CONTRIBUTION OF PERIVASCULAR ADIPOSE TISSUE TO CORONARY VASCULAR DYSFUNCTION

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DEDICATION

Each of us, famous or infamous, is a role model for somebody, and if we aren't, we should behave as though we are. Cheerful, kind, loving, courteous. Because you can be sure someone is watching and taking deliberate and diligent notes.

~Maya Angelou

This thesis is dedicated to my parents who inspire me to always reach for my goals, and to my wife for her unwavering love and support throughout my education.

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ABSTRACT

Gregory Allen Payne

CONTRIBUTION OF PERIVASCULAR ADIPOSE TISSUE

TO CORONARY VASCULAR DYSFUNCTION

The epidemic of obesity and associated cardiovascular complications continues to grow at an alarming rate. Currently, obesity is thought to initiate a state of chronic inflammation, which if unresolved potentially causes cardiovascular dysfunction and disease. Although poorly understood, release of inflammatory mediators and other cytokines from adipose tissue (adipocytokines) has been proposed to be the molecular link between obesity and coronary artery disease. Furthermore, the anatomic location of adipose has been increasingly recognized as a potential contributor to vascular disease. Importantly, the development of coronary atherosclerosis, a key component of heart disease, is typically found in segments of coronary arteries surrounded by perivascular adipose tissue. Accordingly, the goal of this project was to determine how perivascular adipose tissue affects coronary artery function and elucidate the critical mechanisms involved. Initial studies assessing arterial function were conducted with and without perivascular adipose tissue. Preliminary results demonstrated that factors released by perivascular adipose tissue effectively impaired coronary endothelial function both *in vitro and in vivo*. This observation was determined to be caused by direct inhibition of nitric oxide synthase (NOS), a critical enzyme for the production nitric oxide. Attenuation of endothelium-dependent vasodilation was independent of changes in superoxide production, smooth muscle response, or peroxide-mediated vasodilation. Additional

studies revealed that perivascular adipose-induced impairment of NOS was due to increased inhibitory regulation by the β isoform of protein kinase C (PKC-β). Specifically, perivascular adipose-derived factors caused site specific phosphorylation of nitric oxide synthase at Thr-495. Additional experiments investigated how perivascular adipose-derived factors contributed to coronary artery disease in an animal model of obesity. Results from these studies indicated that perivascular adipose-derived leptin markedly exacerbated underlying endothelial dysfunction, and significantly contributed to coronary endothelial dysfunction through a PKC-β dependent mechanism. Findings from this project confirm epicardial perivascular adipose tissue as a local source of harmful adipocytokines. In addition, perivascular adipose-derived leptin was demonstrated to be a critical mediator of coronary vascular dysfunction in obesity. Together, the results strongly suggest that perivascular adipose tissue is a key contributor to coronary artery disease in obesity.

Johnathan D. Tune, Ph.D., Chair

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Chapter 1

The Epidemic of Obesity and Metabolic Syndrome

Within the United States, the incidence of obesity has undoubtedly reached epidemic proportion. Since 1980, obesity rates among adults within the United States have more than doubled, signaling the rapid development of a health crisis for all ethnic and socioeconomic groups throughout the nation. Sadly, while our acknowledgement and appreciation for the severity of this problem has increased, little evidence suggests that current efforts have substantially deterred weight gain across the country. Defined as a body max index (BMI) of ≥ 30 kg/m^2, approximately 33% of adult men and 35% of adult women within the United States were considered obese in 2006[1]. As of 2009, still more than a third of the population (or 72 million individuals) is definably obese, with many more considered overweight[2].

Perhaps even more concerning is the dramatic increase in childhood obesity. In the United States, more than 16% of children and adolescents are obese, representing a threefold increase over the past three decades[2]. Lastly, these detrimental dietary and behavioral practices have managed to extend far beyond our domestic borders. In particular, recent estimates indicate that there are approximately 1 billion individuals worldwide who are either overweight or obese (BMI ≥ 25 kg/m^2)[3]. Together, these statistics exemplify the dire situation confronting both adults and children worldwide.

While the growing prevalence of obesity alone is problematic, even more troubling is the expected increase in obesity-associated cardiovascular disease. Since the landmark Framingham Heart Study first began to identify key factors contributing to cardiovascular disease, subsequent investigations have further demonstrated obesity-induced increases in the incidence of coronary atherosclerotic disease, cardiomyopathies, myocardial infarction, sudden death, congestive heart failure, and

stroke[4-10]. For example, men with a BMI of ≥ 30 kg/m^2 are at a tremendous risk of death from cardiovascular disease (relative risk of 2.90)[11]. Furthermore, individuals who are either overweight or obese are at greater risk for a number of harmful clinical conditions (Figure 1-1)[12]. Importantly, cardiovascular pathologies have long been among the leading causes of death nationwide. Hence, the relatively sharp increase in obesity promises to further exacerbate the incidence and mortality of these deadly conditions, and apply additional strain on our healthcare system.

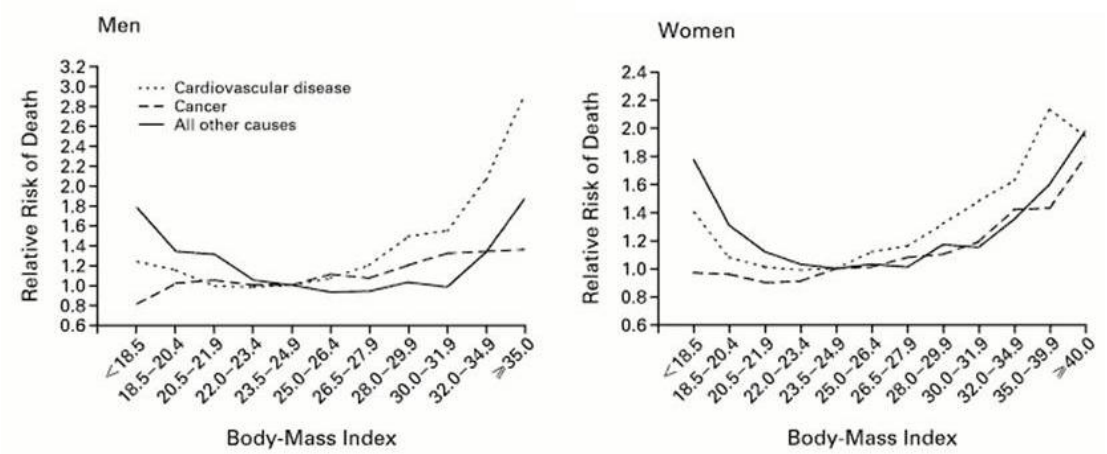

Figure 1-1 ***Health risks associated with elevated body mass index.*** *Relative risk of death from cardiovascular disease, cancer, and other causes among men and women are shown. Note the sharp increase in cardiovascular risk associated with body mass indices greater than approximately 25*[11]*.*

Current hypotheses, however, speculate that simple measurement of BMI may not be sufficient to provide a detailed cardiovascular risk assessment for individual patients[13, 14]. Specifically, results from the International Day for the Evaluation of Abdominal Obesity (IDEA) confirmed that waist circumference (as opposed to BMI) is a stronger indicator of cardiovascular disease and type II diabetes in normal, overweight, and obese patients[14, 15]. These results suggest that general weight gain alone is not solely responsible for disease, and that abdominal (or visceral) adiposity has particularly deleterious effects on cardiovascular health. This conclusion has subsequently been supported by several investigations reporting differing risks associated with abdominal

versus peripheral obesity[16-18]. Hence, while increases in total body weight may serve as a convenient indicator of cardiovascular risk, other mechanisms (either dependent or independent of body weight) are clearly contributing to the progression of obesity-induced cardiovascular disease.

