different short axis views at different levels of the ventricle compensates for irregularities in ventricular shape and greatly increases accuracy of chamber volume measurements (283). All of these values were measured at both systole and diastole using Visual Sonics software (Markham, ON). With these values,

end systolic and diastolic volumes in units of cm^3 (equivalent to ml) were each calculated for the left ventricle using: *(Equation I)* $V = (A_1+A_2)\, h + ((A_3 h)/2) + (\pi/6(h^3))$

End systolic volume was subtracted from end diastolic volume to give stroke volume (V_S) in milliliters. Heart rate in beats per minutes for each rat was recorded at three different times throughout one imaging period and then averaged. Cardiac output (CO) in milliliters/minute was then determined using heart rate (f_H) and stroke volume (V_S): (Equation II) $CO = f_H * V_S$. Ejection fraction (%) for each rat was also calculated using stroke volume (V_S) and end diastolic volume (EDV): (Equation III) $E_f = V_S / EDV$

In order to measure left-ventricular free wall thickness, a clip in parasternal long axis view was obtained for all experimental groups. At least three individual images were exported from the clip at both systole and diastole using Premiere Elements 2.0 (Adobe, San Jose, CA) and then left-ventricular wall thickness determined using ImagePro 6.0 software (Bethesda, MD). Heart rate (f_H in beats per minute) was calculated from ECG traces during the blood pressure experiment.

3.3.4 Total RNA isolation and quantitative RT-PCR for NF-κB, AP-1 and JNK

The left ventricle was homogenized and quantitative RT-PCR done as we previously reported (187, 200, 202). Briefly, triplicate samples of 1 µl of cDNA each was ran using a template of 3.2 pmol of primers for NFκB (forward, 5'CATGCGTTTCCGTTACAAGTGCGA-3' and reverse 5'TGGGTGCGTCTTAGTGGTATCTGT-3'), AP-1 (forward,5'AGCAGATGCTTGAGTTGAG A G CCA3' and reverse 5'TTCCATGGGTCCCTGCTTTGAGAT-3'), JNK (forward 5′AAGCAGCAAGGCTACTCCTTCTCA-3′ and reverse 5′ATCGAGACTG CTGTCTGTGTCT GA-3′) and glyceraldehyde-3-phosohate dehydrogenase (5'TCTGGGATGGAATTGTGAGGGA

GA-3') in a final volume of 25 µl. Sequences of all primers were confirmed by the National Research Council of Canada, Saskatoon.

3.3.5 Western immunoblotting

The left-ventricular tissue was homogenized and centrifuged as previously described (187, 199, 200, 202). Primary antibodies (Santa Crutz Biotechnology, CA, USA) including ED-2 (sc-58956), ED-1 (sc-59103), phosphatidylinositol-3-kinase (PI3K) (sc67306), protein kinase-B (PKB) (sc9118) and insulin-receptor-substrate-1 (IRS-1) (8299) were used. Densitometric analysis was done with UN-SCAN-IT software (Silk Scientific, Utah, USA). GAPDH antibody (Sigma St Louis, MO, USA) was used as a control to ascertain equivalent loading.

3.3.6 Left-ventricular histology

The middle portion (midpapillary level) of the left ventricle of heart was separated, fixed in 10 % formalin phosphate buffer for 48 hrs., processed and paraffin embedded as we previously reported (187). Then sections of 5 µm thicknesses were cut and stained with hematoxylin and eosin for histological analysis. The left-ventricular sections were obtained from the middle portion of the left ventricle to avoid differences in regional cardiomyocyte size in different regions of the left ventricle. The cardiac sections were scanned using a microscope (Aperio Scan Scope Model CS, Aperio Technology Inc, CA, USA) and analyzed using Aperio Image Scope V11.2.0.780 software (Aperio, e-Pathology Solution, CA USA). Left-ventricular myocyte width longitudinal myocyte thickness was measured randomly in 20-30 cardiac muscle fibers from each left-ventricular tissue section. Muscle fiber thickness was quantified and analyzed between different groups. For consistency, myocytes were positioned perpendicularly

to the plane of the section with a visible nucleus and cell membrane clearly outlined. Unbroken areas were selected for measurement. All sections were imaged at 40X zoom (40X; 0.50 µm/pixel) in Aperio Image Scope using length measurement tool (µmeter).

Statistical analyses

All data were expressed as means ± SEM from at least six independent experiments unless otherwise stated. Statistical analyses were done using unpaired Student's *t*-test and analyses of variance in conjunction with Bonferroni test for repeated measures where appropriate. Group differences at the level of $p<0.05$ were considered statistically significant.

3.4 Results

3.4.1 Hemin therapy abated pericardial adiposity and restored normoglycemia in ZDF rats

ZDF rats were severely hyperglycemic with fasting glucose levels of 24.6 ± 3.1 mmol/L (**Table 3-1**), whereas their age/sex matched littermates' control-ZL were normoglycemic (7.2 ± 0.8 mmol/L). The 8-week regimen of hemin to ZDF reduced the elevated glycemia to physiological levels (24.6 ± 3.1 vs. 6.8 ± 1.3 mmol/L; $p<0.01$), whereas the co-treatment of hemin and the HO-blocker, SnMP abolished the effect of hemin, suggesting a role of the HO system on glucose homeostasis. Similarly, hemin treatment significantly reduced pericardial adiposity (1.85 ± 0.2 vs. 0.79 ± 0.13 g/Kg body weight, $p <0.01$) and cardiac hypertrophy (3.8 ± 0.3 vs. 2.4 ± 0.14 g/Kg body weight; $p<0.01$) in ZDF, whereas the co-administration of hemin and SnMP nullified the effect, suggesting a role of the HO system on the regulation of pericardial adiposity and cardiac hypertrophy. The vehicle dissolving hemin and SnMP had no effect on blood glycemia, pericardial adiposity and heart weight (**Table 3-1**).

Hemin therapy also affected ZL rats though less intensely. A slight but significant reduction of blood glucose, cardiac hypertrophy and pericardial adiposity was observed in ZL rats. These effects were abolished by SnMP (**Table 3-1**). In hemin-treated ZDF rats, blood glucose, cardiac hypertrophy and pericardial adiposity were reduced by 72.3, 36.8 and 57% respectively whereas in ZL rats these same parameters were reduced by 11.1, 14.8 and 25.4% respectively, suggesting greater selectivity of the actions of hemin in diseased conditions like the situation in ZDF rats, whereas less active in healthy status as in the case of ZL rats.

The HO inducer, hemin and HO blocker, SnMP also affected body weight. A slight body-weight loss (<10%) was observed in hemin- and SnMP-treated animals (**Table 3-1**). In ZL+Hemin, ZDF hemin, and ZF+Hemin+SnMP the loss of body weight was 2.5, 5.3 and 8.1% respectively. Although body-weight loss can affect blood glucose levels, it is unlikely in this case since the slight body-weight loss in hemin- and SnMP-treated were accompanied by opposite effects on glucose levels (**Table 3-1**). Accordingly, we observed a decrease of glucose levels in hemin-treated animals, but an increase in SnMP-treated animals suggesting that the HO system may be endowed with intrinsic anti-diabetic effects. The loss of body weight may not be due to toxicity as we recently showed that several indices of toxicity including plasma gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were within normal range (200).

Hemin therapy also improved cardiac hemodynamics (**Table 3-2)**. In hemin-treated ZDF, systolic blood pressure was reduced by 13.95%, while cardiac output increased by 8.2%. Interestingly, the lowering of systolic blood pressure was associated with a 12.2% decrease in total peripheral resistance, suggesting reduced afterload to the left ventricle (420). Correspondingly, a reduction of 8.9% in the rate of left-ventricular pressure development

(+dP/dt) was observed (**Table 3-2)**. Furthermore, hemin increased the left-ventricular ejection fraction by 5.4%, and this effect was accompanied by a 2.2 % reduction of left-ventricular systolic pressure (LVSP). Therefore, increased cardiac output coupled to the concomitant reduction of total peripheral resistance, +dP/dt and LVSP are indicative of improved cardiac function in hemin-treated ZDF.

3.4.2 Hemin therapy enhanced HO-1 and HO activity but abated ET-1 and 8-isoprostane in the left ventricle of ZDF rats

To investigate the role of the HO system in the improved cardiac function and insulin-signaling in ZDF, we measured HO activity, ET-1 and 8-isoprostane. Our results indicate that the basal levels of HO-1 and HO-activity in control-ZDF were significantly reduced by 2.13- and 1.98-fold respectively as compared to control-ZL rats. Hemin therapy markedly increased the depressed levels of HO-1 and HO activity in ZDF rats by 8.1- and 10.56-fold respectively (**Figs. 3-1A and 3-1B)**, whereas the co-treatment with the HO inhibitor, SnMP nullified the effects of the HO inducer, hemin. Similarly, treatment with SnMP alone depleted the basal levels of HO-1 and HO activity (**Figs.3-1A and 3-1B**). Hemin therapy also enhanced the levels of HO-1 and HO activity in ZL rats, although a greater increment was observed in hemin-treated ZDF animals (**Figs. 3-1A and 3-1B**). The higher magnitude of HO-signaling may be responsible for the more intense reduction of glycemic levels in ZDF rats as compared to ZL rats (**Table 3-1**). Alternatively, the less-preponderant increase of HO activity in ZL rats may suggest greater stability of the HO system in healthy conditions.

Given that elevated oxidative stress is among the causative factors of insulin resistance and cardiac dysfunction, we measured 8-isoprostane, an important marker of oxidative stress

(379). In ZDF rats, the basal levels of left-ventricular 8-isoprostane were markedly elevated, suggesting enhanced oxidative stress (**Fig. 3-1C**). Interestingly, hemin therapy significantly reduced 8-isoprostane by 57.6%. Contrarily, in SnMP+ZDF treated animals, the effect of hemin on 8-isoprostane was annulled and 8-isoprostane was reversed to comparable levels as observed in control-ZDF rats. On the other hand, in SnMP-treated animals, the levels of left-ventricular 8-isoprostane were further increased, suggesting that oxidative stress is further potentiated by blockade of basal HO activity (**Fig. 3-1C**). Hemin therapy also reduced 8-isoprostane in ZL rats, although less-intensely as compared to ZDF as only a 28.9% reduction was observed in hemin-treated ZL rats as compared to 57.6% hemin-treated ZDF rats.

Since 8-isoprostane stimulates ET-1 (378), and both ET-1 and 8-isoprostane are involved in the oxidative destruction of tissue, we also assessed ET-1 in the left ventricle. Our results indicate that the levels of ET-1 in ZDF rats were 2.7-fold higher than in control-ZL rats. Interestingly, hemin therapy greatly attenuated the elevated levels of left-ventricular ET-1 in ZDF rats, while SnMP abolished the effect of hemin (**Fig. 3-1D**). Hemin therapy also reduced ET-1 levels in ZL rats albeit to a lesser extent as compared to ZDF rats. Accordingly, a reduction of 25.2% of ET-1 was observed in hemin-treated ZL rats as compared to 54.2% hemin-treated ZDF rats.

Therefore, the preponderant increase of HO activity in hemin-treated ZDF as compared to hemin-treated ZL (**Figs. 3-1A**), coupled to the more accentuated reduction of left-ventricular 8-isoprostane (**Fig. 3-1C**) and ET-1 (**Fig. 3-1D)** may account for the greater anti-diabetic effect (**Table 3-1**) and improved cardiac effects (**Table 3-2**) in hemin-treated ZDF.

3.4.3 Hemin therapy suppressed pro-inflammatory cytokines that deregulate glucose metabolism cardiac function

TNF-α, IL-6 and IL-1β are cytokines that impair cardiac function and glucose metabolism (5, 421, 422), so we investigated whether the improvement in cardiac function and glucose metabolism in hemin-treated ZDF would be accompanied by reduction of these cytokines. Our results indicate that the levels of TNF-α, IL-6 and IL-1β in the left ventricle of control-ZDF rats were significantly elevated by 4.5-, 9.1- and 2.5-fold respectively as compared to control-ZL rats (**Fig. 3-2**). Treatment with hemin markedly reduced TNF-□, IL-6 and IL-1□ by 71.2, 51.3 and 56.8% respectively. In contrast, the co-application of the HO-inhibitor, SnMP with hemin reversed the effects of hemin (**Fig. 3-2A, 3-2B and 3-2C**), suggesting a role of the HO system in the regulation of these inflammatory cytokines. Hemin therapy also reduced the levels of TNF-α, IL-6 and IL-1β in the ZL rats, although less intensely. A reduction of 35.4, 37.0, 28.5% of TNF-α, IL-6 and IL-1β respectively was observed in hemin-treated ZL rats as compared to 71.2, 51.3 and 56.8% in hemin-treated ZDF rats.

3.4.4 Hemin abated transcription factors that impair insulin-signaling and cardiac function

Many inflammatory and oxidative transcriptions factors including NF-κB, AP-1 and JNK are implicated in tissue damage and insulin resistance (414, 423). In ZDF rats, quantitative real-time RT-PCR analyses indicated that the levels of NF-κB, AP-1 and JNK in the left ventricle were strikingly elevated (**Fig. 3-3**). Treatment with hemin reduced NF-κB and AP-1 by 2.5- and 2.9-fold respectively, while the HO inhibitor, SnMP, nullified the effects of hemin (**Fig. 3-3A and 3-3B**). Moreover, treatment with SnMP alone further enhanced NF-κB and AP-1 in ZDF rats by 20% and 31.9% respectively, suggesting the involvement of basal HO activity in the

regulation of these oxidative/inflammatory mediators. Furthermore, in ZDF rats, the basal expression of left-ventricular JNK, a substance that suppresses insulin biosynthesis (414), was markedly increased by 4.75-fold, but was abated by hemin (**Fig. 3-3C**). Hemin therapy also reduced NF-κB, AP-1 and JNK in ZL rats by 30.5, 24.8, and 26.1% respectively which were less-intense as compared to hemin-treated ZDF where the reduction of NF-κB, AP-1 and JNK were 59.3, 65.6 and 57.8%, suggesting greater selectivity of hemin in the diseased condition.

3.4.5 Hemin therapy abated inflammation but potentiated insulin-signaling agents

Given that macrophages are amongst the fundamental sources of many of the circulating inflammatory molecules in obesity, and are postulated to be causal in the development of insulin-resistant T2D (5, 412), we used specific markers (ED1 and ED2) to quantify the M1 pro-inflammatory phenotype (ED1) and the M2 anti-inflammatory phenotype (ED2). Our Western immunoblotting and relative densitometric analyses indicated that the expression of the pro-inflammatory M1 phenotype in control-ZDF was significantly elevated (**Fig. 3-4A**), but interestingly was abated by 40.1% by hemin therapy. On the other hand, the anti-inflammatory phenotype, M2, was significantly reduced in control-ZDF (**Fig. 3-4B**), but interestingly was enhanced by 61.3% by hemin therapy, suggesting that hemin may preferentially favor macrophage polarization towards the M2 anti-inflammatory phenotype as an alternative mechanism to counteract tissue insult.

Given that IRS1, PI3K and PKB are important proteins implicated in the insulin signal transduction pathway (Ndisang, 2010), we investigated the effect of hemin therapy on these proteins. Our results indicate that the expression of IRS1 in control-ZDF was depressed (**Fig. 3-4C**). However, hemin therapy greatly enhanced the expression of IRS1 bv 2.3-fold. Similarly

hemin therapy significantly increased the expressions of PI3K (**Fig. 3-4D**) and PKB (**Fig. 3-4E**) by 3.5-and 2.8-fold respectively.

3.4.6 Hemin therapy reduced longitudinal muscle fiber thickness in ZDF rat

Longitudinal muscle fiber thickness is a common pathophysiological feature of cardiac myocyte hypertrophy (187, 424, 425). In untreated ZDF rats, enlarged cardiomyocytes with increscent nuclei were evident, as compared to normal cardiomyocytes in ZL-control rats (**Fig. 3-5A**). The inter myofibril space was reduced in ZDF-control rats as compared to the age/sex-matched ZL rats (**Fig. 3-5A**). Indeed, a 44 % increase in cardiomyocyte longitudinal fiber thickness was observed in the ZDF-control rats as compared to ZL rats (21.9 ± 0.89 vs 15.2 ± 0.49 μmeter, $p<0.01$, n=6) (**Fig. 3-5B**). Interestingly, hemin treatment reduced cardiomyocyte longitudinal fiber thickness by 25 % as compared to ZDF control (18.1 ± 0.76 vs 21.9 ± 0.89 μmeter, $p<0.05$, n=6). However, this reduction did not reach the basal level of cardiomyocyte longitudinal thickness of the ZL rats (15.2 ± 0.49 μmeter) (**Fig. 3-5B**).