With these limitations of BMI measurements in mind, investigators have sought to define a set of obese risk factors better associated with cardiovascular disease. Consequently, research throughout the past decades has accumulated evidence of a clustering of cardiovascular risk factors referred to as "metabolic syndrome" (MetS)[15, 19-21]. Patients with MetS classically display a constellation of symptoms including hypertension, insulin resistance, truncal obesity and dyslipidemia[22]. Clinically, patients displaying an elevated waist circumference (≥ 40 for men, 35 for women), elevated triglycerides (≥ 150 mg/dL), reduced HDL cholesterol (< 40 mg/dL for men, 50 for women), elevated blood pressure (> 130/85 mmHg), and elevated fasting glucose (≥ 110 mg/dL) are considered to suffer from MetS[23]. Estimates suggest that more than 30 % of the adult population within the United States exhibits characteristics of this pre-diabetic, metabolic disorder[23-25]. Importantly, earlier investigations have established that each component of MetS is an independent risk factor for cardiovascular disease[5], and that patients are subject to a 61% increased risk of cardiovascular events[4]. Hence, a large portion of the population may be living at increased risk of cardiovascular disease before any clinical signs (i.e. morbid obesity or type II diabetes mellitus) are readily apparent.

Despite this growing epidemic, the pathophysiologic mechanisms underlying the link between obesity and cardiovascular disease remain poorly understood. Consequently, the long-term goal of our research is to delineate the central mechanisms of obesity-induced coronary vascular disease with the intention of identifying novel therapeutic targets to reduce the incidence of cardiovascular complications in obese patients. The specific focus of this work was to better understand how adipose tissue, an

active endocrine organ, directly affects coronary vascular function under both lean and obese conditions. In particular, dysfunction of normal coronary vascular function is suggested to be the initiating event in progression of several weight related cardiomyopathies (i.e. coronary artery disease and myocardial infarction)[26]. The central premise of the following studies is that local perivascular adipose tissue surrounding the large conduit vessels of the heart is a significant regulator of coronary vascular function and contributor to the development of coronary artery disease in obese patients with the MetS.

Adipose Tissue, Inflammation, and Cardiovascular Disease

Adipose tissue has become the subject of considerable research interest in recent years. Results throughout the past decade have prompted many investigators to recognize the important relationships among adipose tissue, obesity, chronic inflammation, and cardiovascular disease. For many years, adipose tissue was thought to be a relatively inactive signaling organ. While brown adipose tissue has long been associated with thermogenesis and body temperature regulation in newborn infants, the more abundant white adipose tissue was thought to exclusively function as an energy storage depot for fatty acids[27, 28]. In the late 1980's and early 1990's, however, it was discovered that adipocytes secrete two signaling proteins associated with inflammation. Namely, the complement-related factor adipsin[29] and the pro-inflammatory cytokine tumor necrosis factor α (TNF-α)[30] were recognized to be produced by adipose tissue. Together, these initial discoveries challenged long standing assumptions about the physiologic nature of adipose tissue.

The critical change in perspective on the physiological role of adipose tissue occurred with the identification of the adipose-derived hormone leptin[31]. Subsequent investigations demonstrated that leptin is associated with the regulation of energy balance through endocrine signaling to the hypothalamus[32-34]. Importantly, investigators

have more recently documented that leptin either directly contributes, or is associated with several complications of obesity[35]. These include coronary endothelial dysfunction[36], coronary heart disease[37], and hypertension[38-40]. These leptin-associated pathologies are hallmarks of the MetS, and therefore provide evidence of a molecular link between obesity and cardiovascular disease. Together, the discovery of adipose-derived adipsin, TNF-α, and leptin serve as the foundation for the current theory of adipose tissue as an active endocrine, paracrine, and inflammatory organ[41].

Several investigations have subsequently identified numerous agents produced either by adipocytes or within adipose tissue (Figure 1-2). Many of these adipose-derived cytokines (or adipokines) have been shown to influence a wide spectrum of hemodynamic, metabolic, and immunologic factors. These include changes in insulin sensitivity (i.e. adiponectin and resistin), inflammation (i.e. IL-8 and monocyte chemotactic protein 1), vascular reactivity (i.e. leptin, resistin, TNF-α, adiponectin), and coagulation (i.e. plasminogen activator inhibitor 1)[36, 41-45]. The widespread and sometimes detrimental activity of these signaling molecules has resulted in the current "adipokine hypothesis" linking obesity and cardiovascular disease. To date, there are well over 50 adipokines that have been identified with more undoubtedly to follow. Table 1-1 summarizes a few of these adipokines that are proposed to be linked with obesity, MetS, and cardiovascular disease.

Table 1-1 *Perivascular adipose tissue expression and vascular effect of keyadipokines associated with cardiovascular disease.*

	PROPOSED PHYSIOLOGIC FUNCTION	PROPOSED DISEASE ASSOCIATIONS	EFFECT ON VASCULAR REACTIVITY	EPICARDIAL ADIPOSE TISSUE EXPRESSION	CITATIONS
Leptin	Energy status messenger Regulator of SNS to control energy expenditure Short-term adapter during starvation	Obesity CAD Atherosclerosis HTN DM Type II Hepatic Disease	Endothelial Dysfunction Oxidatice stress Pro-thrombotic VSMC migration and proliferation	Markedly elevated epicardial adipose expression in comparison to omental adipose form obese patients with CAD (**2.5 fold increase**)	34, 46-49
Resistin	Questionable link between obesity and DM Type II Pro-inflammatory	Insulin resistance CAD	Endothelial Dysfunction Endothelin-1 release Increased VCAM-1	Elevated with obesity. Epicardial expression similar to omental adipose from obese patients with CAD	43, 48-50
PAI-1	Inhibits plasminogen activators and fibrinolysis Pro-inflammatory Promote adipose development	Obesity DM Type II Chronic inflammation	Correlated with impaired vascular reactivity in the setting of MetS	Markedly elevated epicardial adipose expression in comparison to omental adipose form obese patients with CAD (**2.5 fold increase**)	49, 51, 52
Adiponectin	Insulin-sensitizer Anti-inflammatory Fatty acid oxidation and lipid metabolism	Obesity CVD CAD Insulin resistance DM Type II Dyslipidemia	Increased CFR Endothelial improvement	Reduced in all adipose tissues from obese patients with CAD, but significantly greater reduction observed in epicardial adipose (**2 fold decrease**).	42, 47, 48, 53, 54
MCP-1	Chemokine Monocyte trafficking Inflammation	Obseity Atherosclerosis CAD CVD	Thought to propagate neointimal thickening	Elevated epicardial adipose expression, but significantly less than omental adipose expression.	44, 55, 56

IL-6	Acute-phase inflammatory reaction Anti-inflammatory Lipolysis Increase CNS activity	Insulin resistance DM Type II Obesity Cachexia CVD	No known direct effect, but associated with: ROS Endothelial Dysfunction	Markedly elevated epicardial adipose expression in comparison to omental adipose form obese patients with CAD (**9.5 fold increase**)	44, 47, 49
TNF-α	Pro-inflammatory cytokine	Obesity Inflammation Auto-immunity Insulin Resistance DM CVD CAD	ROS Endothelial Dysfunction	Elevated with obesity. Epicardial expression similar to omental adipose from obese patients with CAD	44, 45, 57

CAD- coronary artery disease; **CVD**-cardiovascular disease; **HTN**-hypertension; **SNS**-sympathetic nervous system; **CNS**-central nervous system; **DM**-diabetes mellitus; **CFR**-coronary flow reserve; **ROS**-reactive oxygen species

Notably, many adipokines have been either directly or indirectly associated with inflammation[41, 44, 58]. It is therefore not surprising that obese patients have elevated inflammatory markers, and display a phenotype characteristic of chronic inflammation[59]. In fact, obesity is strongly associated with elevated C-reactive protein (CRP), TNF-α, IL-6, and cellular adhesion molecules (CAMs)[60]. Importantly, these inflammatory proteins have each been associated with increased vascular risk and cardiovascular disease[61, 62]. The expansion and growth of adipose depots during obesity is further linked with inflammation due to increased monocyte infiltration and activation within adipose tissue[63]. In the setting of obesity, adipocytes therefore provide a wealth of pro-inflammatory factors, while adipose tissue in general offers a supportive environment for inflammatory cells to mount an immune response and further propagate chronic inflammation[59]. Hence, both the inflammatory and endocrine nature of adipose tissue provides a potential link between cardiovascular disease and obesity.

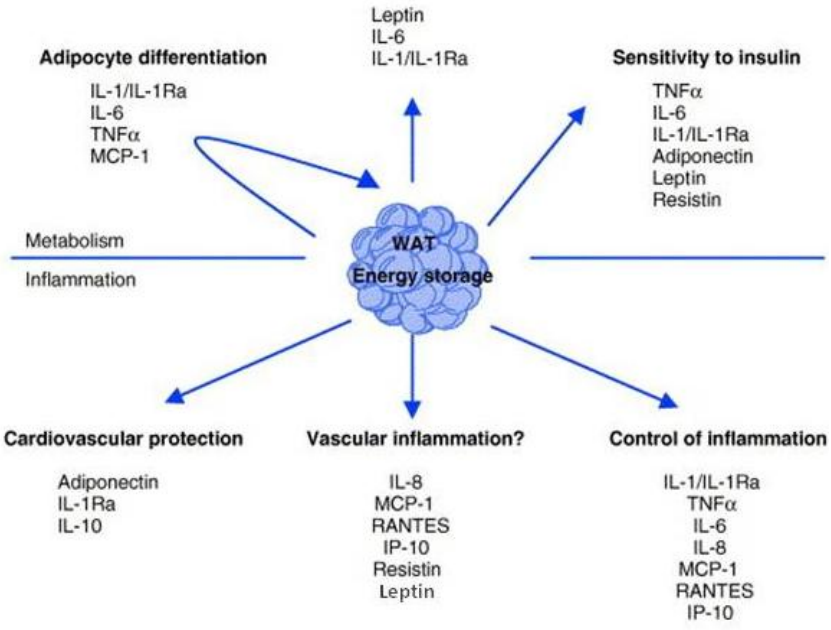

Figure 1-2 ***Expression and function of some known adipokines.*** *Adipokines are linked to a wide variety hemodynamic, metabolic, and inflammatory factors. Adipose tissue is therefore a highly active and regulated secretory organ. Expression of various adipokines has been shown to be linked with obesity-related metabolic and vascular complications. Adipokines are therefore proposed to be the molecular link between obesity and cardiovascular disease. TNF-α (tumor necrosis factor α); IL (Interleukin); RANTES (regulated upon activation, normal T cell expressed and secreted); MCP-1 (monocyte chemotactic protein 1)*[44]*.*

Lastly, it should pointed out that different adipose depots have distinct metabolic characteristics, leading to individual differences in the impact of obesity on cardiometabolic disease. During obesity, abdominal visceral adipose tissue is suggested to confer greater risk of cardiovascular complications in comparison to subcutaneous adipose tissue[64-66]. This is due to a few important anatomic and metabolic factors. By its anatomic location, the expanded abdominal visceral adipose depot has easy access to the liver via the portal circulation (Figure 1-3) [58, 66]. As a result, the liver receives a increased amount of free fatty acids (FFA) from visceral adipose tissue, leading to increased production of lipoproteins, clotting factors, and systemic inflammatory factors[67, 68]. Likewise, abdominal visceral adipose tissue provides a unique source of adipokines that may directly impact vascular disease and atherogenesis. While it is not

clear how much abdominal adipose tissue contributes to systemic increases of inflammatory markers and harmful adipokines, these findings suggest that the anatomic location of adipose tissue is an important determinant of obesity-associated cardiovascular disease. Additional studies, however, are needed to further elucidate a relationship between anatomic location of adipose tissue and disease.

In the end, while our understanding of a link between adipose tissue and cardiovascular disease remains limited, adipokines appear to hold a critical role in disease progression. Notably, the majority of these signaling molecules display altered expression patterns in the setting of obesity[41, 47, 58, 69]. In addition, previous studies also suggest that the anatomic location of adipose tissue contributes to cardiovascular disease. Future investigations are needed to better delineate clear relationships and mechanisms underlying adipokine production, anatomic location, and obesity-associated cardiovascular disease.