3.5 Discussion

The present study demonstrates for the first time that upregulating the HO system with hemin preferentially favors macrophage polarization towards the M2 phenotype that dampens inflammation, while suppressing the M1 pro-inflammatory phenotype. Another novel observation is that hemin therapy suppresses pericardial adiposity in ZDF rats, a model of insulin resistance, dyslipidemia and hyperglycemia (200). Pericardial adiposity is associated with elevated inflammatory/oxidative insults and dyslipidemia that adversely affect cardiac function, especially in individuals co-morbid with obesity and insulin resistance (8, 381, 415).

Interestingly, hemin therapy significantly reduced pericardial adiposity, attenuated macrophage infiltration and pro-inflammatory/oxidative mediators, such as NF-κB, AP-1 JNK, TNF-α, IL-6 and IL-1β (5, 414, 421-423), but enhanced important proteins involved in the insulin signal transduction pathway such as IRS-1, PI3K, and PKB (5), with corresponding reduction of hyperglycemia in ZDF.

Although one study had previously reported the role of HO-1 promoter in macrophage polarization (426), the expression levels of M1 and M2 phenotypes were not measured, so our study provides solid evidence on the role of the HO system on macrophage polarization. Importantly, the preferential enhancement of the M2 phenotype may be considered an alternative anti-inflammatory mechanism through which the HO system counteracts inflammatory insult. Interestingly, the HO system may reduce tissue inflammation through other mechanisms including the suppression of inflammatory cytokines like TNF-α, IL-6 and IL-1β (5, 421, 422). Consistently, in hemin-treated ZDF rats, TNF-α, IL-6 and IL-1β were markedly reduced. Similarly, other pro-inflammatory/oxidative mediators such as 8-isoprostane, NF-κB, AP-1 and JNK (414, 423) were greatly attenuated by hemin therapy, whereas the HO inhibitor, SnMP, nullified the hemin effects and exacerbated oxidative/inflammatory insults causing further impairment in glucose metabolism.

Hemin therapy also improved cardiac hemodynamics. In particular, systolic blood pressure was significantly lowered while cardiac output increased in hemin-treated ZDF. The lowering of systolic blood pressure and LVSP observed were accompanied by the reduction of total peripheral resistance, and these effects would reduce the afterload to the left ventricle and thus prevents the onset of ventricular dysfunction (420, 427, 428). An abnormal left-ventricular function would affect the cardiac performance and contribute to the symptoms associated with

cardiac failure (429). With the reduction of systolic blood pressure in hemin-treated ZDF, left-ventricular cardiomyocytes would contract less vigorously. This was evidenced by the reduction of +dP/dt. Therefore, by not generating a high pressure gradient to maintain adequate blood circulation, the workload and oxygen consumption of the left ventricle would be reduced and the risk of cardiovascular related morbidity and mortality would be curtailed (420, 427-429). Another beneficial effect of hemin treatment to ZDF rats was the increase in left-ventricular ejection fraction. Interestingly, this was accompanied by a reduction of LVSP. Thus, the concomitant increase in ejection fraction and cardiac output accompanied by the parallel reduction of systolic blood pressure, LVSP, +dP/dt and total-peripheral resistance would translate into improved cardiac function in hemin-treated ZDF.

Several mechanisms may be responsible for the improvement of cardiac hemodynamic parameters by hemin. These include vascular contractility (430), ventricular contractility (80), and the reduction of cardiac damage by hemin (185, 187, 193, 424, 425). In the present study, hemin therapy significantly reduced longitudinal muscle fiber thickness, a common pathophysiological feature of cardiac myocyte hypertrophy (187, 424, 425). Similarly, an upregulated HO system by hemin has been shown to correspondingly reduce cardiac histopathological lesions such as longitudinal muscle fiber thickness, cross-sectional muscle fiber thickness, scarring, muscular hypertrophy, coronary arteriolar thickening and fibrosis, (185, 187, 193) which would result in a healthier heart with improved ventricular contractility and improved hemodynamics (80). Furthermore, the HO system generates a vasodilator like carbon monoxide, a stimulator of cGMP that modulates both vascular contractility (80, 430) and ventricular contractility thus hemodynamics (80).

Generally, cardiac function is influenced by multiple factors and contractility is only one of such factors (420). By keeping after-load constant, any increase in cardiac function will increase left-ventricular force development (increase in +dp/dt). An increase in ejection fraction or cardiac output resulting from improvement in cardiac function can be accompanied by a subsequent decrease in pre-load (427). This is particularly true when the increase in cardiac function is accompanied by a decrease in after-load (total-peripheral resistance). The decrease in pre-load will cause a decrease in the resting fiber (sarcomere) length and reduce the ventricular force development which is manifested by the decrease of left-ventricular +dp/dt (max) (420, 427). Although the present study indicates that upregulating the HO system with hemin significantly reduced total-peripheral resistance and lowered systolic blood pressure in ZDF rats, the other hemodynamic parameters measured such as cardiac output, ejection fraction, LVSP and +dP/dt were improved by 8.26, 5.37, 2.28 and 8.94% respectively, and significance was not attained. Therefore the hemodynamic data presented in this study should be cautiously interpreted, and future investigations would be needed for a more comprehensive understanding on the effects of hemin therapy on cardiac hemodynamics. Nevertheless, the HO system has been shown to modulate vascular contractility and ventricular contractility (80, 430) and improve ejection fraction (431), whereas blockade of the HO system resulted to a decrease in cardiac output and an increase in total-peripheral resistance (432). Collectively these studies and the present findings strongly suggest that the HO system may contribute towards the improvement of cardiac hemodynamics and cardiac function.

Hemin therapy also enhanced the HO system and abated NF-κB, AP-1 JNK, TNF-α, IL-6 and IL-1β in ZL-control rats, although the magnitude was smaller as compared to ZDF rats with depressed HO-activity. The reasons for this selective effect of HO are not fully understood.

However, it is possible that since ZL rats are healthy animals with normal/functional insulin-signaling, the HO system may be more stable as compared to ZDF which have depressed HO activity. Importantly, the selectivity of the HO system in diseased conditions could be explored against the co-morbidity of insulin-resistant diabetes and obesity. Nevertheless, future studies will be done to investigate the selective effects of the HO system on ZL-control rats. Although we previously reported the insulin sensitizing effect of hemin in the gastrocnemius muscle of ZDF, tissue-specific response is a well-known phenomenon in the pathophysiology of insulin resistance and impaired glucose metabolism, and different tissues may respond distinctly to the same stimuli, indicating that a physiological response in one tissue may not necessarily be the same in another tissue (433, 434). Whether the reported effects were unique for the gastrocnemius muscle or universal for other tissues is critical for understanding the role of hemin in insulin resistance and glucose metabolism. Therefore, studying the effect of an upregulated HO system in the left ventricle of ZDF rat is important for the advancement of knowledge in this area. Moreover, the effects of hemin therapy on left-ventricular IRS-1, PI3K and PKB in ZDF-rat, a model with diabetic cardiomyopathy (374, 416) remains poorly understood. Interestingly, the present study unveils that the restoration of normoglycemia in hemin-treated ZDF was accompanied by the concomitant potentiation of left-ventricular IRS-1, PI3K and PKB, and the improvement of cardiac function.

3.6 Conclusion

Collectively, our study unveils the beneficial effect of the HO system on pericardial adiposity, impaired insulin-signaling, diabetic cardiomyopathy and suggest that the suppression of inflammatory/oxidative mediators are among the multifaceted mechanisms by which the HO

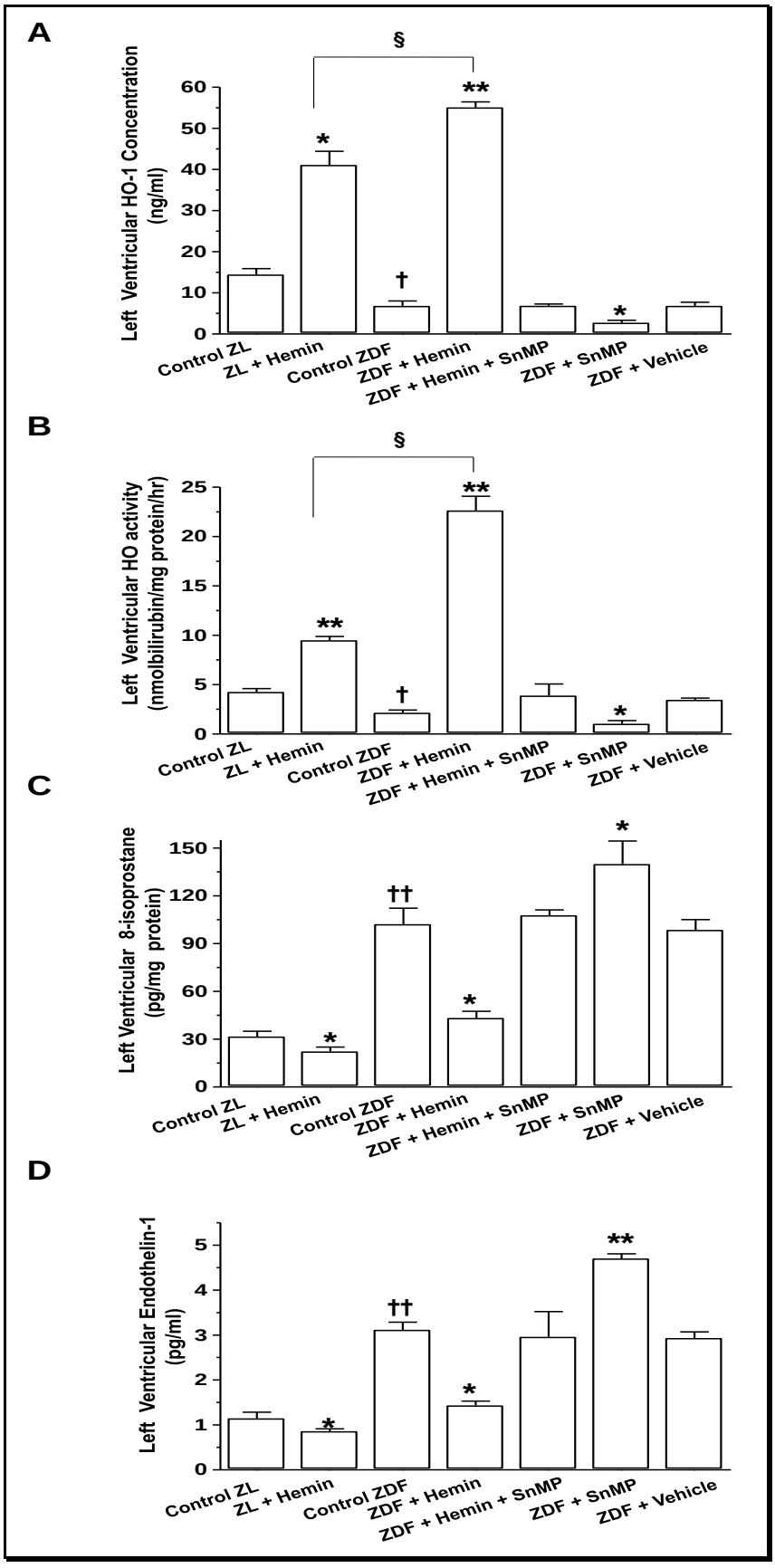
system maintains homeostasis in physiological milieu. Given that NF-κB activates TNF-α, IL6 and IL-1β (5), while TNF-α, JNK and AP-1 impair insulin-signaling (5), the high levels of these cytokines and inflammatory/oxidative mediators in the chronic conditions of obesity and diabetes would create a vicious cycle that when added to the oxidative insults generated by 8-isoprostane and ET-1 (435) would exacerbate tissue insult and compromise its function. Therefore, the concomitant reduction of pro-inflammatory cytokines and macrophage infiltration coupled to the potentiation of insulin-signal transduction agents such as IRS-1, PI3K, PKB and the improved cardiac hemodynamics may account for enhanced glucose metabolism and improved cardiac function in hemin-treated ZDF. Importantly, the novel findings of our study includes: **(i)** the preferential polarization of macrophages towards anti-inflammatory M2-phenotype in cardiac tissue as evidenced by increased expression levels of the M2-phenotye and the parallel reduction of the M1-proinflammatory phenotype; **(ii)** the suppression of pericardial adiposity; and **(iii)** the hemin-induced improvement of cardiac function in ZDF rat, a model of obesity, insulin resistance, type-2 diabetes with cardiomyopathy (374, 416).

With the escalation of obesity, diabetes and hypertension in industrialized and developing countries, the incidence of cardio-metabolic complications including diabetic cardiomyopathy and heart failure will increase. Cardio-metabolic complications are multifactorial diseases and a wide variety of different pathophysiological factors including inflammatory/oxidative insults are involved. The present study highlights the ability of hemin therapy to suppress inflammatory/oxidative mediators and improve insulin-signaling in T2D. Impaired insulin-signalling is not only an important etiological factor in the pathogenesis of T1D and T2D, but also an important patho-physiological driving force that is capable of dictating the dynamics and progression of the disease as well as its ultimate evolution in to complications like diabetic

cardiomyopathy. Therefore, the findings reported here could serve as a useful tool for the formulation of novel therapeutic agents against diabetes, pericardial adiposity and related complications like diabetic cardiomyopathy. Interestingly, our study may have great translational potential as the drugs used (hemin and SnMP) may have therapeutic application. Both hemin and SnMP may have application in clinics as hemin has been approved by the FDA against porphyria (55, 56), while SnMP has successfully completed phase-III clinical trials for possible use against neonatal jaundice (436-438).

Acknowledgements

This work was supported by the Heart & Stroke Foundation of Saskatchewan of Canada to Dr. Joseph Fomusi Ndisang. The authors are grateful to James Talbot for technical assistance.


A
§
Left Ventricular HO-1 Concentration (ng/ml)
60
50
40
30
20
10
0
*
**
†
*
Control ZL
ZL + Hemin
Control ZDF
ZDF + Hemin
ZDF + Hemin + SnMP
ZDF + SnMP
ZDF + Vehicle
B
§
Left Ventricular HO activity (nmolbilirubin/mg protein/hr)
25
20
15
10
5
0
**
**
†
*
Control ZL
ZL + Hemin
Control ZDF
ZDF + Hemin
ZDF + Hemin + SnMP
ZDF + SnMP
ZDF + Vehicle
C
Left Ventricular 8-isoprostane (pg/mg protein)
150
120
90
60
30
0
*
*
††
*
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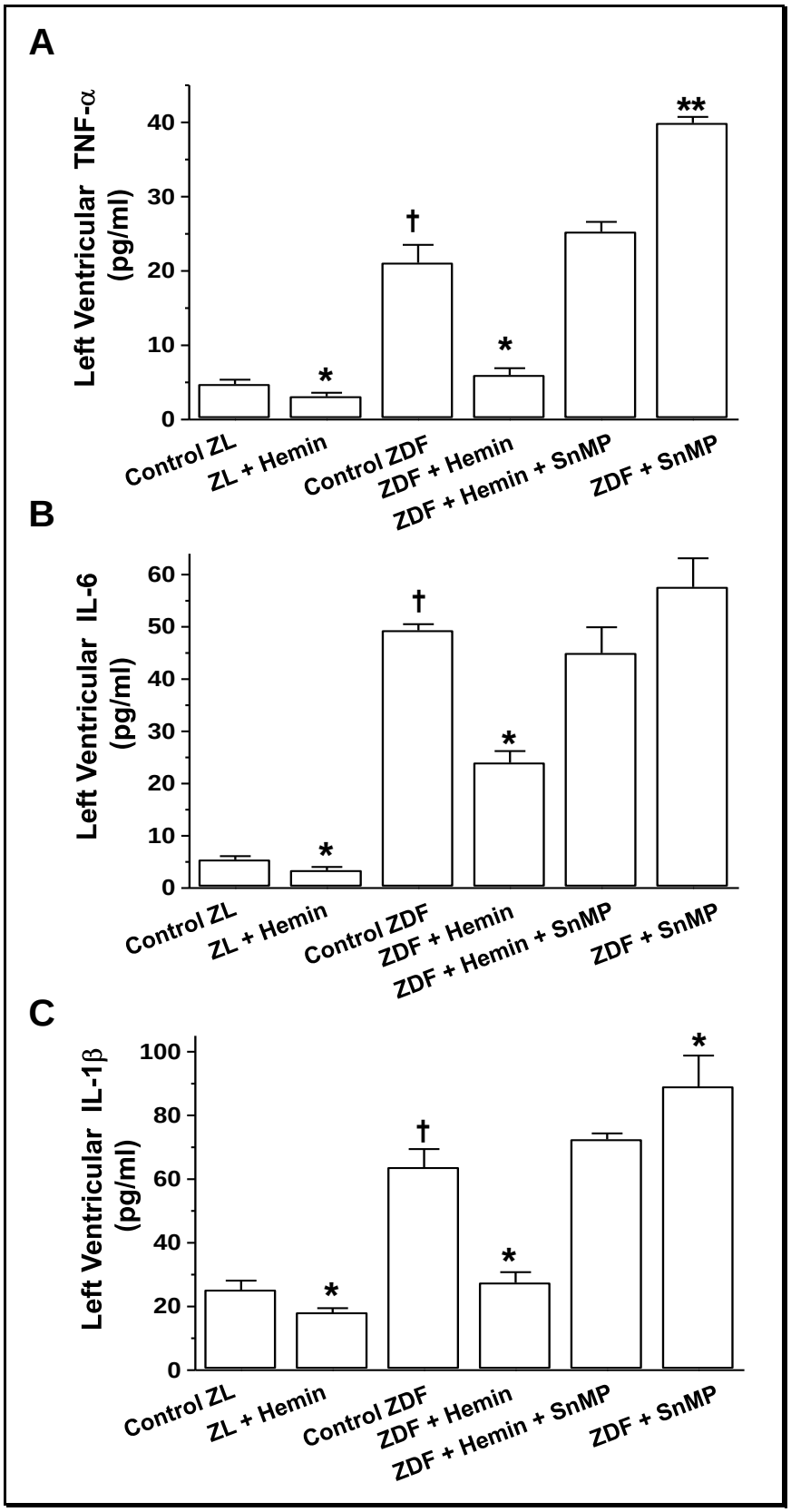
Control ZL
ZL + Hemin
Control ZDF
ZDF + Hemin
ZDF + Hemin + SnMP
ZDF + SnMP
ZDF + Vehicle
D
Left Ventricular Endothelin-1 (pg/ml)
5
4
3
2
1
0
*
††
*
**
Control ZL
ZL + Hemin
Control ZDF
ZDF + Hemin
ZDF + Hemin + SnMP
ZDF + SnMP
ZDF + Vehicle