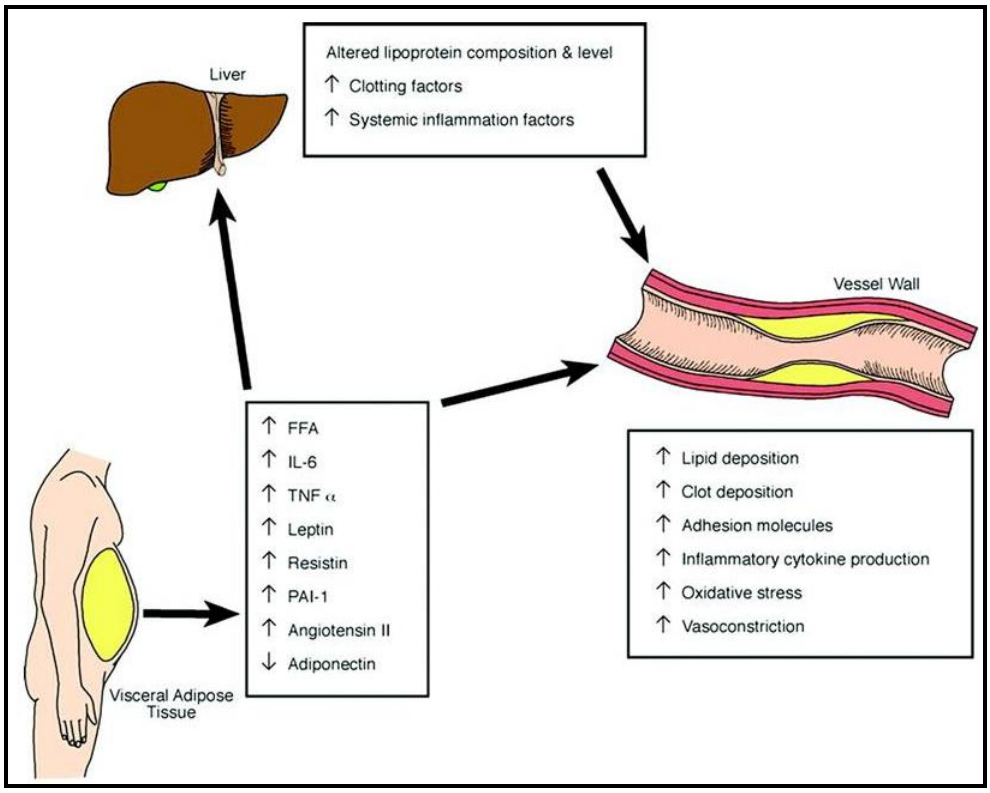

Figure 1-3 ***Contribution of visceral adipose tissue to obesity-induced vascular disease.*** *Adipokines work directly at the vessel wall and through the liver to promote an atherogenic environment. Adipokines derived from visceral adipose tissue have favored access to the liver through the portal circulation. At the liver, adipose tissue–derived factors influence the composition and level of circulating lipoproteins and the levels of systemic inflammatory and clotting system components. Adipose tissue–derived factors also can directly regulate gene expression and function of endothelial, arterial smooth muscle, and macrophage cells in the vessel wall. FFA (free fatty acids); PAI (plasminogen activator inhibitor)*[58]*.*

Obesity and Coronary Vascular Disease

While adipokines have received considerable research interest, investigators have also sought to understand the overall (or net result) effect of obesity on coronary vascular function. There are many plausible mechanisms by which an increase in adipose tissue (or adipokines production) could adversely affect coronary vascular function and health. These include changes in blood pressure regulation, glucose tolerance, lipid metabolism, and chronic inflammation[58]. While each of these mechanisms are important, emerging evidence suggests that the cornerstone of

coronary vascular disease is the initial disruption of normal vascular function. Indeed, dysfunctional coronary blood flow regulation has been demonstrated to precede any changes in metabolism or inflammation[70]. Hence, although our understanding of the cellular and molecular mechanisms controlling obesity-induced coronary disease is limited, considerable evidence suggests that dysfunction of the coronary circulation is a primary component of disease progression.

The etiology of coronary vascular dysfunction, however, is both multi-factorial and heavily dependent on which segment(s) of the vascular tree are affected. In particular, unique pathology can occur in the small arterioles (located deep within the myocardium) and/or in the much larger conduit arteries (i.e. branches of the right and left main coronary arteries). Hence, to better understand how adipokines (and other suspected agents) may contribute to vascular disease, it is important to first understand how obesity in general affects the coronary circulation. Investigating the effect of obesity at the level of both the micro- and macrovasculature, therefore, is critical to fully understand the mechanisms underlying obesity-induced coronary disease.

Microvascular Coronary Disease and Obesity

Disruption of coronary microvascular function as a consequence of obesity is thought to underlie the increased morbidity and mortality to cardiovascular diseases commonly observed in obese patients[71]. Adequate coronary circulation is critical for optimal cardiac function. The myocardium has a very limited anaerobic capacity, and is highly dependent on a continuous supply of oxygen from the coronary circulation to meet metabolic demands[72-74]. If this need for oxygen is not adequately met, the resulting underperfusion/ischemia substantially diminishes cardiac function within seconds[75-78]. For this reason, myocardial oxygen consumption (MVO_2) is closely matched with oxygen delivery (i.e. coronary blood flow) during normal physiologic conditions. Disruption of this

delicate feedback system, as observed in both obesity and MetS[79], is therefore potentially detrimental to cardiac function and cardiovascular health.

Mounting evidence suggests that the ability to match coronary blood flow to myocardial oxygen demand is diminished with obesity. While measures of resting coronary blood flow from both lean and obese subjects demonstrate little to no difference[70, 79-84], obese patients display a "hyperdynamic circulation" characterized by elevated cardiac output, larger stroke volume, and increased cardiac mass[85-88]. These hyperdynamic changes increase MVO_2 by altering the key determinants of myocardial metabolism (i.e. heart rate, arterial pressure, ventricular work, and wall stress)[79, 83, 89]. Therefore, despite similar resting blood flow measures, myocardial oxygen demand is increased with obesity. Accounting for these differences reveals that obese subjects have a depressed cardiac index and reduced contractile performance[83, 85, 88, 90]

In contrast to resting coronary blood flow, data from human patients clearly demonstrate diminished coronary flow reserve (the difference between maximal and baseline coronary blood flow) with obesity and MetS[70, 80-82, 84]. These findings suggest that obese patients have a limited capacity to vasodilate and provide sufficient oxygen during situations of increased myocardial metabolic demand. Specifically, results from Kiviniemi et. al. demonstrate that waist-to-hip ratio is a negative, independent predictor of coronary blood flow reserve in young, healthy men (Figure 1-4)[70]. Furthermore, the onset and severity of microvascular dysfunction appears to parallel the progressive increase of truncal fat. The observed reduction in flow reserve could be related to impaired vasodilator capacity, enhanced vasoconstrictor responsiveness, and/or structural remodeling of the coronary microvasculature[91, 92].

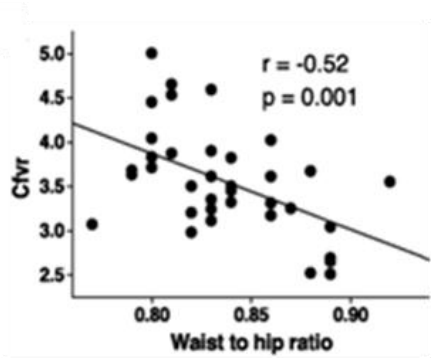

Figure 1-4 ***Waist-to-hip ratio is a negative, independent predictor of coronary blood flow reserve.*** *The correlation of waist-to-hip ratio with coronary flow velocity reserve (cfvr) in young, healthy men is shown. Blood flow velocity was measured by transthoracic echocardiography*[70]*.*

To further investigate this observed impairment of coronary blood flow regulation, our laboratory explored the control of coronary blood flow in conscious, instrumented control and chronically high-fat-fed dogs at rest and during graded treadmill exercise[79]. When fed a high-fat diet, these animals displayed many of the characteristics of MetS including obesity, insulin resistance, dyslipidemia, hyperleptinemia, and impaired blood pressure regulation[79, 89, 93]. We documented that the MetS significantly attenuated exercise-induced coronary hyperemia. In particular, slopes of coronary blood flow vs. MVO_2 and coronary conductance vs. MVO_2 relationships were significantly depressed in obese dogs relative to normal controls (Figure 1-5A). In addition, there was a significant parallel downward shift in the relationship between coronary venous PO_2 and MVO_2 in the high-fat-fed dogs (Figure 1-5B), suggesting loss of a tonic vasodilator mechanism and/or activation of a tonic vasoconstrictor mechanism[73, 74]. These data are consistent with coronary flow responses to cardiac pacing in obese patients with the MetS[80].

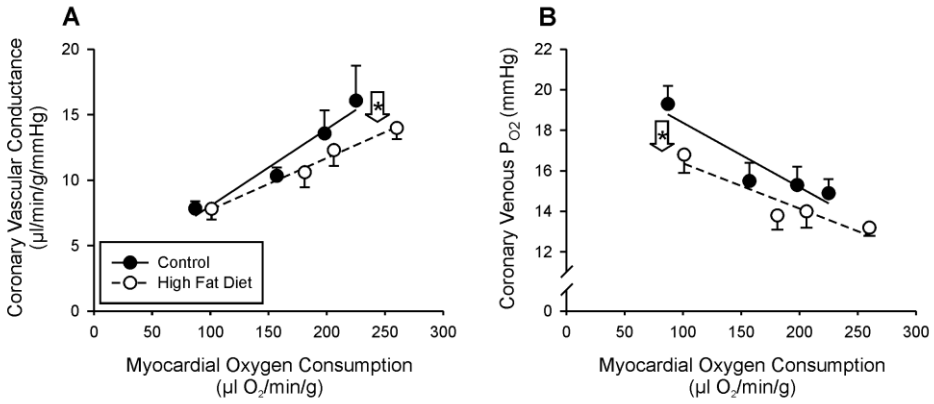

Figure 1-5 ***Effects of the MetS on control of coronary blood flow at rest and during increases in myocardial metabolism.*** *Data are adapted from Setty et al., 2003[79]. Coronary blood flow was measured from a flow probe around the left circumflex artery; blood was sampled from the aorta and coronary sinus. MVO_2 was calculated from the arterial-coronary venous O_2 difference and coronary flow. A plot of coronary conductance vs. MVO_2 and demonstrates that the MetS significantly attenuates vascular response to increased metabolic demand (A). Coronary blood flow was converted to coronary vascular conductance, as dogs fed a high fat diet were hypertensive and thus had a greater driving force for flow. This impairment of coronary blood flow control forced the myocardium to increase O_2 extraction (decrease coronary venous PO_2 at a given MVO_2) to meet the metabolic requirements of the heart (B).*

Together, these findings demonstrate that the MetS impairs the ability of the coronary microcirculation to match myocardial oxygen supply with oxygen demand. This could be related to a variety of pathophysiological conditions associated with obesity and the MetS. These potential conditions include alterations in endothelial-dependent control of coronary blood flow, activation of vasoactive neural-hormonal pathways, as well as dysfunction of microvascular ion channels (Figure 1-6). Importantly, coronary dysfunction in the MetS has been associated with sensitization of vasoconstrictor pathways, impaired K^+ channel function, and augmented L-type Ca^{2+} channel activity in smooth muscle of resistance arteries[71]. Hence, dysfunction of the coronary microcirculation could result in various cardiomyopathies, and represents one critical (and multifaceted) component of obesity-induced vascular disease. The mechanism and contribution of each of these conditions to cardiovascular disease is complex (and not further discussed within this text); however, it is important to note that the precise cellular

and molecular mechanisms underlying these obesity-associated conditions are not completely understood.

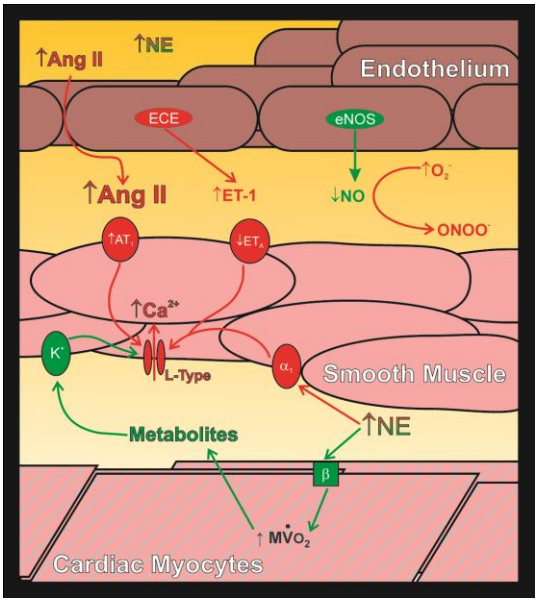

Figure 1-6 ***Scheme of mechanisms associated with coronary microvascular dysfunction in obesity and MetS.*** *Vasoconstrictor pathways are shown in red while vasodilator pathways are depicted in green. The MetS is associated with impaired coronary endothelial function, activation of vasoactive neural-hormonal pathways, as well as dysfunction of microvascular ion channels. Ang II (angiotensin II); NE (norepinephrine); ECE (endothelin converting enzyme); eNOS (endothelial nitric oxide synthase)*[71]*.*

Macrovascular Coronary Disease

In contrast to the coronary microvasculature, the larger epicardial arteries of the heart contribute very little to blood flow regulation[94-97]. In principal, these major coronary arteries serve as conduits for arterial blood flow between the ascending aorta and the smaller resistance arteries deep within the myocardium. While endothelial dysfunction and intensified vasoconstriction appear to be hallmarks of obese coronary circulation, these mechanisms may cause dramatically different pathologies in coronary conduit vessels as opposed to the microcirculation. In particular, coronary atheroclserosis (or coronary artery disease) is the primary obesity-associated pathology observed in large

coronary arteries. In addition, more hazardous atherosclerotic lesions typically occur in the larger coronary arteries as opposed to distal branches and microvessels[98].

Research throughout the past decades has intensely sought to understand the cellular and molecular mechanisms underlying atherosclerosis. Initially, atherosclerosis was viewed as the accumulation of lipids within the arterial wall; however, many investigations have demonstrated the disease to be much more complex. In fact, coronary artery disease is currently understood to be a highly complex cellular and molecular inflammatory response[41, 58]. For this reason, identifying critical mediators of obesity-induced atherosclerosis has proven difficult.

In the 1970's, it was proposed that endothelial injury was the initiating event in the development of atherosclerosis[99]. This initial proposal has now developed into the "response-to-injury" hypothesis of atherosclerosis. This hypothesis states that chronic endothelial dysfunction precipitates the cascade of cellular events that result in arterial plaque formation (Figure 1-8)[100, 101]. Specifically, endothelial dysfunction is thought to increase platelet and inflammatory cell adhesion along the arterial wall. This results in both a pro-inflammatory and pro-coagulant environment at the endothelium. If the inflammatory response is not quelled, increased vascular permeability results allowing leukocytes to migrate beneath the endothelial layer. In doing so, the inflammatory response continues resulting in the activation of vascular smooth muscle cells to proliferate and migrate towards the arterial lumen. In the setting of obesity, a vicious cycle of inflammation, lipid deposition, and smooth muscle proliferation results in early "fatty streaks" and atherosclerotic lesions. If unabated, these lesions can progress into complicated, stenotic plaques with necrotic cores that are susceptible to rupture[100-104].