Figure 3-1: Effects of the HO inducer, hemin and the HO inhibitor, SnMP, on left-ventricular HO-1, HO activity, 8-isoprostane and ET-1 of ZDF and ZL rats. (**A**) The basal HO-1 levels in ZDF rats were lower as compared to ZL-control rats. Hemin therapy increased HO-1 concentration, while the HO blocker, SnMP annulled the hemin effect. Hemin also enhanced HO activity in ZL rats, but less intensely as compared to ZDF rats. (**B**) The basal HO activity in ZDF rats was depressed as compared to ZL-control rats, but was enhanced by hemin while the HO blocker, SnMP annulled the hemin effect. Hemin also enhanced HO activity in ZL rats, but less intensely as compared to ZDF rats. Hemin reduced (**C**) 8-isoprostane and (**D**) abated ET-1 in ZDF and ZL-control rats, but SnMP abolished the effect. The vehicle dissolving hemin and SnMP had no effect on HO activity, 8-isoprostane and ET-1. Bars represent means ± SE; *n=6* rats per group (*$p<0.05$, **$p<0.01$ vs. all groups; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ vs. Control-ZL rats).


A
Left Ventricular TNF-α (pg/ml)
0
10
20
30
40
*
†
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Control ZL
ZL + Hemin
Control ZDF
ZDF + Hemin
ZDF + Hemin + SnMP
ZDF + SnMP
B
Left Ventricular IL-6 (pg/ml)
0
10
20
30
40
50
60
*
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Control ZL
ZL + Hemin
Control ZDF
ZDF + Hemin
ZDF + Hemin + SnMP
ZDF + SnMP
C
Left Ventricular IL-1β (pg/ml)
0
20
40
60
80
100
*
†
*
*
Control ZL
ZL + Hemin
Control ZDF
ZDF + Hemin
ZDF + Hemin + SnMP
ZDF + SnMP

Figure 3-2: The effects of hemin and the HO inhibitor, SnMP, on left-ventricular inflammatory cytokines. Hemin therapy reduced (**A**) TNF-α, (**B**) IL-6 and (**C**) IL-1β, in ZDF rats and ZL-controls, while SnMP nullified the effects of hemin. Bars represent means ± SE; *n=6* rats per group (*p<0.05, **p<0.01 vs. all groups; †p<0.01 vs. Control-ZL rats).

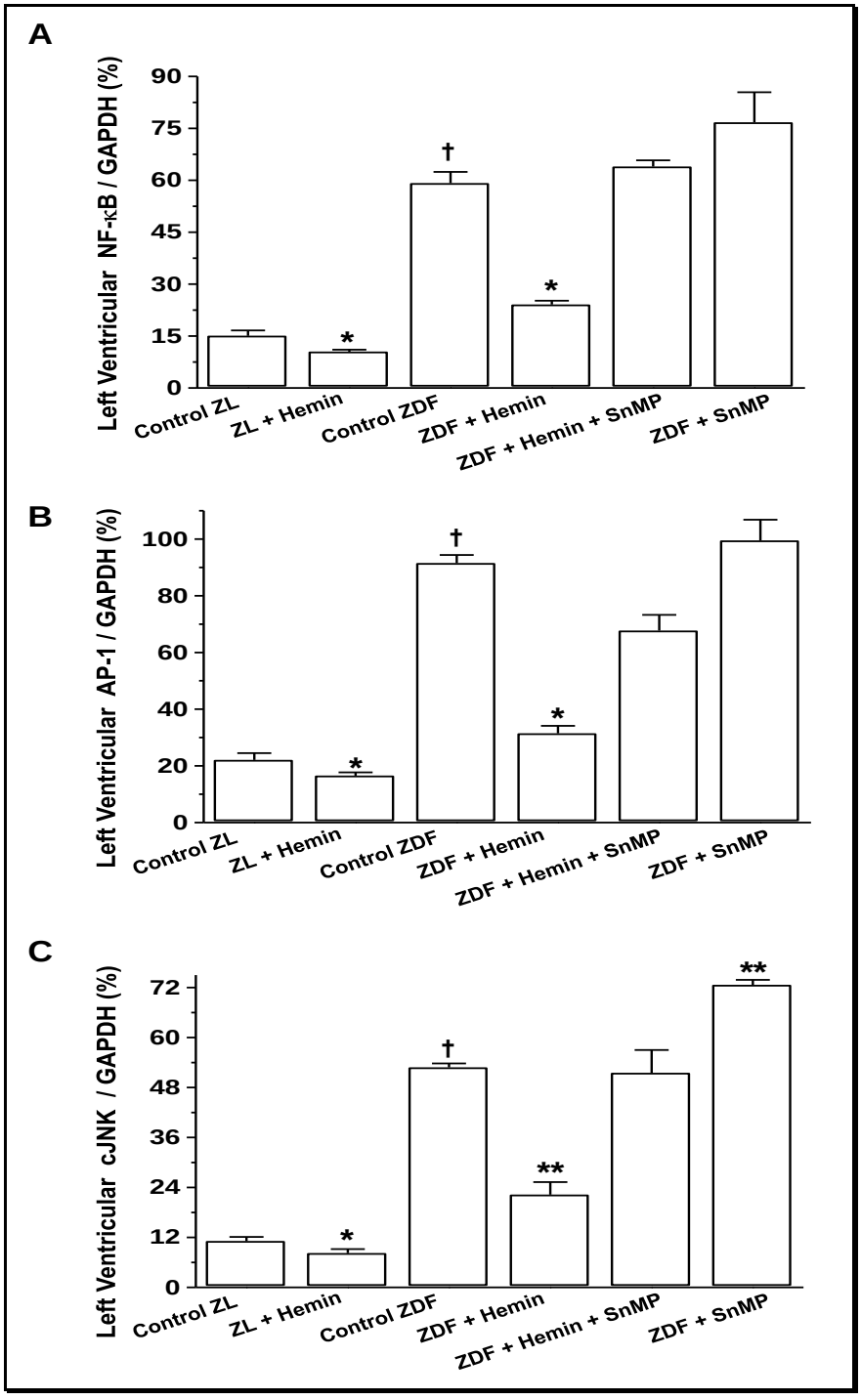


Figure 3-3: The effects of hemin and the HO inhibitor, SnMP, on left-ventricular NF-□B, AP-1 and JNK. Quantitative real-time RT-PCR indicated hemin therapy suppressed the elevated basal mRNA expression of (**A**) NF-κB, (**B**) AP-1 and (**C**) JNK in ZDF and ZL rats, but SnMP annulled the hemin effect. Bars represent means ± SE; *n*=6 rats per group (*p<0.05, **p<0.01 vs. all groups; †p<0.01 vs. Control-ZL rats).

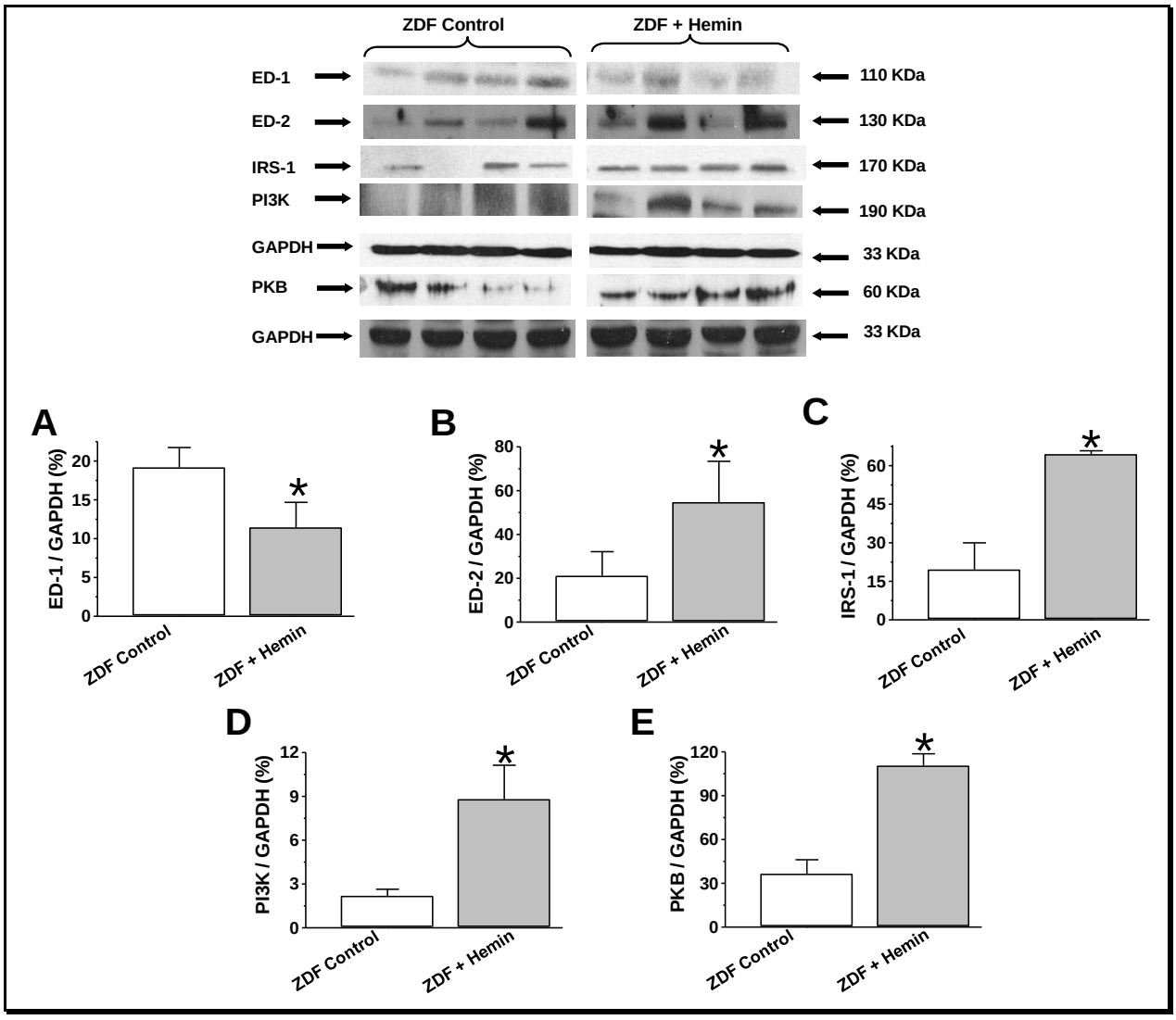


Figure 3-4: Effect of hemin on left-ventricular ED-1, ED-2, IRS-1, PI3K and PKB in ZDF rats. Representative Western immunoblots and relative densitometry indicates that hemin therapy (**A**)

suppressed ED-1, but enhanced (**B**) ED-2, (**C**) IRS-1, (**D**) PI3K and (**E**) PKB in ZDF. Bars represent means ± SE; *n=4* rats per group (*p<0.05 vs. control-ZDF).

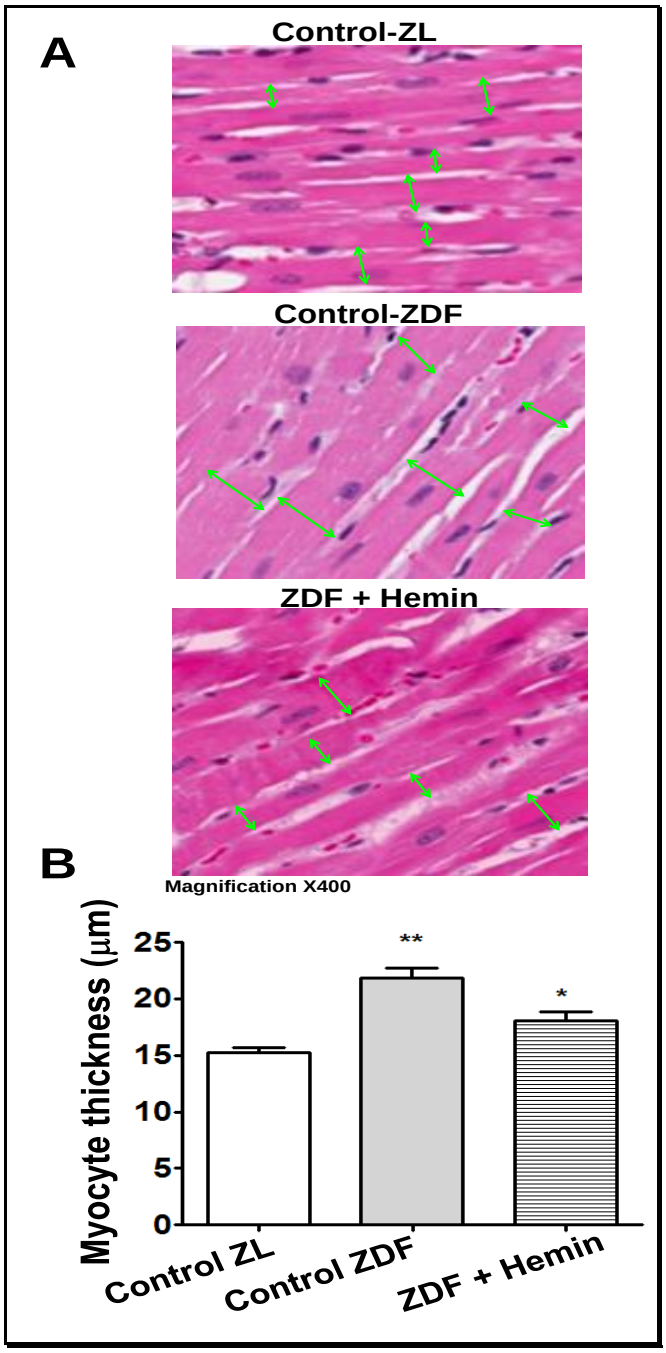


Figure 3-5: Effect of hemin on longitudinal muscle fiber thickness is ZDF. (**A**) Representative images of histological sections revealed that hemin therapy attenuated longitudinal muscle fiber thickness is in ZDF. In untreated ZDF rats, enlarged cardiomyocytes with increscent nuclei were

evident as compared to normal cardiomyocytes in age/sex-matched the ZL-control rats. (**B**) Semi-quantitative analyses revealed that hemin therapy markedly reduced longitudinal muscle fiber thickness is ZDF rats. Bars represent means ± SE; *n=4* rats per group (*p<0.05 vs. control-ZDF and control-ZL; **p<0.01 vs. control-ZL).

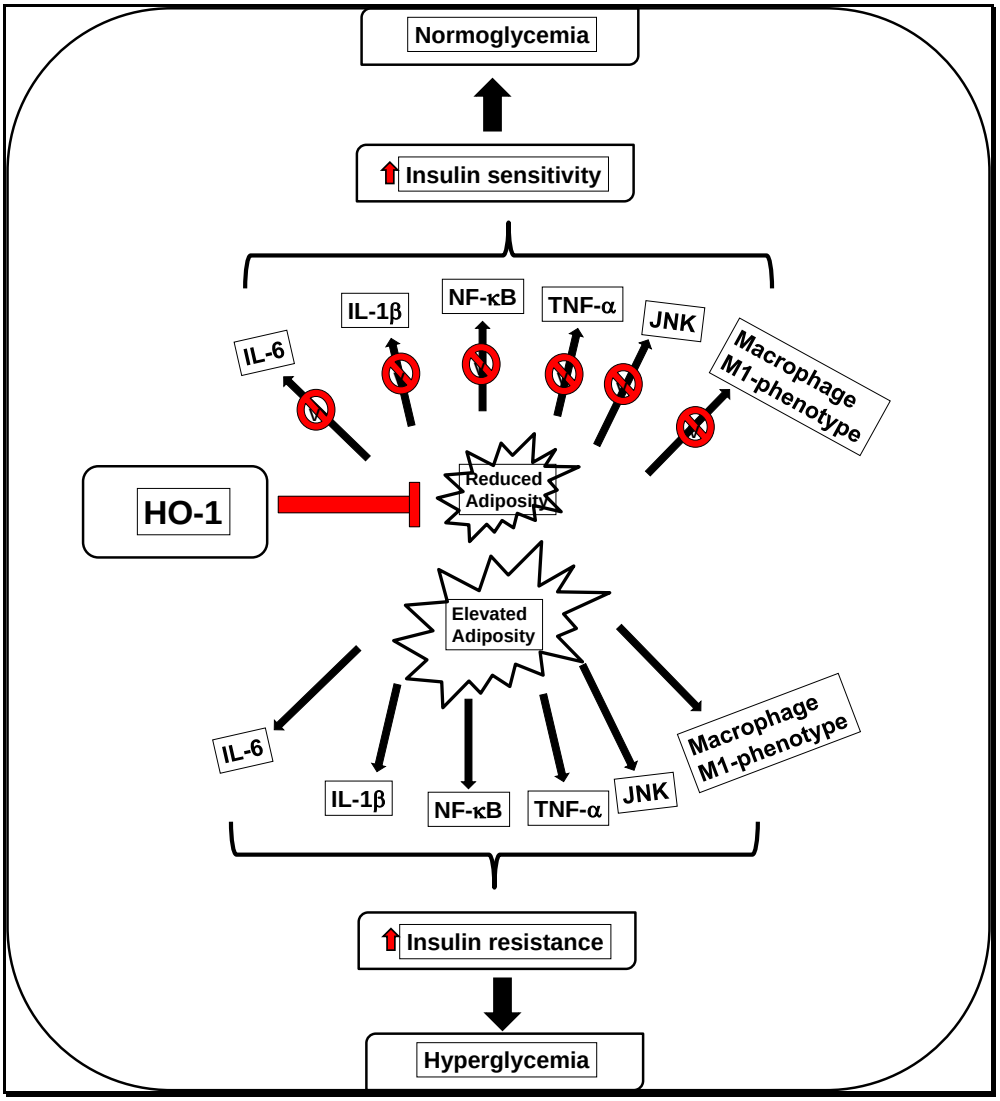


Figure 3-6: Schematic representation of the actions of hemin. Hemin therapy enhances HO-1, which in turn reduces adiposity and pro-inflammatory cytokines including IL6, IL-1β, NF-κB, TNF-α, JNK and M1-phenotype macrophage infiltration. Correspondingly, insulin sensitivity increases and normoglycemia is restored.

Physiological variables	Animal groups				
	Control ZL	**ZL+ Hemin**	**Control ZDF**	**ZDF + Hemin**	**ZDF + Hemin +SnMP**
Body weight (g)	363.7 ± 5.4	354.5 ± 9.5	383.6 ± 5.4	363.4 ± 6.5†	352.5 ± 8.2#
Fasting glucose (mmo/L)	7.2 ± 0.5	6.4 ± 0.3*	24.6 ± 3.1	6.8 ± 1.3**	19.2 ± 2.8#
Cardiac hypertrophy (g/Kg body weight)	2.7± 0.2	2.3 ± 0.1*	3.8 ± 0.3	2.4 ± 0.14**	3.4 ± 0.3#
Pericardial adiposity (g/Kg body weight)	1.3 ± 0.1	0.97 ± 0.05*	1.85 ± 0.2	0.79 ± 0.13**	1.72 ± 0.5#

*†p<0.05 vs controls; *p<0.05 vs Control ZDF or control ZL, #p<0.05 vs ZDF + Hemin n=8 per group*

Table 3-1: Effect of hemin and stannous mesoporphyrin (SnMP) on physiological variables in ZDF and ZL rats.

Hemodynamic Parameters						
Animals	Systolic BP (mmHg)	Cardiac output (ml/min)	Ejection Fraction (%)	LVSP (mm Hg)	+dP/dt	Total Peripheral Resistance (mmHgmin/ml)
Control ZDF	137.6 ± 3.7	82.06 ± 9.36	62.35 ± 0.93	135.05 ± 7.31	2828.74 ± 194.41	1.32 ± 0.14
ZDF+Hemin	118.4 ± 2.5*	88.76 ± 4.67	65.70 ± 1.21	131.97 ± 2.66	2575.86 ± 103.35	1.16 ± 0.08*
% improvement in ZDF+Hemin	-13.95 [1]	8.26	5.37	-2.28 [1]	-8.94 [1]	-12.16 [1]

Table 3-2: Echocardiographic and invasive hemodynamic measurements

[1] The negative percentage changes in left-ventricular systolic pressure (LVSP) and left-ventricular pressure development (+dP/dt) are well correlated to the reduction of total peripheral resistance and systolic blood pressure, all of which are indicative of improved cardiac function (420). (*$^{*}p<0.05$ vs Control ZDF, n=6-8 per group).*

CHAPTER 4

General discussion

The research presented in this thesis strongly underscore the novel and beneficial effects of an upregulated HO system by hemin against impaired insulin signaling and cardiomyopathy in obese ZF rats and the mechanisms by which HO improves dysfunctional insulin signaling, glucose metabolism, pericardial adiposity and altered cardiac structure and function (Chapter 2-3). The potentiation of ANP and adiponectin by hemin-induced upregulated HO in cardiac tissue of ZF rats is an important and novel cardio-protective mechanism for attenuation of inflammatory and oxidative mediators, suppression of pro-inflammatory M1 macrophage phenotype, attenuation of ECM/profibrotic proteins and the improvement in impaired cardiac hemodynamic and echocardiographic parameters. Therefore, this chapter includes a general discussion about overall research results in terms of their importance in understanding the novel and beneficial role of the hemin-induced upregulated HO system against obesity and cardiomyopathy. The present study for the first time highlights the novel role of the HO system on macrophage polarization in the cardiac tissue of ZF rats. Importantly, my thesis work provides novel insights on the beneficial effects of concomitant potentiation of HO, ANP and adiponectin against cardiomyopathy and obesity in ZF rats.

Although, the beneficial role of hemin-induced upregulated HO against inflammation, insulin resistance, oxidative stress, pericardial adiposity and cardiac hypertrophy are discussed briefly, several challenging questions remain to be addressed. Some of these questions will be addressed in the ensuing section below.

4.1 Is obesity induces inflammatory events as primary response or is it a consequence of insulin resistance?

Chronic low-grade inflammation and oxidative stress are common denominators that links obesity with diabetes, insulin resistance, dyslipidemia and cardiovascular diseases (14, 15, 27, 29, 58, 68, 69, 133, 149, 150, 175, 178, 215). The inflammatory response induced during the obese condition is quite distinct from classical inflammation that is elicited as a defense mechanism against various tissue insults (24). Under the obese state, a metabolic inflammatory trigger is induced in response to increased levels of glucose, oxidized low-density lipoproteins and FFA. This metabolic trigger is detected by adipocytes and they respond by activating inflammatory pathways that lead to an impaired metabolic homeostasis, a reduced metabolic rate and disruption of insulin signaling (1, 5, 14, 76, 77, 124, 133, 142, 186, 439-441). However, it is still not very clear exactly how obesity elicits an inflammatory response. It is still not clear whether obesity induced inflammation is a primary response or is it a consequence of insulin resistance. It is suggested that inflammatory signals are initiated within the adipose tissue itself by adipocytes and other immune cells, as adipocytes are the very first cells that are affected by excess body fat (142). But how do these hypertrophied adipocytes elicit inflammatory signals? Several mechanisms have been proposed to explain this query. The mechanism for obesity-induced inflammation might be by the altered metabolic signals produced by hypertrophied adipose tissue in response to excess glucose, low-density lipoproteins and FFA. As a consequence, the metabolic function of adipose tissue is compromised due to the overproduction of pro-inflammatory cytokines and chemokines such as TNF-α, IL-6, IL-1β, MCP-1 (1, 5, 15, 76, 77) and inflammatory transcription factors such as NFκB and JNK by resident and recruited macrophages (1, 5, 14, 77, 124, 134, 142, 186, 439-441). This leads to localized inflammation in

adipose tissue followed by the development of systemic inflammation and other obesity-related complications including insulin resistance and cardiovascular diseases (14, 15, 27, 29, 58, 68, 69, 149). Another mechanism that has been proposed is increased infiltration of macrophages into fatty adipose tissue that contributes to the increased production and expression of pro-inflammatory mediators that propagate inflammatory signaling cascades (137-139). Moreover, increased macrophage infiltration during the inflammatory process was reported to be associated with a macrophage polarization event where macrophages polarizes from the anti-inflammatory M2 to the pro-inflammatory M1 phenotype (19, 130, 137-140). In addition, it is suggested that enlarged adipose tissue became hypoxic which in turn triggers inflammatory signaling cascades (134, 137-139). Another important factor that contributes to inflammation in obesity is oxidative stress. The condition of over nutrients in adipose tissue leads to high levels of intracellular ROS generation which in turn activates inflammatory pathways and subsequent endothelial injury (178, 214, 215). It is proposed that obesity induces stress signals inside adipocytes that leads to the deposition of misfolded proteins inside the ER. The ER stress generated stimulate the unfolded protein response pathway and activates an inflammatory signaling cascade through the activation of JNK and NFκB pathway along with the suppression of the IKK-β pathway (1, 3, 5, 15, 67, 76). This altogether results in the elevated production of pro-inflammatory cytokines and chemokines that leads to the gradual deterioration of insulin sensitivity (1, 5, 15, 67, 77). It is interesting to know that blocking of inflammatory mediators such as TNF-α and JNK provides protection against insulin resistance in obese human and obese animal models (145). Based on these findings, it can be concluded that obesity contributes to both chronic low-grade inflammation and insulin resistance and inflammation is not just the consequence of obesity but

rather is a primary event for obesity related insulin resistance, hyperglycemia, and hyperlipidemia (145).

Modulatory effects of HO, ANP and adiponectin

The highlight of the present study is the beneficial role of an upregulated HO, ANP and adiponectin by hemin in ameliorating obesity induced inflammatory/oxidative insults and associated cardiometabolic complications in obese ZF rats (Chapter 2). An interplay exists between HO, ANP and adiponectin, as mutual stimulatory effects have been reported between HO and ANP (196, 359, 442). Furthermore, both ANP and HO have been shown to stimulate adiponectin (202, 319, 397, 443, 444). Interestingly, ANP has inhibitory effects on inflammatory cytokines such as TNF-α, IL-6, IL-1β (322). In addition, adiponectin was shown to attenuate macrophage infiltration by abating NFκB and TNF-α and promote macrophage polarization towards the anti-inflammatory M2 phenotype (334, 336). Furthermore, both ANP and HO exhibit endogenous cytoprotective effects by virtue of their anti-inflammatory, anti-oxidant and anti-proliferative properties (3-5, 32-54, 319, 322, 324). Together, these evidence strongly suggest that the therapeutic agents that can potentiate the HO-ANP-adiponectin axis would be of great benefit, as they have potential to counteract the adverse effects of inflammatory/oxidative insults and insulin resistance associated with obesity and cardiomyopathy.

4.2 Mechanisms underlying the suppression of visceral adiposity by hemin

Visceral adiposity is an independent risk factor for progression and development of cardiovascular disease (1-12, 14, 15, 80). Initially, under the normal physiological state deposition of excessive fat takes place in non-ectopic tissue such as subcutaneous adipose tissue

(7, 71-73). However, during obesity, once the maximal limit of subcutaneous adipose tissue is achieved, increased FFA and other lipids than start to deposit in ectopic tissue such as heart, liver, kidney, muscles and the vasculature (1-5, 77, 241, 258). Thus, the deposition of perivascular, pericardial, and epicardial ectopic fat in heart exerts adverse effects in both an endocrine and paracrine manner (72, 253). Visceral adiposity like pericardial fat due its close anatomical location to coronary circulation has more serious consequences compared to subcutaneous fat and is a greater risk factor for heart disease and associated complications (12, 355, 356). As a consequence, excess pericardial fat eventually results in elevated myocardial inflammation, increased oxidative stress, cardiac hypertrophy and excessive deposition of ECM/profibrotic proteins and the subsequent development of insulin resistance in cardiac tissue (72, 75, 79, 81, 129, 150, 253, 260, 261, 265, 287-289). This suggests that agents that reduce visceral adiposity, inflammation and attenuates oxidative stress would be beneficial to counteract obesity induced cardiometabolic complications.

As a parallel project to the research outlined in Chapter 2 our study provides another novel observation that hemin therapy suppressed pericardial adiposity in obese ZDF (Chapter 3) and ZF rat models (54). The data presented in our most recent publication (330) showed that treatment with hemin improves pericardial adipocyte morphology and function in obese ZF rats as evidenced by elevated adiponectin levels and reduced levels of inflammatory and oxidative mediators, reduced expression of osteopontin and TGF-β as well as attenuation of pericardial adipocyte hypertrophy. Interestingly, hemin-mediated potentiation of proteins of repair and regeneration such as beta-catenin, Oct3/4, and Pax2 is suggestive of another important novel mechanism for the restoration of adipocyte morphology and function in the pericardial adipose tissue of obese ZF rats.

In this prospective study, I showed for the first time that HO-1 induction by hemin polarizes macrophages towards the anti-inflammatory M2 phenotype and suppresses the M1 pro-inflammatory phenotype and thereby contributes towards the suppression of inflammation in cardiac tissue of ZF rats (Chapter 2 and Chapter 3). Interestingly, our study demonstrated that administration of hemin to ZF rats and ZDF rats and to their counterpart ZL rats increased HO activity, HO-1 concentration and cGMP levels in cardiac tissue. In contrast, co-administration of the HO inhibitor SnMP and HO inducer hemin suppressed HO-1 and HO activity and reduced cGMP levels. This suggests that increased cardiac HO-1 concentration, HO activity and cGMP levels were associated with a corresponding downregulation of inflammatory/oxidative mediators. In addition, hemin therapy improved cardiac structure and function as evidenced by suppressed cardiac hypertrophy, cardiac lesions and reduction in the ECM/pro-fibrotic proteins as well as improved cardiac hemodynamics (Chapter 2 and Chapter 3). Taken together, data from the present thesis and recent publication suggest that hemin treatment suppressed pericardial adiposity, improved pericardial adipose tissue morphology and function as well as improved cardiac function. Suppression of inflammatory and oxidative mediators, reduction of ECM proteins, suppression of pericardial adipocyte and cardiac hypertrophy and elevated adiponectin levels are some of the suggested mechanisms through which the HO system suppressed visceral adiposity and improved cardiac function.

4.3 Role of the HO system in preventing cardiac hypertrophy and improvement in cardiac morphological lesions and function

Cardiac hypertrophy and cardiac fibrosis is a pathophysiological driving force for heart failure (129, 150, 265, 287-289). In response to various stress stimuli myocytes grow either in

length and/or width as a result of increased cardiac afterload (287, 445). Since, upregulation of the HO system by hemin suppressed inflammatory/oxidative markers, macrophage infiltration and potentiated insulin signaling, I examined whether the cytoprotective role of hemin can be extended to cardiac tissue in the form of improvement in cardiac morphological lesions and function.

In Chapter 2 and Chapter 3, I explored the effects of hemin treatment on cardiac structure and function. In ZF and ZDF rats, hemin treatment significantly reduced left-ventricular hypertrophy, cardiac fibrosis and cardiomyocyte longitudinal muscle fiber thickness and collagen deposition. However, co-administration of hemin and SnMP nullified the cytoprotective effects of hemin. Histological and morphometric analysis revealed that obesity-induced interstitial and perivascular collagen deposition, scarring of cardiomyocytes and cardiac fibrosis as observed in ZF rats was significantly attenuated by the hemin treatment (Chapter 2). As a parallel project, another novel and important finding of our study was that hemin therapy successfully reduced pericardial adipocyte hypertrophy as observed in untreated ZF rats and restored it to a level comparable to their ZL counterparts (54). The present research and previous studies support the existence of inter-organ crosstalk between the heart and pericardial adipose tissue (75, 78, 79). Thus, the movement of inflammatory/oxidative mediators and other atherogenic factors from the pericardial fat to the myocardium or vice versa in a paracrine manner might be suggestive of a mechanism for the subsequent development of insulin resistance and related cardiac complications (72, 129, 150, 260, 261, 265, 287-289). Altogether, the data from the present study and recent publication (54), strongly suggests that hemin-mediated suppression of pericardial adiposity and the parallel reduction of pro-inflammatory cytokines and chemokines may attenuate the adverse effects of inflammatory mediators on the

myocardium derived from pericardial adipose tissue through a paracrine mechanism. Although, further research is needed to establish this interconnection, the results obtained with hemin treatment in the present study have given important suggestions for future work in this direction.