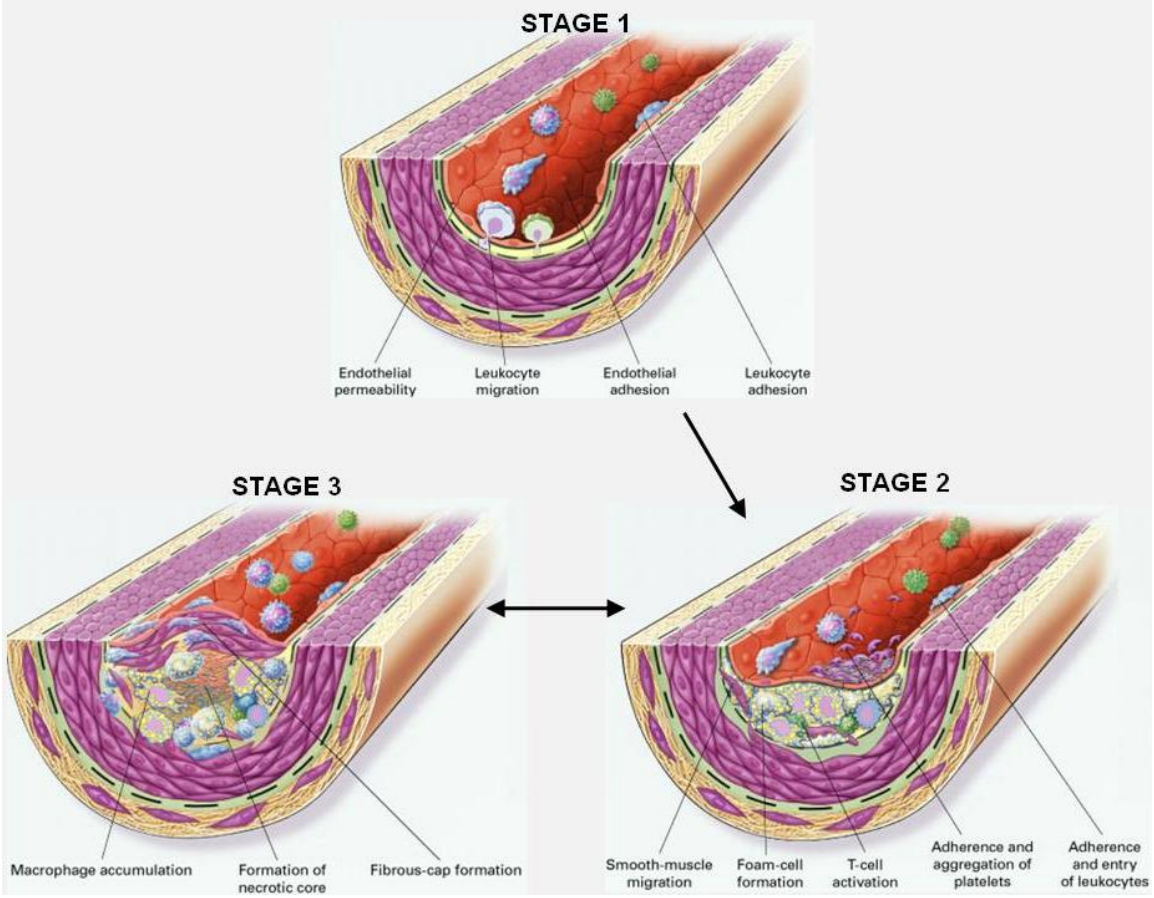

Figure 1-7 ***Pathogenesis of atherosclerosis.*** *Endothelial dysfunction is proposed to be the initiating event in the development of atherosclerosis.* ***STAGE 1:*** *The earliest changes that precede the formation of lesions of atherosclerosis take place in the endothelium. These changes include increased endothelial permeability to lipoproteins and other plasma constituents. This is mediated by altered nitric oxide, prostacyclin, platelet-derived growth factor, angiotensin II, and endothelin activity. Up-regulation of leukocyte adhesion molecules and endothelial adhesion molecules leads to the migration of leukocytes into the artery wall.* ***STAGE 2:*** *Fatty streaks initially consist of lipid-laden monocytes and macrophages (foam cells) together with T lymphocytes. Later they are joined by various numbers of smooth-muscle cells. The steps involved in this stage include smooth-muscle migration, T-cell activation, foam-cell formation, and platelet adherence and aggregation.* ***STAGE 3:*** *As fatty streaks progress to intermediate and advanced lesions, they tend to form a fibrous cap that walls off the lesion from the lumen. This represents a type of healing or fibrous response to the injury. The fibrous cap covers a mixture of leukocytes, lipid, and debris, which may form a necrotic core. These lesions expand at their shoulders by means of continued leukocyte adhesion and entry. The necrotic core represents the results of apoptosis and necrosis, increased proteolytic activity, and lipid accumulation. If unabated, atherosclerotic plaques can grow large enough to impede blood flow (flow-limiting stenosis) or rupture causing rapid thrombosis and occlusion. Modified from Ross, NEJM, 1999*[104].

The endothelium is therefore a critical regulator of vascular permeability, inflammatory cell adhesion, and platelet activation. Thus, endothelial cells provide a protective barrier for undisturbed blood flow and inappropriate inflammatory reactions. Disruption of this protective barrier, is generally detrimental to normal vascular reactivity, homeostasis, and blood flow regulation[102, 103]. Furthermore, in the setting of high shear stress and turbulent blood flow (i.e. large coronary arteries), vessels are particularly vulnerable to injury if the vascular endothelium is impaired or dysfunctional[98, 105]. Collectively, the onset of obesity or MetS (i.e. inflammation, hyperlipidemia, hyperglycemia, and hypertension) compromises the natural endothelial response to blood flow and potentially initiates the development of atherosclerosis, particularly in large coronary arteries (Figure 1-7)[105, 106].

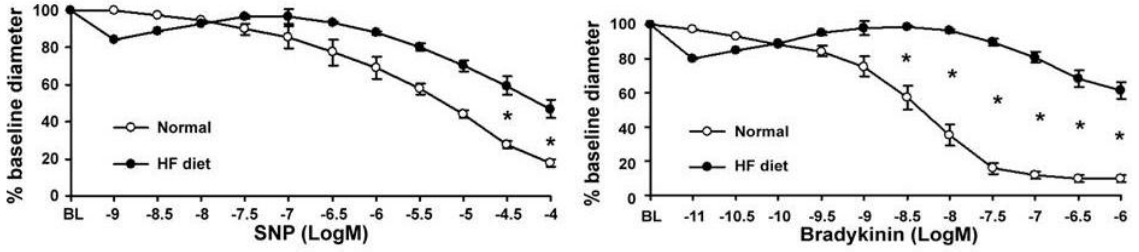

Figure 1-8 ***Obesity causes coronary endothelial dysfunction in conduit coronary arteries.*** *Left circumflex coronary arteries from normal and high fat fed swine were used to test endothelium-dependent and endothelium-independent vascular responses. Coronary artery vasorelaxation in response to endothelium-independent stimulus sodium nitroprusside (SNP; left) was modestly decreased by high fat feeding. In contrast, response to endothelium-dependent stimuli bradykinin (right) was dramatically reduced by high fat feeding (*P < 0.001). Modified from Galili O, AJP, 2007[106].*

While much is currently known about the development of atherosclerosis, we still lack a comprehensive understanding of the relationship between obesity and macrovascular disease. In particular, current investigative results have failed to provide a stepwise mechanism linking the endocrine nature adipose tissue with coronary heart disease. In addition, there is limited evidence explaining why atherosclerosis is primarily a macrovascular disease. These observations strongly suggest that additional

mechanisms are at work. Despite these knowledge gaps, endothelial dysfunction appears to be the pivotal starting point of coronary artery disease.