Myocardial remodeling is an important pathophysiological process characterized by abnormal deposition of interstitial collagen and over expression of extracellular matrix proteins such as TGF-β, fibronectin and collagen that leads to increased myocardial stiffness and reduced myocardial contractility (129, 248, 265, 281, 283, 286, 292). This suggests that attenuation of cardiac fibrosis in turn might improve cardiac function. Another important observation of my thesis work is that hemin treatment improved cardiac function through a mechanism that involves the reduction of ECM/profibrotic proteins including TGF-β, fibronectin and collagen IV. Collagen IV plays an important role in cardiac hypertrophy and fibrosis (21, 446), In addition, TGF-β mobilizes ECM through the induction of collagen and fibronectin, and contributes to cardiac injury and fibrosis (21). Thus, reduction in TGF-β expression will in turn suppresses fibronectin and collagen protein expression. Interestingly, my work also showed that hemin treatment in ZF rats reduced the expression of collagen IV and TGF-β by 2.8 fold and 3.4 fold compared to 6.9 fold and 4.6 fold above basal expression of collagen IV and TGF-β in untreated ZF rats. In a similar manner the 7.5 fold increase in basal expression of fibronectin in ZF rats was significantly suppressed 4.5 fold in hemin treated ZF rats (Chapter 2).

To further confirm the cardioprotective effects of an upregulated HO system by hemin, I assessed the effect of hemin on markers of heart failure such as osteopontin and osteoprotegerin. Osteopontin and osteoprotegerin have been implicated to play pathophysiological roles in various biological processes such as adipose tissue inflammation, fibrosis, tissue remodeling, hypertrophy and insulin resistance (294, 295, 297-309, 311, 447). Osteopontin and

osteoprotegerin are highly activated in response to various inflammatory molecules such as LPS, TNF-α, IFN-γ, TGF-β, angiotensin-II, IL-1β (295, 297, 299, 309). It is suggested that the elevated expression of osteopontin and osteoprotegerin in the circulation and in cardiac tissue is a predictor for impaired cardiac function and subsequent heart failure (295, 297, 298, 303, 309, 311). In Chapter 2, I have shown that in ZF rats, basal expression of osteopontin and osteoprotegerin in cardiac tissue was significantly elevated by 4.6 and 7.1 fold, respectively, as compared to ZL controls. However, hemin therapy in ZF rats reduced the expressions of osteopontin and osteoprotegerin by 3.5 and 3.3 fold, respectively. Similarly, our recent publication showed that hemin treatment significantly reduced the expression of osteopontin in pericardial adipose tissue of ZF rats (54).

The novel and central observation of my thesis work is that hemin treatment improved cardiac structure and function as evidenced by improvement in various hemodynamic and echocardiographic parameters in ZF rats and ZDF rats (Chapter 2 and Chapter 3). It should be noted that cardiomyocyte hypertrophy and myocardial fibrosis are microscopic changes which are observed at initial stages in heart failure (286, 292). Later, macroscopic changes such as increased left-ventricular wall thickness, altered cardiac hemodynamics, impaired diastolic function and subsequent systolic dysfunction becomes more prominent (287, 445). My thesis data showed that hemin treatment suppressed cardiac hypertrophy, fibrosis and left-ventricular longitudinal muscle fiber thickness (Chapter 2 and Chapter 3). Therefore, another possible mechanism that might be responsible for improved cardiac function by an upregulated HO system include improved hemodynamic and echocardiographic parameters that eventually leads to a healthier heart accompanied by improved ventricular contractility (448). Interestingly, my thesis data showed that hemin treatment significantly reduced left-ventricular diastolic wall

thickness and left-ventricular systolic wall thickness and decreased mean-arterial pressure, arterial-diastolic pressure and arterial-systolic pressure in ZF rats and ZDF rats (Chapter 2 and Chapter 3). Furthermore, reduction in left-ventricular developed pressure, +dp/dt, and total peripheral resistance along with enhanced cardiac output was observed in hemin treated ZF rats (Chapter 2). Reduction in the pressure gradient that is required to maintain adequate systemic blood supply will reduce the left-ventricular workload and oxygen consumption and decrease the risk for cardiovascular abnormalities (428). Altogether, from these results, it can be inferred that the improvement in hemodynamic and echocardiographic parameters by hemin decreased left-ventricular afterload and thereby, protects against the onset of ventricular dysfunction that would affect cardiac performance and lead to heart failure (80).

Overall, my study clearly showed the beneficial effects of upregulated HO by hemin against obesity-induced altered cardiac structure and function. Suppression of cardiac hypertrophy, fibrosis and ECM/pro-fibrotic proteins are some of the mechanisms by which the HO system improved cardiac function in obese ZF rats.

4.4 Role of the HO system against inflammation

Excessive inflammation and oxidative stress leads to dysfunctional insulin signaling and compromised cardiac function (14, 15, 27, 29, 58, 68, 69, 133, 149, 150, 175, 178, 215). In response to obesity, adipose tissue reciprocates through the process of adipocyte hypertrophy and hyperplasia (1-5, 241). As obesity and corresponding adipocyte enlargement progresses, the insufficient blood supply leads to the process of necrosis/apoptosis of adipocytes and increased macrophage infiltration into the adipose tissue. As a consequence, the metabolic function of adipose tissue is compromised due to the overproduction of pro-inflammatory cytokines and

chemokines such as TNF-α, IL-6, IL-1β, and MCP-1 and inflammatory transcription factors such as NFκB, AP-1 and JNK (13, 67, 76, 142, 150). This leads to localized inflammation in adipose tissue followed by the development of systemic inflammation which is an underlying mechanism for obesity-related cardiovascular complications including insulin resistance (14, 15, 27, 29, 58, 68, 69, 149, 161, 241, 448).

During my Ph.D. research, I investigated whether hemin had any effect on the levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β (Chapter 2). Interestingly, data from Chapter 2 and Chapter 3 revealed that the elevated levels of TNF-α, IL-6, IL-1β as observed in the left ventricle tissue of ZF and ZDF rats were successfully reduced in hemin-treated animals. This is consistent with recent publications from our lab which indicate that hemin therapy not only suppressed the elevated levels of TNF-α, IL-6 and IL-1β but also other pro-inflammatory and oxidative mediators such as NF-kB, AP-1, and JNK (4, 5, 53, 202). Similar results were observed in hemin treated lean controls, however, the reduction in the levels of TNF-α, IL-6 and IL-1β was found to be less intense compared to ZF and ZDF rats treated with hemin. In contrast, coadministration of hemin and the HO blocker SnMP nullified the effects of hemin, indicating the regulatory role of the HO system on these inflammatory cytokines. Thus, suppression of inflammatory mediators might serve as one of several mechanisms by which hemin-induced HO improved cardiac function, as high levels of pro-inflammatory cytokines have been implicated in abnormal glucose metabolism and impaired cardiac function (14, 115, 161, 241, 448).

4.5 Mechanism underlying modulation of macrophage polarization by the HO system

The strength of my thesis work is that it provides, for the first time, the credential for the role of an upregulated HO system on macrophage polarization in cardiac tissue of ZF rats. At the

time when my Ph.D. research was initiated, there were not many publications on the role of HO on macrophage polarization especially in cardiac tissue. Although, Weis et al., 2009 previously performed an *in vitro* analysis to demonstrate the role of the HO-1 promoter in macrophage polarization, the expression levels of classical M1 and alternative M2 phenotype markers were not demonstrated. Therefore, I analyzed the effects of hemin on the pro-inflammatory M1-phenotype and anti-inflammatory M2-phenotype macrophage markers in left-ventricular tissue of ZF rats.

During the pathological condition of visceral obesity, increased macrophage infiltration leads to insulin resistance (19, 130, 137-140). My thesis results showed that hemin treatment successfully suppressed the pro-inflammatory cytokines implicated in the inflammatory process. Therefore, it was expected that suppression of these inflammatory mediators would be accompanied by a corresponding decrease in macrophage infiltration as cytokines and chemokines including IL-6, IL-1β, TNF-α, MCP-1 and MIP-1α were known to promote macrophage infiltration (19, 151). As expected, western blot analysis showed that in cardiac tissue of ZF rats hemin therapy significantly reduced ED-1 expression, which is a marker of macrophage infiltration to a level comparable to the healthy ZL control rats (Chapter 2). This result was further confirmed by immunohistochemical analysis done by using the ED-1 antibody; where there was a significant reduction in ED1-positively stained dark brown macrophages in hemin-treated ZF rats compared to unhealthy ZF controls (Chapter 2). Treatment with hemin also suppressed the elevated levels of MCP-1 and MIP-1α, chemokines that promote macrophage infiltration, in the left ventricle tissue of ZF rats (Chapter 2). However, the co-administration of SnMP with hemin reversed the protective effect of hemin, suggesting the role of the HO system in macrophage infiltration.

After this important observation, we examined the underlying mechanisms responsible for HO-mediated suppression of macrophage infiltration. It is known that macrophages have the potential to exist in different activation states (127, 135, 139, 151, 154, 156-160). Depending upon different kind of stimuli, macrophages are activated and in turn express distinct surface receptors and produce distinct chemokines and cytokines that lead to diverse macrophage pro-inflammatory and anti-inflammatory functions (151, 157, 160). Macrophages are broadly classified into: the classically activated pro-inflammatory M1 phenotype and the alternatively activated anti-inflammatory M2 phenotype (151, 157, 160). The present study unveils that upregulation of the HO system by hemin selectively polarizes macrophages towards the anti-inflammatory M2 phenotype in cardiac tissue of ZF and ZDF rats with corresponding attenuation of the pro-inflammatory M1 phenotype, indicating a novel mechanism through which hemin therapy attenuates obesity induced cardiac inflammation (Chapter 2 and Chapter 3). This was confirmed by western bolt and relative densitometry analysis of specific markers for the M1 and M2 phenotype of macrophages. In the left ventricle tissue of ZF and ZDF rats, basal expression of ED1 which is a specific marker for pro-inflammatory M1 phenotype of macrophages (381), was greatly elevated compared to lean controls. The basal expression of anti-inflammatory macrophage M2 phenotype markers including ED2, CD14, CD206 and CD36 was significantly reduced in untreated ZF and ZDF rats. Interestingly, treatment with hemin potentiated the depressed expression of anti-inflammatory ED2, CD206, CD36, and CD14 markers, whereas it suppressed the elevated expression of pro-inflammatory ED1 markers (Chapter 2 and Chapter 3), suggesting the role of the HO system in selective polarization of macrophages towards the M2 phenotype to attenuate inflammation.

Taken together, in addition to the suppression of pro-inflammatory cytokines and chemokines, the hemin mediated polarization of macrophages towards the M2 phenotype might be considered as a novel anti-inflammatory mechanism employed by the HO system to counteract inflammatory insult associated with insulin resistance and obesity.

4.6 Role of the HO system against insulin resistance

Visceral adiposity and chronic low grade inflammation are pathophysiological driving forces for insulin resistance and cardiomyopathy (1, 5, 8, 15, 18, 150, 261, 266). Since hemin treatment successfully suppressed visceral adiposity and attenuated inflammation, it was believed that these beneficial effects of hemin would be accompanied by the improvement in insulin signaling and glucose metabolism. In general, IRS1, PI3K, GLUT4 and PKB are important proteins of insulin signal transduction pathways and potentiation of these proteins in turn potentiate insulin signaling (128, 167, 168). Therefore, during my PhD research work, I investigated the effect of hemin on the expression of IRS1, PI3K, GLUT4 and PKB proteins (Chapter 2 and Chapter 3). The basal expression of IRS1, PI3K, and GLUT4 was found to be depressed in ZF rats (Chapter 2). Similarly, in obese ZDF rats, a rat model characterized by insulin resistance and overt hyperglycemia, the basal expression of IRS1, PI3K, and PKB was suppressed (Chapter 3). However, treatment with hemin greatly enhanced the expression of these proteins. Moreover, in obese ZF rats glucose intolerance was observed as when challenged with a bolus injection of glucose, only a slight glucose stimulated insulin release was observed. (Chapter 2) In contrast, when hemin-treated ZF rats and ZL rats were challenged with a bolus injection of glucose, there was a high release of glucose stimulated insulin, suggesting hemin improved glucose tolerance. Similarly, hemin treated ZF rats showed reduction in insulin

resistance. Co-administration of SnMP with hemin nullified the beneficial effects of hemin treatment. To further assess the beneficial role of hemin on glucose metabolism the effect of hemin on adiponectin was examined. Adiponectin is an important insulin-sensitizing and anti-inflammatory adipokine (219, 220, 222, 329-338) that plays key roles in glucose metabolism. An inverse relationship was observed between the plasma levels of adiponectin and disease conditions including obesity, diabetes and cardiovascular diseases (219, 221, 222, 331, 332, 334). Consistent with previous findings (196, 202), the present study revealed that treatment with hemin elevated the depressed basal adiponectin levels in both ZF rats and ZL rats (Chapter 2). In contrast, treatment with SnMP reversed the effects of hemin and further reduced the depressed levels of adiponectin.

Taken together, my study clearly showed that the hemin-induced upregulated HO system enhanced insulin-sensitizing adiponectin, potentiated important proteins of the insulin signaling pathway and thereby, improved insulin signaling and glucose metabolism.

4.7 Role of the HO system against oxidative stress

Increased oxidative stress plays a crucial role in tissue remodeling, cardiac dysfunction and insulin resistance (178, 214, 215). Hence, in order to maintain metabolic homeostasis, enhancement of the anti-oxidant defense system that counteracts deleterious effects of increased ROS generation is largely needed. The anti-oxidant effect of an upregulated HO system has been accepted by various reports (3-5, 32-54). Interestingly, in the present study the observation that hemin therapy suppressed 8-isoprostane, the marker of oxidative stress, is consistent with the antioxidant effect of the HO system acknowledged by several studies (3-5, 32-54). In general, increased ROS generation leads to β-oxidation of tissue phospholipids and produces isoprostanes

including 8-isoprostane (225, 226). Urinary 8-isoprostane is a stable and important biomarker of elevated oxidative stress (224-228). Under physiological conditions, basal levels of 8-isoprostane is detected in plasma and urine samples; however, its level is elevated during increased oxidative stress conditions (225, 226). In the present study, the elevated levels of 8-isoprostane was suggestive of increased oxidative stress in the untreated ZF rats and ZDF rats. However, when treated with hemin, there was a marked reduction in levels of 8-isoprostane in ZDF and ZF rats (Chapter 2, Chapter 3 and Chapter 4). Hemin treatment also reduced the levels of 8-isoprostane in ZL controls but the magnitude of reduction was less compared to treated ZF and ZDF rats. In contrast, administration of SnMP along with hemin nullified the effects of hemin and restored the levels of 8-isoprostane comparable to untreated animals. Furthermore, in SnMP alone treated animals, the levels of 8-isoprostane was increased more compared to untreated ZF and ZDF rats, indicating that the increase in oxidative stress might be due to the suppression of basal HO activity via the HO blocker SnMP in ZF and ZDF rats.

Since, 8-isoprostane stimulates ET-1 (196, 378) and both ET-1 (26, 230-235) and 8-isoprostanes (225, 226) were implicated in oxidative stress mediated tissue injury, the effect of hemin on ET-1 was assessed. The left-ventricular levels of ET-1 in untreated ZF and ZDF rats were highly elevated but significantly reduced by hemin treatment. In contrast, SnMP+hemin treatment resulted in elevated ET-1 levels, suggesting the anti-oxidant role of the HO system. One of the possible mechanisms that accounts for the antioxidant effect of the HO system is that treatment with hemin leads to enzymatic degradation of heme via HO-1 into its catabolic products such as bilirubin, biliverdin and ferritin, all of which possess the capacity to scavenge superoxide radicals. Thus, the reduction of ROS generation in hemin treated animals prevented

oxidation of tissue phospholipids and thereby, contributed to the reduction of 8-isoprostane levels.

4.8 Crosstalk between ANP and the HO system

An important observation from my thesis work is the upregulation of ANP by hemin treatment. ANP is a cytoprotective endogenous molecule that plays an important role in blood pressure homeostasis and exhibits anti-inflammatory, anti-proliferative, natriuretic and diuretic effects (315-324). Moreover, it is reported that ANP attenuates cardiac hypertrophy (316), promotes peripheral vasodilation and suppresses cardiac and vascular remodeling (315, 317). In addition, ANP is known to stimulate various cytoprotective heat shock proteins including HO-1 (400, 401, 449). Both HO and ANP has mutual stimulatory effects. On one hand, HO upregulates ANP (359, 442), similarly, ANP also potentiates HO (400, 401, 449). The exact mechanism for the ANP mediated cytoprotective action is not clear. However, it was shown that ANP exhibits its beneficial effects via the guanylate cyclase-coupled A-receptor and guanosine 3', 5'-cyclic monophosphate (cGMP) (449). ANP exerts its anti-inflammatory effects through the attenuation of pro-inflammatory cytokines including IL-6, IL-1β and TNF-α (359, 449). Interestingly, ANP inhibits TNF-α induced inflammatory pathways through the activation of the second messenger cGMP (450). In addition, ANP was shown to reduce fibrosis via the inhibition of expression of ECM proteins TGF-β and fibronectin (387, 450, 451).

My research work showed that hemin treatment successfully abated inflammation and exhibited cardioprotection through the attenuation of pro-inflammatory/oxidative mediators, decreased macrophage infiltration, and suppression of ECM proteins such as collagen-IV, TGF-β and fibronectin. Since, HO can potentiate ANP (359, 442), it is suggested that the cytoprotective

action of the HO system might be attributed indirectly through the potentiation of ANP. In Chapter 2, my thesis results revealed that in untreated ZF rats the basal left-ventricular levels of ANP were markedly depressed by 1.7 fold, but significantly upregulated by 3.3 fold in hemin-treated animals. Moreover, it is well documented that both ANP and ET-1 display reciprocal relationships and exhibit opposing effects (452, 453). For instance, while ANP is anti-inflammatory, reduces insulin resistance and abates fibrosis by suppressing TGF-β1 and fibronectin (314, 316, 322, 324), ET-1 promotes insulin resistance by stimulating inflammatory/oxidative mediators and participates in fibrosis via the induction of fibronectin in conjunction with TGF-β1 (231, 232, 235, 237, 238).. Therefore, potentiation of ANP through hemin may be regarded as another mechanism for hemin-dependent suppression of ET-1.

Altogether, since both ANP and HO exhibit endogenous cytoprotective effects by virtue of their anti-inflammatory, anti-oxidant and anti-proliferative properties (3-5, 32-54, 319, 322, 324), it can be inferred that the combined beneficial effects of both the HO system and ANP ameliorated insulin resistance, improved glucose metabolism and cardiac function.

4.9 Crosstalk between adiponectin and the HO system

Adiponectin is an adipokine that plays major roles in tissue homeostasis (219, 220, 222, 329-339). Owing to its anti-diabetic, antioxidant, anti-atherogenic and insulin sensitizing effects, it is cytoprotective against the pathological conditions of obesity, insulin resistance and cardiovascular disease (220, 329, 334, 336, 337). Evidence is there to support the idea that low levels of adiponectin are a predictor of cardiovascular disease (72, 220, 329, 331). High levels of adiponectin were demonstrated to be cardioprotective against impaired cardiac function, cardiac ischemia reperfusion injury (72, 329) and cardiomyopathy (329, 337), whereas its deficiency

aggravated cardiac injury as observed in the adiponectin knockout mouse model (219, 332). Another important observation of my thesis work was the hemin-dependent upregulation of depressed basal levels of adiponectin in left-ventricular tissue of ZF rats (Chapter 2). However, treatment with SnMP along with hemin resulted in further depression of the basal levels of adiponectin. This suggests that hemin treatment enhanced adiponectin levels in ZF rats. This is consistent with recent reports from our lab and other studies that demonstrated that the HO-mediated upregulation of adiponectin levels contribute to improved insulin sensitivity and glucose metabolism (202, 444, 446). In addition, my thesis work showed that in ZF rats hemin treatment potentiated insulin signaling and improved glucose tolerance through the suppression of inflammatory/oxidative mediators and selective polarization of macrophages towards the anti-inflammatory M2 phenotype. Moreover, it is well documented that adiponectin is anti-inflammatory in function and reduces the expression of pro-inflammatory cytokines TNF-α and IL-6 (334, 336). Interestingly, adiponectin also promotes macrophage polarization towards the anti-inflammatory M2 phenotype (220, 329, 334, 336).

All together, these findings indicate that an interplay exists between adiponectin and the HO system, as both HO and adiponectin are cardioprotective and upregulation of both contribute to attenuation of insulin resistance and improved glucose metabolism (38, 39, 44, 162, 205, 329-331, 334, 337, 340, 347-352). Thus, hemin-mediated potentiation of adiponectin might serve as another important mechanism for improved glucose metabolism and cardiac function in ZF rats.

4.10 Synergistic effects between the HO system, ANP and adiponectin on glucose metabolism and cardiomyopathy

The key observation of my PhD research work indicates that the upregulation of HO system by hemin and corresponding enhancement in adiponectin and ANP levels is an important cytoprotective mechanism for improved glucose metabolism and cardiac function in hemin-treated ZF rats (Chapter-2). No other study has reported the effects of an upregulated HO system on ANP or adiponectin in cardiac tissue of ZF rats and the corresponding link between HO-ANP-adiponectin axis and the levels of IL-6, TNF-α, IL-1β, MCP-1, TGF-β, and fibronectin and macrophage infiltration. An interplay exists between HO, ANP and adiponectin, as mutual stimulatory effects have been reported between HO and ANP. On one hand, the HO system upregulates ANP (359, 442), similarly, ANP also potentiates the HO system (400, 401, 449). In addition, both ANP and HO were known to stimulate adiponectin (202, 319, 397, 443, 444). Based on these findings, it is possible that the elevated levels of adiponectin in hemin treated animals (Chapter-2) was either a consequence of direct stimulation by an upregulated HO system or through the indirect enhancement of ANP levels. Either way, the underlying mechanisms responsible for potentiation of adiponectin in response to HO and ANP still need to be clarified. It is well documented that ANP increases the levels of the anti-inflammatory and insulin sensitizing protein adiponectin in the circulation (397, 443). Moreover, ANP action is mediated largely by cGMP (449) and adiponectin is also reported to stimulate the second messenger cGMP (399). Furthermore, the HO system generates a metabolite CO, a vasodilator and a stimulator of cGMP that regulates vascular contractility (42, 45). Studies have shown that on exposure to chronic inflammatory and oxidative insults, decreased levels of adiponectin and HO-1 contribute to the development of insulin resistance (41, 219-223, 454). Interestingly, an

increase in HO-1 levels was shown to be associated with a parallel increase in adiponectin levels that exhibit cytoprotective effects accompanied by improved insulin sensitivity and preserved endothelial function in different animal models (53, 196, 202, 359, 444, 446, 454). Furthermore, since both ANP and HO exhibit endogenous cytoprotective effects by virtue of their anti-inflammatory, anti-oxidant and anti-proliferative properties (3-5, 32-54, 319, 322, 324), it is suggested that the mutual stimulatory effects between the HO system and ANP, in turn, ameliorate insulin resistance and potentiate cardioprotection in synergistic manner.

Taken together, the novel observation of my thesis work clearly shows that the potentiation of the HO-ANP-adiponectin axis by hemin in ZF rats has beneficial effects against obesity induced cardiomyopathy and improved glucose metabolism through the mechanisms that involved the attenuation of pro-inflammatory chemokines/cytokines and oxidative mediators such as IL-6, TNF-α, IL-1β, MCP-1, ET-1, suppression of ECM deposition and macrophage infiltration.

4.11 Conclusion

In conclusion, my thesis has presented a detailed study on the novel effects of upregulating the HO system by hemin on cardiomyopathy in ZF rats and the multifaceted mechanisms by which hemin therapy improves insulin signaling, glucose metabolism and cardiac function in obese ZF rats. My results clearly showed for the first time that the enhancement of ANP and adiponectin by hemin-induced upregulated HO system is an important cardio-protective mechanism for reduction in insulin resistance and improved cardiac hemodynamics in ZF rats.

The present study for the first time unveils:

(i) a novel role of an upregulated HO on selective polarization of macrophages towards an anti-inflammatory M2 phenotype with parallel suppression of the pro-inflammatory M1 phenotype in the cardiac tissue of ZF rats.

(ii) hemin-induced suppression of heart failure proteins such as osteopontin and osteoprotegerin implicated in inflammation, tissue remodeling and hypertrophy in the cardiac tissue of ZF rats

Other important observations from this study include:

(iii) The hemin-dependent attenuation of pro-inflammatory chemokines/cytokines (TNF-α, IL-6, IL-1β, MCP-1, MIP-1α) and oxidative mediators (8-isoprostane and ET-1).

(iv) The hemin-dependent reduction of ECM/pro-fibrotic proteins like collagen-IV, fibronectin, TGF-β and suppression of cardiac lesions, fibrosis and left-ventricular and cardiomyocytes hypertrophy.

(v) Hemin-induced potentiation of ANP, adiponectin and important proteins of the insulin signaling pathway including IRS1, PI3K and GLUT4 accompanied by reduction in glucose intolerance, insulin resistance and improved cardiac hemodynamics in ZF rats.

Collectively, these data suggests that an upregulated HO system by hemin and the corresponding suppression of inflammatory/oxidative mediators, reduction of the pro-inflammatory M1 macrophage infiltration, attenuation of ECM/heart failure proteins including osteopontin and osteoprotegerin, reduction in cardiac hypertrophy and potentiation of the components of insulin signaling, ANP and adiponectin are among the several mechanisms by which the HO system counteracts cardiometabolic complications arising from obesity.

4.12 Significance

Chronic low grade inflammation and increased oxidative stress are underlying mechanisms that links obesity with cardiovascular disease and insulin resistance (3, 5, 14, 15, 24, 27, 29, 58, 68, 69, 127, 149, 255, 256). Although, considerable efforts have been made in elucidating mechanisms implicated in obesity-related cardiovascular disease, new therapeutic tools with novel mechanisms of action to counteract the adverse effects of obesity-related cardiometabolic complications, is greatly needed. In this context, the present study strongly underscore the beneficial effects of concomitantly upregulating the HO, ANP and adiponectin by hemin against impaired insulin signaling and cardiomyopathy. Collectively, the findings reported in the present study provide insights for designing novel therapeutic strategies that can synergistically potentiate the HO-ANP-adiponectin axis to counteract cardiometabolic complications related to obesity and thereby, have great translational potential. In addition, the drug hemin used as HO-inducer have therapeutic applications, as it has been already approved by the Food and Drug Administration for use against porphyria (55, 56).

4.13 Future directions

As a natural extrapolation of the findings reported so far, several important thematic issues can be further developed and elucidated in future studies.

1. My study showed that treatment with hemin in both healthy ZL-control rats and insulin resistant obese ZF rats enhanced HO-1, adiponectin and ANP and suppressed IL-6, IL-1β, TNF-α, MCP-1, MIP-1α, 8-isoprostane, ET-1 and lowered HOMA-IR index (Chapter 2). Interestingly, we observed that the magnitude of suppression of these inflammatory/oxidative mediators in normal ZL control rats was smaller as compared to

unhealthy obese ZF rats. The exact mechanism for this hemin-mediated HO effect in ZL and ZF rats is still not clear and needs to be resolved. One of the possible explanations for this selective effect of HO is that the HO system is more stable in ZL rats that are normal and healthy animals with a normal HO system and functional insulin signaling, as compared to unhealthy, obese and insulin resistant ZF animals which have compromised cardiac function with depressed HO-1 and HO-activity. Exploring the putative alternative pathways for the selectivity of the HO system in diseased conditions might help in better understanding of the HO system that can be explored as a therapeutic tool against the co-morbidity of obesity and cardiomyopathy.

2. The present study, our recent publications and other clinical studies have well acknowledged the beneficial role of HO system in response to various pathophysiological stress conditions (3-5, 32-54). However, the activation of HO by different stress stimuli only results in the transient increase in HO activity that falls below the threshold level required for the activation of downstream signaling components such as CO, bilirubin and biliverdin through which the HO system confers cytoprotection. CO is a physiological signaling molecule with anti-inflammatory, anti-apoptotic, anti-proliferative and vasorelaxing properties (41, 45, 50, 208, 430, 455). The proposed mechanism for the vasoregulatory effect of CO is through the activation of the soluble guanylyl cyclase (sGC)/cGMP pathway. Interestingly, CO mimics nitric oxide (an important gasotransmitter) in several biological functions (39, 52, 456). Both nitric oxide and CO shares similar vasodilatory properties via binding to the heme moiety of sGC and function through the activation of similar sGC/cGMP pathways with a corresponding

increase in cGMP levels (43, 52). It is important to determine, whether their actions are additive, opposite, overlapping or synergistic (456). It is documented that CO is chemically more stable compared to NO and has a longer half-life. However, as a vasodilator CO is 1000-fold less potent than NO. Although, CO and nitic oxide functions similarly as a vasorelaxing agent both display variations in their biological availability (35, 52, 456). Hence, to activate the HO/CO system above the threshold level, HO inducers like hemin are needed to boost the activity of the sGC/cGMP pathway (4, 53, 54, 195, 196, 359). Inspite of these findings, some important questions need to be answered. Besides CO/sGC what are other alternative pathways for HO-mediated elevated cGMP levels? What is the optimal level of HO-1 expression that is sufficient to stimulate CO and bilirubin to exhibit a beneficial role? Future studies directed to explore these questions might help us to better understand the HO/CO system in order to exploit it as a therapeutic tool against cardiac complications related to obesity.

3. An important observation from my research is the potentiation of the HO-ANP-adiponectin axis by hemin in ZF rats. Although, an interplay between HO, ANP and adiponectin have been reported (202, 319, 397, 400, 401, 442-444, 449), the underlying mechanisms for the potentiation of HO-ANP-adiponectin axis by hemin is unknown. Several questions still remain puzzling:

   (i) What is the exact mechanism for the enhancement of ANP by HO or vice versa?

   (ii) How does upregulation of HO increase adiponectin levels?

   (iii) Does adiponectin stimulate HO?

   (iv) How does ANP stimulate adiponectin?

(v) On the other hand, does adiponectin stimulate ANP?

(vi) Would the combination of both adiponectin and ANP stimulate HO differently than when each substances is given alone?

(vii) Does the combination of both HO and adiponectin stimulate ANP?

(viii) Since HO and ANP are known to stimulate adiponectin release, (202, 319, 397, 443, 444), it remains unclear if it is a direct stimulation by the HO system or indirectly through the enhancement of ANP?

With so many unanswered questions, future research is needed to discover mechanisms responsible for the synergistic potentiation of either adiponectin by HO and ANP or the potentiation of HO by ANP and adiponectin as well as the potentiation of ANP by HO or adiponectin. The elucidation of these complex interactions will help us to better understand and explore the HO-ANP-adiponectin axis as a new therapeutic target against obesity and cardiomyopathy.

Collectively, the findings reported in the present study open new avenues for further investigation on HO inducers that can be exploited to design novel therapeutic strategies to counteract cardio-metabolic complications associated with obesity.

Appendices

Appendix A, appendix B, appendix C below are three of my first-authored published manuscripts. These manuscripts provides up-to-date and detailed knowledge of the functional significance of the HO system and its downstream signaling molecules including bilirubin, CO and ferritin as potential therapeutic tools for effective management of hypertension, type-1 diabetes and associated cardio-metabolic complications. In ***appendix A***, the current state of knowledge and future directions of research in the area of obesity-related cardiomyopathy and nephropathy have been acknowledged.

Appendix A: **Tiwari S**, Ndisang JF. The Role of Obesity in Cardiomyopathy and Nephropathy**.** ***Curr Pharm Des. 2014***; 20(9):1409-17.

Appendix B**: Tiwari S**, Ndisang JF. The Heme Oxygenase System and Type-1 Diabetes**.** ***Curr Pharm Des. 2014***; 20(9):1328-3.

Appendix C**: Tiwari S** and Ndisang JF. Heme Oxygenase System and Hypertension: a comprehensive insight. ***Curr Pharm Des. 2014***; 20(9):1354-69.

Appendix D1: *Classification of obesity*

Obesity is a chronic condition of nutrient overload and the only tissue that stores excess nutrients is adipose tissue. Adipose tissue is mainly composed of adipocytes that are specialized cells and store excess nutrients in the form of lipids (23, 62, 260). Adipose tissue either produces new adipocytes or enlarges the existing ones to meet excess nutrient demand. In addition,

adipose tissue consists of preadipocytes, endothelial cells, fibroblasts and a variety of immune cells including, leucocytes and macrophages (62, 133, 260). Based on appearance, adipose tissue can be classified into white adipose tissue and brown adipose tissue. White adipose tissue is a predominant site of energy storage mainly in the form of triglycerides and is more distinct. In contrast, brown adipose tissue is dense and highly vascularized exhibiting brown coloration and regulates body temperature through non-shivering thermoregulation (23, 133). On the basis of anatomical location, white adipose tissue is further classified into visceral adipose tissue and subcutaneous adipose tissue (62, 251, 253, 354).

1.1 Visceral adipose tissue and subcutaneous adipose tissue

Distribution and anatomical location of fat tissue is an important feature in the pathophysiology of obesity and associated complications (62, 251, 253, 354). White adipose tissue is classified into two major depots namely, subcutaneous and visceral adipose tissue (62, 251, 253, 354). Anatomically, visceral adipose tissue is mainly found within the abdominal cavity and mediastinum surrounding internal organs such as heart, pancreas and kidneys, whereas subcutaneous adipose tissue is found between the muscle and dermis (62, 354). Visceral adipose tissue is mainly comprised of tightly packed unilocular adipocytes which are metabolically more active compared to subcutaneous adipocytes and is more susceptible to lipolysis by catecholamine (62, 457). Besides adipocytes, visceral adipose tissue is composed of stromal vascular components such as preadipocytes, ECM proteins (collagen, fibronectin, osteopontin and metalloproteinase), fibroblasts and endothelial cells (62, 260, 458). During obesity, visceral adipose tissue is the most altered tissue (4, 5, 71, 459). Alterations occur in both adipocytes and stromal vascular components in response to the increased storage of triglycerides.