Adipokines and Coronary Endothelial Function

Although adipokines have been proposed to be the molecular link between obesity and cardiovascular disease[107], much work is still needed. The versatile activity of adipokines and the multifaceted nature of coronary artery disease has made discovering specific, disease causing adipokines especially difficult. Furthermore, it is fair to speculate that adipokines do not contribute equally throughout the entire pathogenesis of vascular disease. Together, these obstacles have delayed both our scientific and clinical understanding of obesity-associated diseases.

Despite these difficulties, researchers have attempted to link adipokines with various components of cardiovascular disease. Given the suggested importance of the endothelium, several studies have linked specific adipokines with endothelial dysfunction. In particular, results from our laboratory and others have demonstrated that both leptin (Figure 1-9) and resistin (Figure 1-10) impair endothelial function[36, 43, 106, 108, 109]. Specifically, we found that concentrations of leptin in the obese range (approximately ≥ 10 ng/ml) impair acetylcholine-mediated coronary vasodilation in open-chest dogs (*in vivo*) and isolated circumflex coronary artery rings (*in vitro*)[36]. Leptin administration alone had no effect on coronary vascular tone or hemodynamics. Interestingly, physiologic (~4 ng/ml) concentrations of leptin had no effect on acetylcholine-mediated coronary artery relaxation (Figure 1-9). In this study, leptin concentrations in the obese range did not affect endothelium-independent coronary vasodilation to sodium nitroprusside (data not shown). Thus, the effect of leptin on coronary vasomotor responses was specific for endothelial-mediated dilation in both conduit and resistance coronary arteries, indicating direct effects on NO production.

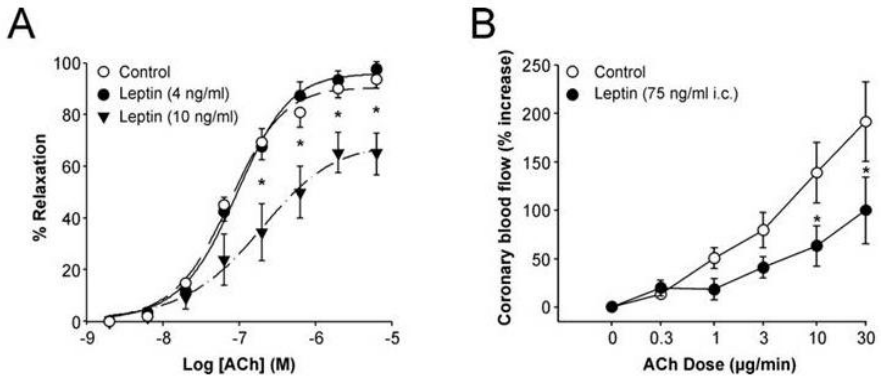

Figure 1-9 ***Leptin induces significant coronary endothelial dysfunction.*** *Leptin impairs acetylcholine-mediated coronary artery relaxation of isolated canine coronary canine coronary artery rings at a concentration of 10 ng/ml but not 4 ng/ml (A). Leptin significantly reduced endothelium-dependent coronary vasodilation to acetylcholine in open-chest anesthetized canines (B). Modified from Knudson et al., AJP, 2005*[36].

In the same way, Dick *et al.*[43] studied the hemodynamic effects of intracoronary administration of resistin both *in vivo* and *in vitro*. Intracoronary administration of resistin, like leptin, did not significantly affect coronary vasomotor tone or systemic hemodynamics. However, resistin did significantly attenuate coronary blood flow increases and arterial dilation to bradykinin (but not acetylcholine, Figure 1-10). Thus, both resistin and leptin can cause significant endothelial dysfunction when acutely administered both *in vivo* and *in vitro*[36, 43]. These findings are important because decreased nitric oxide (as well as other endothelium-derived vasodilators) in some degree regulate cellular adhesion, vascular permeability, and other precipitating events in the initiation of atherosclerosis[104].

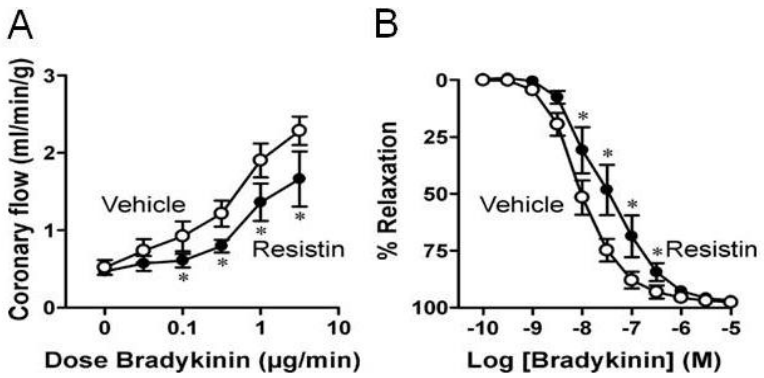

Figure 1-10 ***Resistin induces significant coronary endothelial dysfunction.*** *Resistin significantly attenuated coronary endothelium-dependent vasodilation to bradykinin, but not acetylcholine, in open-chest anesthetized dogs (B) and isolated coronary artery rings (A). Modified from Dick et al., AJP, 2006*[43]*.*

In addition to resistin and leptin, other adipokines have also been linked with endothelial dysfunction and cardiovascular disease. Accumulating evidence suggests that the inflammatory cytokine TNF-α plays a pivotal role in the disruption of macrovascular and microvascular circulation both in vivo and in vitro[45, 57]. In particular, advanced glycation end-products (AGE) / receptor for AGEs (RAGE), lectin-like oxidized low-density lipoprotein receptor (LOX)-1, and nuclear factor kappaB (NF-kappaB) activity play key roles in increasing TNF-alpha expression[57, 110-112]. The increase in TNF-alpha expression induces the production of reactive oxygen species (ROS), resulting in endothelial dysfunction in many pathological conditions.

Interestingly, adiponectin has been associated with endothelial improvement/protection through multiple means, and is significantly decreased during obesity[42]. First, the anti-inflammatory nature of adiponectin may directly attenuate the detrimental effects of other adipokines (i.e. leptin, resistin, or TNF-α)[42, 48]. Second, studies by Date *et al.* and Takano *et al.* suggest a relationship between adiponectin and coronary endothelial response. Results from Date *et al.* demonstrate an association between plasma adiponectin and coronary flow reserve, while Takano *et al.* showed a

negative correlation between adiponectin and coronary endothelial response to acetylcholine[53, 54].