As a result, adipocytes undergo hypertrophy and enlargement of visceral adipose tissue takes place (1-5). Due to the expansion of visceral adipose tissue, dysfunctional adipocytes and ECM remodeling, infiltration of macrophages and other immune cells occurs that activates adverse morphological and inflammatory responses in visceral adipose tissue (3, 5, 15, 124-127, 133). In addition, enlarged visceral adipose tissue is found to be associated with elevated levels of pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α (3, 5, 15, 124-127, 133). In contrast, low levels of anti-inflammatory adipokines such as adiponectin was reported in visceral adipose tissue compared to subcutaneous adipose tissue (72, 220, 329, 331, 337). Thus, excess accumulation of visceral adipose tissue accounts for a pro-inflammatory state (4, 5, 71, 75, 459). Although, both visceral and subcutaneous adipose tissue contribute to pathological conditions associated with obesity, however, excess visceral adipose tissue is more deleterious than subcutaneous adipose tissue (2, 25, 62, 460). Various studies confer that excess deposition of visceral adipose tissue leads to a higher risk for obesity associated metabolic and cardiovascular complications including, dyslipidemia, insulin resistance and diabetes (1-14). Studies showed that the removal of abdominal subcutaneous adipose fat from obese individuals with insulin resistance had no effect on metabolism, while, insulin sensitivity was improved with the removal of visceral adipose fat in these subjects (461, 462). Thus, excess visceral fat is a major risk factor for obesity associated adverse metabolic outcomes (3, 5, 15, 124-127, 463).

Appendix D 2*: *Adipokines in obesity related metabolic disorders

1.1 Leptin

First identified in 1994 by Zang et al. leptin is an adipokine mainly secreted by adipocytes that plays a major role in energy metabolism (18, 75). Leptin regulates energy metabolism

through its direct or indirect actions on the brain. It is proposed that leptin stimulates distinct centers in the hypothalamus which in turn releases neuropeptides and neurotransmitters that reduce food intake, enhance the energy expenditure as well as modulate glucose and lipid metabolism (18, 75). Leptin possess anti-diabetic properties and was reported to attenuate hyperglycemia in obese mice (464, 465). Moreover, treatment with leptin reversed hepatic steatosis and improved insulin resistance in lipodystrophic patients diagnosed with relatively decreased levels of circulating leptin (466). However, it should be taken into account that during obese conditions leptin therapy is highly ineffective due to leptin resistance. Leptin resistance is a condition, where high levels of leptin are unable to exert anorexic effects (18, 467) owing to unresponsiveness towards the hormone (75, 459, 468). The underlying mechanisms responsible for leptin resistance during obesity are still not clear. However, it is suggested that SOCS3 and protein tyrosine phosphatase 1B (PTP1B) are among the possible signaling molecules involved in the regulation of leptin resistance (75). It is suggested that the different metabolic effects of leptin are mediated by multiple signaling pathways (18). For example, leptin exerts its anti-diabetic effects through the stimulation of PI3K/Akt pathway while, its anorexic effects are governed by the activation of JAK-STAT signaling pathway (18). Thus, an understanding of the mechanisms underlying leptin resistance will be helpful in exploring new therapeutic strategies to target leptin against obesity and associated metabolic disorders.

1.2 Resistin

Resistin is an adipokine that belongs to a family of cysteine-rich proteins termed as resistin like molecules (RELMs) (78, 469). Resistin expression was first identified in adipose tissue of obese mice (78). The name resistin has been derived due to its resistance towards

insulin action. Various rodent models of obesity and insulin resistance were found to be associated with elevated levels of resistin (75, 470). However, improved glucose metabolism was observed in resistin knockout mice fed a high fat diet (471), suggesting the possible role of resistin in the pathogenesis of obesity-mediated insulin resistance and type-2 diabetes (75, 470). The underlying mechanism responsible for the pathological role of resistin in various disease conditions is still not very clear. However, an in vitro study using a pre-adipose 3T3-L1 cell line demonstrated that resistin impaired normal insulin signaling via the alteration of expression of an insulin receptor as well as IRS1 and IRS2 (472). Furthermore, resistin has been reported to modulate AMP-activated protein kinase (AMPK) activation that plays important roles in insulin sensitization during insulin signaling (75). However, in humans, the role of resistin regarding insulin resistance is uncertain. Epidemiological studies demonstrated the association between high levels of resistin and increased risk for insulin resistance, type-2 diabetes, myocardial infarction and atherosclerosis (18, 75). In addition, resistin was shown to stimulate the production of several pro-inflammatory cytokines and chemokines including, IL-6, TNF-α, MCP-1 all of which are implicated in the development of insulin resistance during obese conditions (18, 75). This suggests that in human obesity, resistin alters insulin signaling indirectly through the elevation of inflammatory cytokine production (473). Thus, the therapeutic approach that targets resistin inhibition will be of great relevance for the treatment of obesity.

Appendix D 3: *Altered cardiac structure and function in obesity*

1.1 Hemodynamic alterations

Obesity independent of other cardiovascular risk factors such as hypertension, atherosclerosis and myocardial infarction can alter both diastolic and systolic function (1, 14, 23,

71, 244-252, 254). Obesity has detrimental effects on cardiac diastolic function (129, 265, 289). It is reported that impaired diastolic function often precedes systolic dysfunction and can occur either independently or in combination with systolic dysfunction (129, 265, 289, 474). Diastolic dysfunction is characterized by alterations in left-ventricular filling indices that results in abnormal diastolic pressure in response to reduced ventricular compliance due to the enlargement in the left-ventricular mass and impaired pressure and volume load (129, 265, 289). Abnormal load conditions including increased afterload and duration of obesity also contribute to compromised left-ventricular systolic function (129, 289, 474). In addition, increased cardiac output and heart rate was also found to be associated with obesity (129, 265, 289, 474). It is suggested that the elevated heart rate in obese patients might be due to the suppressed parasympathetic tone instead of increased sympathetic activity (129, 265, 289). Moreover, increased extracellular volume and higher blood flows to adipose and non-adipose tissues with increased venous return and cardiac output has been observed during obese conditions (129, 265, 289). Thus, obesity-mediated impaired cardiac hemodynamics and ventricular function contribute to the pathogenesis of cardiomyopathy.

1.2 Cardiac hypertrophy

Cardiac hypertrophy is one of the important clinical features associated with heart failure (129, 287-289). In general, obesity is an independent risk factor for left-ventricular hypertrophy independent of conventional risk factors (1, 14, 23, 71, 244-252, 254, 288). The severity and duration of obesity influences the degree of left-ventricular hypertrophy and myocardial remodeling (129, 254, 288). Myocardial remodeling and fibrosis, cardiomyocytes hypertrophy and thickening of cardiac myofibrils are characteristic features of left-ventricular hypertrophy

(129, 287-289). Studies have shown that compared to lean controls increase in both left-ventricular cavity size and wall thickness were observed in obese subjects. However, wall thickness increased more than cavity size, indicating the prevalence of concentric hypertrophy more than eccentric hypertrophy during obese conditions (288). However, obesity has been found to be associated with both concentric and eccentric forms of cardiac hypertrophy. An increase in right ventricular wall thickness and volume were also found to be associated with obese individuals (129, 257, 265). Obesity induced altered hemodynamic changes in response to increased blood volume and corresponding venous return amplifies cardiac load and triggers a series of hemodynamic, neurohormonal and molecular factors. This result in increased left-ventricular mass and wall thickness, elevated left-ventricular pressure, ventricular dilatation as well as decreased systolic performance that eventually leads to the development of left-ventricular hypertrophy (129, 150, 265, 287-289). Moreover, obesity associated hypertension further exacerbate the condition of left-ventricular hypertrophy and myocardial remodeling (60, 150, 254).

1.2.1 Concentric and eccentric hypertrophy

In response to pressure overload caused by pathological insults including hypertension, myocardial infarction and aortic stenosis, the heart triggers a concentric hypertrophic response to counter balance the increased wall stress and intraventricular pressure (129, 257, 265, 286, 287). The heart responds to increased left-ventricular systolic wall stress and high systolic pressure by adding new sarcomeres units (which are repeating micro-anatomical units of myofibrils of cardiomyocytes) in parallel to the previous sarcomeres. This leads to the development of increased left-ventricular wall thickness and smaller cavities (286, 287). The increased

myocardial stiffness and reduced compliance, eventually leads to left-ventricular diastolic dysfunction and diastolic heart failure (286, 293). It is reported that as a consequence of concentric hypertrophy, there is a prolongation in the early filling phase of diastole accompanied by reduced rate and volume of rapid filling (286, 287).

In contrast, pathological insults such as chronic mitral regurgitation and aortic regurgitation cause ventricular dilation due to volume overload and increases diastolic wall stress. As a result, myocytes lengthen by adding new sarcomeres in a series to existing sarcomeres that develops into eccentric cardiac hypertrophy (286, 287). Volume overload related eccentric hypertrophy in the heart is characterized by large dilated cavities and a relatively thin myocardial wall together with decreased left-ventricular systolic function and performance (286, 287, 293). It is suggested that individuals with systolic heart failure display left-ventricular eccentric hypertrophy (293).

1.2.2 Physiological hypertrophy

In contrast to concentric and eccentric cardiac hypertrophy, the physiological stimulus that induces physiological hypertrophy includes strength and endurance exercise training (286, 287, 293). Another common example for physiological hypertrophy was observed in pregnant females characterized by left-ventricular enlargement with preserved wall thickness and ejection fraction (287, 293). The cardiac structural changes observed during isotonic exercise, such as running, walking, cycling and swimming might be considered a form of eccentric hypertrophy where both chamber enlargement and proportional increase in left-ventricular mass was observed in response to increased venous return to the heart and volume overload (287, 293). However, high level resistance training such as weight lifting is associated with a large pressure load

compare to volume load on the heart and thereby, develops a form of concentric hypertrophy (286, 287). Furthermore, compared to concentric and eccentric hypertrophy, physiological hypertrophy does not lead to left-ventricular dilation or heart failure (286, 287, 293).

Appendix D 4: ***ANP (Synthesis, tissue distribution and functions)***

Initially, ANP was isolated from the atria of heart (313-316). During embryonic development, ANP is highly expressed by both atrium and left-ventricle. Apart from cardiac tissue the pituitary gland, lungs, hypothalamus and kidneys express ANP at lower levels (314). The exact mechanism that governs ANP secretion is still not very clear. However, mechanical stretching and external stimuli exerted by angiotensin II, catecholamine and vasopressin may have stimulatory effects on ANP release (315). Structurally, ANP is 28-amino acid long peptide with a single intra-chain disulfide bond. Initially, ANP is synthesized as a precursor pro-ANP in cardiac cells (313). Upon secretion from atrial cardiomyocytes, pro-ANP is cleaved by a myocardium-specific type-II transmembrane protease to yield biologically active ANP and an inactive amino terminal fragment NT-pro ANP (314, 316). The process is followed by the release of Pro-ANP, NT-pro ANP and ANP in the circulation (313). The biological effects of ANP are mainly mediated by its active form via binding to specific high affinity receptors natriuretic peptide receptor (NPR)-A and NPR-C on the surface of target cells (315, 318). NPR-A is known to be abundantly expressed by several tissues including kidney, adrenal gland, heart, brain, testis, eye, intestine and vascular system (315). Binding of ANP to NRP-A activates intrinsic guanylyl cyclase activity of the receptor that leads to the increase in intracellular secondary messenger cGMP levels (313, 314). It is reported that impaired or absence of NRP-A gene expression results in the development of hypertension, cardiac hypertrophy and ventricular

fibrosis (314). Furthermore, NPR-C is also known as a clearance receptor, as it plays a major role in clearance of ANP from the circulation via a receptor-mediated endocytosis process (315). NPR-C is expressed by several tissues including kidneys, adrenals, lungs, vascular system, intestine, brain, and heart (315). In addition, NPR-C coupled with adenylate cyclase activity reduced cAMP production and promotes phospholipase-C stimulation there by, increasing inositol triphosphate (IP3) and diacylglycerol (DAG) production (315). Binding of ANP to NRP-C promotes endothelial nitric oxide synthase expression as well as inhibition of endothelin stimulated MAPK pathway (315). These findings suggest that ANP and its receptors plays important role in biological processes.

1.1 ANP and inflammation

ANP is anti-inflammatory in function and exerts an inhibitory effects on pro-inflammatory chemokines, cytokines and adipokines (315-324). ANP inhibits the activation of pro-inflammatory transcriptional factors such as NFκB and AP-1 as well as attenuates TNF-α secretion via ANP/cGMP signaling that attenuates p38 MAPK downstream signaling pathway (322). Furthermore, in humans, ANP has been demonstrated to suppress pro-inflammatory chemokines and cytokine production from adipocytes and macrophages of adipose tissue (322). Besides having anti-inflammatory function, ANP has a regulatory role on adipokines related to energy metabolism (319). Studies demonstrated that a positive correlation exists between ANP and adiponectin levels. For example, an increase in ANP levels results in a parallel increase in total and high molecular weight (HMW) adiponectin in humans (319, 443). An ANP-mediated reduced levels of leptin has been observed in human adipocytes (319). Thus, the decreased levels

of ANP and its corresponding effect on other adipokines might be one of the mechanisms that aggravate the metabolic disorders during obesity.

During the inflammatory process, macrophages release large amounts of nitric oxide through iNOS stimulation (450). The interaction of ANP with NPR-A receptors were known to attenuate nitric oxide release from macrophages in an autocrine manner through the subsequent enhancement of intracellular cGMP levels that inhibits the NFκB signaling cascade involved in iNOS synthesis (450). In addition, in vitro experiments showed that ANP attenuates LPS-induced TNF-α and IL-6 synthesis and thereby, protects against inflammatory cell injury (450, 475). TNF-α plays a major role in the pathogenesis of diseases including obesity, insulin resistance, hypertension and atherosclerosis (81, 83, 84, 90). It is demonstrated that ANP/NPR-A show anti-inflammatory effects against TNF-α induced endothelial cell injury through the inhibition of the downstream p38MAPK signaling pathway, increased production of cGMP and mitogen-activated protein kinase phosphatase-1 (MKP-1) (450, 475). ANP is also known to suppress endothelial TNF-α induced MCP-1 production via the inhibition of p38MAPK pathway (450). Interestingly, another mechanism by which ANP inhibits the TNF-α-induced inflammatory pathway through the activation of cGMP mediated HO-1 production, an enzyme that is known to be cytoprotective under different conditions of tissue insults (38, 39, 44, 205, 347-352). In addition, ANP produced locally by macrophages was shown to inhibits the production of LPS-induced cyclooxygenase-2 (COX-2), an enzyme that plays an important pro-inflammatory role during inflammation (450).

Thus, ANP has emerged as an important anti-inflammatory molecule that functions in an autocrine or paracrine manner to counteract the effects of pro-inflammatory chemokines, cytokines and various transcriptional factors associated with inflammation.

1.2 ANP and oxidative stress

Epidemiological studies suggested the anti-oxidant role of ANP against different pathological conditions (315, 323). The exact mechanism by which ANP counteracts the deleterious effects of ROS is not known. However, it is suggested that at physiological concentration, ANP protects against ROS-induced tissue damage by the activation of a NPR-A/cGMP mediated signaling pathway (315). Similarly, in liver tissue ANP was shown to be cytoprotective against oxidative stress through the mechanism of NPR-A/cGMP system activation (315, 449). A recent study reported the cardioprotective and anti-oxidant effect of ANP in cardiomyocytes (315, 324). In patients with heart failure, infusion of synthetic ANP not only improved hemodynamic features but also suppressed free radical generation, thereby, exhibiting protection against oxidative stress injury in cardiomyocytes (315). In addition, the stress stimuli generated by mechanical stretch, pressure overload and angiotensin-II promotes cardiac hypertrophy via ROS generation (315). ANP has been shown to suppress angiotensin-II-mediated oxidative stress and reduced hypertrophic responses in neonatal rat cardiomyocytes (315, 324). Another mechanism for ANP mediated cardioprotection against oxidative stress in vascular smooth muscle cells is via the inhibition of phospholipase-D activity and reduction of intracellular calcium ions (315). Moreover, it is demonstrated that ANP promotes an increase in the plasma levels of adiponectin under pathological condition of type-2 diabetes and heart failure, suggesting another possible mechanism for an ANP-mediated cardioprotective effect (319, 443).

1.3 ANP, glucose metabolism and insulin resistance

ANP plays an important role in glucose homeostasis (319, 476, 477). In isolated rat pancreatic islets, ANP increased insulin secretion in response to glucose and promotes growth of β-cells, whereas, ANP knock-out mice were shown to have reduced β-cell mass, suggesting the regulatory effect of ANP on β-cell function (319, 477). During obesity, metabolic organs such as liver and skeletal muscle are strongly associated with insulin resistance due to excess deposition of lipid content such as triacylglycerol (319). On the other hand, increased lipid oxidation in these metabolic organs improved insulin sensitivity (319, 476). ANP has been reported to improve insulin sensitivity through increased lipid oxidation in liver and skeletal muscles (319). Moreover, there exists a correlation between plasma glucose and insulin levels and ANP levels in circulation. Low levels of natriuretic peptides were found to be associated with high glucose and insulin levels independent of body fat distribution (326). In addition, data from the recent Framingham Heart Study showed that low levels of natriuretic peptides were found to be associated with a greater risk for insulin resistance in both lean and obese individuals (327). Increased levels of natriuretic peptides were reported to be beneficial against insulin resistance and type-2 diabetes (319, 320). Thus, these data suggests that ANP plays a cytoprotective role against the pathological condition of insulin resistance and diabetes.

1.4 Cardioprotective role of ANP

ANP displays a regulatory role in cardiovascular homeostasis and governs blood pressure and extracellular fluid volume (314, 318, 450). It is reported that at basal levels ANP participates in the regulation of blood pressure by enhancing microvascular permeability (314). Moreover, ANP attenuates cardiac hypertrophy, promotes peripheral vasodilation and has inhibitory role in

cardiac and vascular remodeling (315, 317). Depending upon the severity and type of disease various pathophysiological stress conditions acts as a trigger for the release of ANP from atria and ventricles. For example, under normal conditions ANP is mainly secreted from the atria. However, during diseased conditions the ratio of ANP secretion from the left ventricle was reported to be proportional to left-ventricular dysfunction and end diastolic pressure (315, 317). This suggests that release of ANP from the atria and ventricles depends upon the severity of disease condition and represent the extent of cardiac overload. Under stressful conditions of hemodynamic load or myocardial ischemia, ANP was shown to be cardioprotective and reduced cardiac preload and afterload (313, 314) without initiating reflex tachycardia (316). It suppressed total peripheral resistance via its inhibitory effect on the release of rennin, vasopressin and aldosterone (315). It is reported that during hypertension, ANP relaxes vascular smooth muscle cells through the activation of the protein kinase G (PKG) pathway that contribute to reduction in intracellular calcium ions and hyperpolarization of endothelial cells of the vasculature (313, 314).

ANP also possess anti-proliferative properties (450). In vascular smooth muscle cells and cardiomyocytes, ANP promotes anti-proliferative effects against platelet-derived growth factor and insulin-induced cell growth. It is suggested that the anti-proliferative effect of ANP was mediated through the mechanisms that involves the reduction of intracellular cAMP levels and inhibition of MAPK and PKG signaling pathways (315, 317, 450). In a similar manner, ANP-mediated inhibition of PI3K/Akt signaling is another mechanism for ANP anti-proliferative effect and attenuation of ROS production (450).

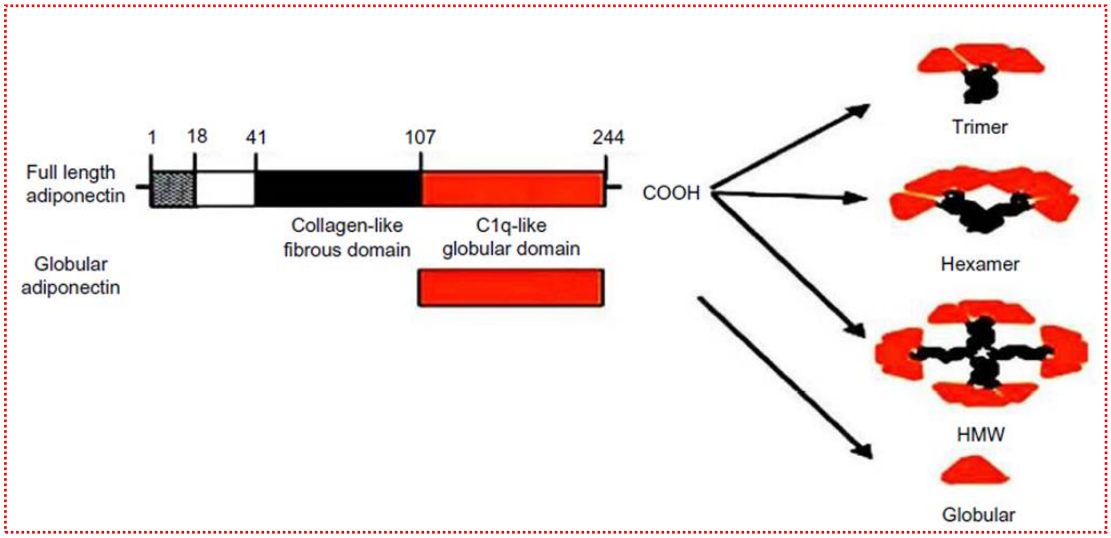
Another mechanism by which ANP can regulate blood pressure is through its action on the RAAS system (314). ANP has been reported to attenuate renin production via a PKG-II

dependent mechanism and suppresses aldosterone production at the adrenal gland (314, 316). Moreover, ANP exerts its diuretic and natriuretic effects through its direct action on renal hemodynamics. It is predicted that in the kidney, ANP increases glomerular filtration rate (GFR) via the dilation of the afferent arteriole and constriction of the efferent arteriole (314). ANP in circulation reduces sodium and water reabsorption through its inhibitory effect on angiotensin II-induced sodium and water transport in the proximal tubules as well as inhibition of aldosterone secretion from the adrenal cortex and cardiac tissue (314, 316). The regulation of extracellular fluid volume and cardio renal homeostasis by ANP demonstrate its cardioprotective properties. Taken together, these findings highlights ANP as an important therapeutic diagnostic tool for cardiovascular and renal disease.

Appendix D 5: ***Adiponectin (receptors, structure and function)***

Structurally, adiponectin is a 30-kDa plasma protein (220) having a globular head at the C-terminal and collagen like domain at the N-terminal (331). In circulation, adiponectin is secreted from adipocytes as three large oligomeric complexes that are modified post-translationally in the endoplasmic reticulum by molecular chaperons (220, 331) **(Figure: Appendix D 5)**. These oligomeric complex formations may be regarded as a mechanism to modulate various adiponectin mediated biological functions (329). The first class is comprised of a low molecular weight (LMW) trimeric adiponectin approximately 90 KDa with basic units. The second class includes approx. 180 KDa middle molecular weight (MMW) hexamers. The third class is of high molecular weight (HMW) isomers (dodecamers and octadecamers) (220, 331). The HMW adiponectin undergoes through extensive post-translational modifications (hydroxylation and glycosylation) at its collagen-like domain to maintain its stability in the

circulation (329, 331). HMW adiponectin is the most biologically active oligomeric isomer that is important in regulation of cardiometabolic complications (331). Furthermore, it is reported that elevated levels of HMW adiponectin is correlated with reduced levels of cardiovascular disease and improved insulin sensitivity (222, 331, 333). Adiponectin is a multipotent adipokine that mediate its biological functions with the help of three receptors namely, adiponectin receptor protein-1 (Adipo-1), adiponectin receptor protein-2 (Adipo-2) and T-cadherin (333, 340). Adiponectin receptors are specific in their distribution and functions and differ with regards to their affinity for adiponectin oligomeric isomers (222). For example, globular adiponectin has affinity for Adipo-1, while full length oligomeric adiponectin binds to Adipo-2 receptors (222). Adipo-1 and Adipo-2 are structurally related transmembrane receptors that have seven transmembrane domains (220). However, both Adipo-1 and Adipo-2 were reported to be functionally and structurally different from G-protein-coupled receptors (220, 222).



Figure, Appendix D 5: Schematic representation of adiponectin structure. Adapted from Vascular *Health and Risk Management* 2015:11 55–70.

Abbreviations: High molecular weight (HMW).

Adiponectin receptors are widely expressed by different cardiac, vascular and immune cell types (329, 340). Adipo-1 is mainly expressed by skeletal muscles, whereas liver is the site for Adipo-2 expression (220, 331). Moreover, both Adipo-1 and Adipo-2 were expressed by endothelial cells and cardiomyocytes (220), suggesting that they have important signaling roles in mediating cardiovascular effects of adiponectin (220, 329, 334, 336, 337, 339). Evidence suggests that adiponectin exhibits its different biological actions through the activation of various signaling molecules such as AMPK, PPARα and p38MAPK and display ceramidase activity (222, 451, 478-480). Adipo-1 and Adipo-2 convert pro-apoptotic ceramides to anti-apoptotic sphingosine-1-phosphate (SIP), thus exhibiting anti-apoptotic effects in cardiomyocytes (222, 451, 478-480). Adipo-1 and Adipo-2 also participate in the modulation of fatty acid oxidation and glucose uptake (222, 331, 451, 478-480). Besides, Adipo-1 and Adipo-2, T-cadherin is a surface cell receptor for full length and oligomeric adiponectin. It functions as a co-receptor with adiponectin receptors to mediate adiponectin signaling pathways in different cell types (329). It is abundantly expressed by myocardium, smooth muscle cells as well as by endothelium (222, 329, 478). T-cadherin is cardioprotective and is essential for adiponectin mediated AMPK phosphorylation (329). In T-cadherin knockout mice, high levels of adiponectin was found in circulation due to the inability of adiponectin to bind with cardiac tissue and display similar defects as observed in adiponectin null mice, suggesting that T-cadherin participates in mediating adiponectin biological action (222, 329, 478). Thus, the specific structure of the three adiponectin isomers, adiponectin receptors and their post translational modifications and distinct expression pattern, altogether participates in mediating a cytoprotective role of adiponectin against obesity and cardiovascular disease.

1.1 Adiponectin and inflammation

Adiponectin possess anti-inflammatory, antioxidant and insulin-sensitizing properties. (219, 220, 222, 329-338). Adiponectin acts in a feedback loop manner and regulates the expression of TNF-α and IL-6. Interestingly, adiponectin can modulate macrophage phenotype and function by promoting macrophage polarization towards anti-inflammatory M2 phenotype (220, 329, 334, 336). Adiponectin regulates inflammatory signals and prevents TNF-α-induced monocyte adhesion and expression of cell adhesion molecules (220, 329). In a recent study in adiponectin knock-out mice, elevated expression of pro-inflammatory macrophage M1 phenotype markers and lower expression of anti-inflammatory M2 markers was observed in adipose tissue and peritoneal macrophages. However, administration of recombinant adiponectin suppressed oxidative stress and increased the expression of anti-inflammatory M2 phenotype markers, thereby, providing protection against systemic inflammation (329). In a similar manner, treatment with globular adiponectin protected adiponectin null mice against micro-vascular inflammation (220). In addition, adiponectin has been demonstrated to augment the secretion of anti-inflammatory cytokines such as IL-10 and IL-1 receptor antagonist in monocytes, macrophages and dendritic cells (329), which might be one of the possible mechanism for anti-atherosclerotic and cytoprotective effects of adiponectin against vascular inflammation (329, 331). It is proposed that adiponectin exerts its anti-diabetic and anti-atherogenic effects due its ability to suppress NFκB activation in response to an inflammatory stimuli (334). It is reported that adiponectin mediated PPARα and AMPK activation promotes suppression of NFκB and COX2 gene expression and protects against inflammatory conditions (334). In contrast, under normal conditions, adiponectin has been shown to favor NFκB activation in various cell types (334). One possible explanation for adiponectin-induced NFκB activation would be that

adiponectin augments IL-6 production through the stimulation of NFκB pathway with the help of receptors distinct from Adipo-1 and Adipo-2, which in turn promotes increased expression of IRS-2 via STAT3 signaling. This altogether results in increased insulin sensitivity (220, 329, 334, 336). Further research is needed to clarify how adiponectin maintains the balance between suppression of NFκB activation induced by inflammatory stimuli and adiponectin mediated NFκB activation under physiological conditions (334).

1.2 Adiponectin and insulin resistance

Insulin resistance associated with obesity is a major risk factor for diabetes and cardiovascular diseases (3, 5, 13-15, 24, 29, 68, 69, 127, 135, 143-149, 170). Elevated FFA levels in the circulation during obesity reduces insulin-induced glucose uptake. This in turn reduces the production of glycogen in skeletal muscle and liver (the principal insulin target organs responsible for regulation of energy metabolism) that subsequently leads to insulin resistance (337). A recent study revealed that in insulin resistant obese mice, the levels of adiponectin as well as expression of its receptors (Adipo-1 and Adipo-2) were significantly reduced in skeletal muscle and adipose tissue in response to the obesity-induced hyperinsulinemia that leads to the dysfunctional adiponectin-mediated AMPK signaling (333, 334, 340). Similarly, in type-2 diabetic patients reduced levels of Adipo-1 and Adipo-2 were reported in the skeletal muscles (334). Thus, during the pathological obese condition, adiponectin sensitivity is significantly reduced due to low circulating levels of adiponectin and reduced expression of its receptors. As a result a vicious cycle is generated leading to hyperinsulinemia and a gradual development of insulin resistance (162, 329, 330, 333, 334, 340). It is well known that adiponectin possess insulin-sensitizing effects and a beneficial role of

adiponectin against insulin resistance and dysfunctional insulin signaling was reported both in vitro and in vivo (162, 329-331, 334, 337, 340). With regards to the molecular mechanism responsible for insulin-sensitizing effects of adiponectin, it is demonstrated that in both liver and skeletal muscle, adiponectin increased glucose uptake and promotes fatty acid oxidation by stimulating the AMPK pathway and inhibiting acetyl coenzyme-A carboxylase (162, 331, 334). However, inhibiting AMPK activation by a blocker reverses the beneficial effect of adiponectin, suggesting that enhancement in glucose uptake and fatty acid oxidation by adiponectin is mediated via AMPK activation (331, 334). Moreover, both Adipo-1 and Adipo-2 were known to play a regulatory role in normal glucose metabolism and fatty acid oxidation (162, 329-331, 334, 337, 340) and serves as a receptors for globular and full length adiponectin (334). It is reported that Adipo-1 activates AMPK, while Adipo-2 activates PPARα ligand activity that results in increased glucose uptake and improved insulin sensitivity in liver (334). Besides liver and skeletal muscle, adiponectin improved insulin signaling in the pancreas. Recent studies showed that adiponectin promotes β-cells survival and function and thereby, improved glucose metabolism in a high fat diet obese mice model (162, 333). Thus, further research on adiponectin and its receptors will prove beneficial in treating obesity-related insulin resistance and glucose intolerance.

1.3 Cardioprotective role of adiponectin

Evidences support the idea that low levels of adiponectin is a predictor for cardiovascular diseases (72, 220, 329, 331, 337). Adiponectin has been reported to be cardioprotective against impaired cardiac function, cardiac ischemia reperfusion injury and cardiomyopathy (72, 220, 329, 331, 337, 481), whereas its deficiency aggravates cardiac injury in adiponectin knock-out

mice model (329, 337). Adiponectin knock-out mice were shown to be more prone to cardiac hypertrophy, impaired cardiac function and aggravated fibrotic response compared to normal control mice (329). The cardioprotective role of adiponectin in both the cardiac and vascular systems is attributed to its anti-inflammatory, anti-atherogenic and anti-fibrotic properties (72, 220, 329, 331, 335). The underlying mechanisms for the protective role of adiponectin against vascular diseases is still not clear, however, it is proposed that adiponectin protects against dysfunctional endothelium via the stimulation of nitric oxide and attenuation of inflammation and oxidative stress (329, 332, 337, 481). For example, it was shown that adiponectin promotes vasodilation of cardiomyocytes via the regulation of nitic oxide synthesis through eNOS and attenuation of nitrative stress. Adiponectin exerts protection against ROS-stimulated cardiac remodeling through the activation of AMPK, p38MAPK and ERK1/2 signaling cascade and inhibition of ERK phosphorylation (338, 339). Moreover, adiponectin-induced AMPK activation and related inhibition of ERK phosphorylation is an important mechanism to counteract the deleterious effect of angiotensin-II and ET-1 induced hypertrophic signals through the suppression of NFκB pathway in cardiomyocytes (478, 481). It is reported that the administration of adiponectin to adiponectin deficient mice and isolated rat heart was cardioprotective against ischemia injury (329). The anti-apoptotic effect of adiponectin is mediated by the activation of AMPK and Akt signaling pathways (451) and increased expression of COX-2 (479). In a similar manner, administration of agonists of adiponectin receptors to diabetic mice exerted protective effects similar to adiponectin mediated by Adipo-1 and Adipo-2 in both liver and skeletal muscle (480). Taken together, these studies support that adiponectin plays a pivotal regulatory role in cardiovascular homeostasis and exerts protection against cardiometabolic complications arising due to obesity.

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