Together, these studies suggest that multiple adipokines may regulate coronary vasomotor function and the pathogenesis of coronary artery disease[42, 46-48, 58]. However, plasma concentrations of many reported adipokines have not proved to be extremely valuable as clinical indicators of cardiometabolic risk. In particular, elevated levels of adiponectin have paradoxically been associated with increased risk and mortality to coronary artery disease, renal disease, and heart failure[113]. Similarly, plasma concentrations of leptin are only moderately associated with coronary heart disease[114]. Again, these findings suggest that our understanding of obesity-associated cardiovascular disease remains limited, and that additional mechanisms are likely responsible.

Perivascular Adipose Tissue and Coronary Artery Disease

As was previously mentioned, the anatomical distribution of adipose tissue is a major determinant of metabolic and cardiovascular diseases. While abdominal adiposity has received considerable attention, there is mounting evidence that local visceral epicardial adipose tissue may contribute to unfavorable cardiometabolic complications[115-118]. This hypothesis is important because it is among the first to suggest an alternative, local pathway in the development of obesity-associated vascular disease. Hence, “local obesity” (as opposed to generalized increases in percent body fat) around the heart may be an important regulator of vascular function and disease progression. Unfortunately, little is currently known about the physiologic and pathophysiologic functions of perivascular adipose tissue.

Perhaps the most noticeable uncertainty regarding the proposed pathologic role of perivascular adipose tissue is determining how factors produced within the adventia are able to traverse the arterial wall. While experimental evidence remains limited,

studies suggest that the coronary vasa vasorum help to traffic harmful adipokines between perivascular adipose tissue and the vascular lumen[119-121]. Above all, results from Herrman *et al.* demonstrated that increased coronary vasa vasorum neovascularization preceded overt coronary endothelial dysfunction and disease in domestic swine fed a high cholesterol diet (Figure 1-11)[120]. These findings suggest an "outside-to-inside" signaling paradigm that further justifies a pathologic role of perivascular adipose tissue[118, 122, 123].

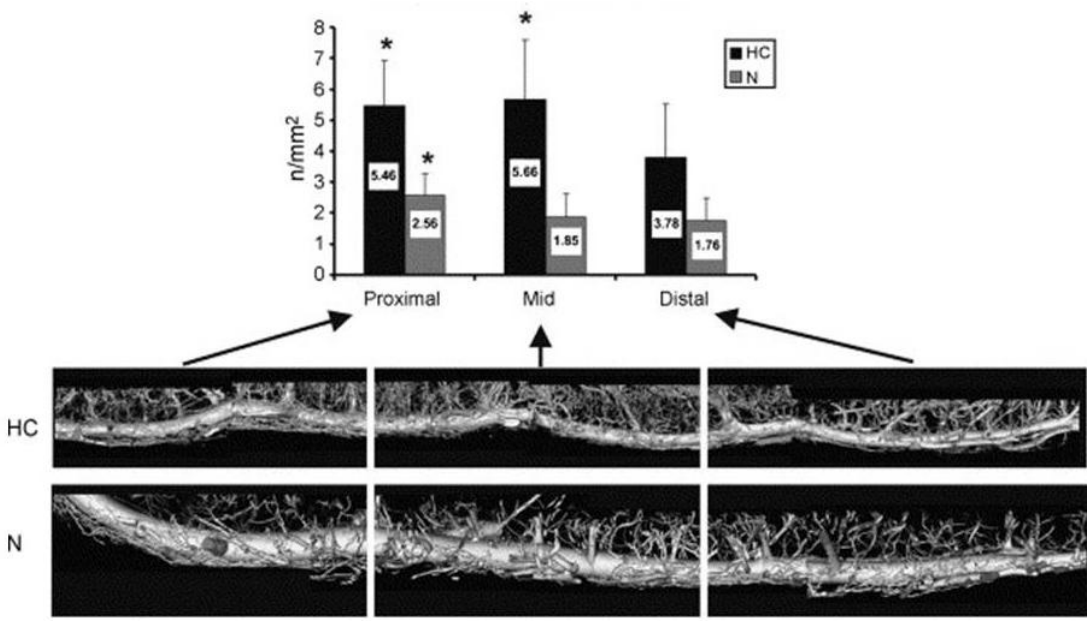

Figure 1-11 ***Increased vasa vasorum density precedes coronary endothelial dysfunction in high cholesterol (HC) fed swine.*** *Bottom: a collage of volume-rendered micro-CT images (scanned in 2-cm increments) adding up to intact left anterior descending (LAD) coronary arteries from a high-cholesterol (HC, black bars) and a control (N, grey bars) pig. The LADs are subdivided in three equal thirds and vasa vasorum parameters are determined and displayed accordingly (here vasa vasorum spatial density). In these particular LADs, proximal portions of the LAD show a higher spatial vasa vasorum (VV) density than the distal portions (*P < 0.001). Modified from Gossl M et al., Atherosclerosis, 2007 [119].*

Initial studies have shown that perivascular adipose tissue significantly attenuates contractile responses of rat aorta[124-126], rat mesenteric arteries[127, 128], and human internal thoracic arteries[129, 130]. In addition, the mechanism of this anti-contractile effect is proposed to be the release of undefined transferable factor(s) (i.e. adipocyte-

derived-relaxing factor) that act via extracellular Ca^{2+}, tyrosine kinases and/or PKA-dependent opening of vascular smooth muscle K^+ channels[124]. While an adipocyte-derived relaxing factor would be potentially beneficial to vascular function, some investigators hypothesize that the vasodilatory capacity of perivascular adipose tissue is decreased in the setting of obesity[131]. While adiponectin was initially proposed to be the mediating agent of this effect, a recent study demonstrated that perivascular adipose tissue from adiponectin gene-deficient mice maintained anti-contractile activity[130]. Hence, no study to date has successfully identified or characterized an adipocyte-derived relaxing factor(s).

In contrast to adipocyte-derived relaxing factors, there are several clinical and correlative observations that suggest perivascular adipose tissue directly contributes to coronary artery disease. In general, perivascular adipose tissue normally surrounds large conduit vessels of the heart. Importantly, coronary atherosclerotic disease primarily occurs in these larger arteries that are encased by adipose tissue. Perivascular adipose volume is also increased in obese patients and animals [116, 132-134]. Interestingly, patients with a myocardial bridge interrupting perivascular adipose tissue have been documented to have limited atherosclerosis within the portion of the vessel underneath the muscle[135]. Together, these observations prompted many to speculate that local epicardial adipose tissue directly contributes to vascular dysfunction and disease.

Clinical findings further support a causative role for perivascular adipose tissue. Results from Baker *et al.*[49] and Cheng *et al.*[47] documented pathogenic adipokine profiles from human perivascular and epicardial adipose tissue. Specifically, Baker *et al.* reported increased macrophage infiltration (and potentially increased inflammation) within epicardial adipose compared to abdominal adipose tissue[49]. Coronary perivascular adipose tissue, therefore, appears to provide a local source of harmful factors and adipokines that may directly influence cardiovascular disease. This

observation is critical as plasma inflammatory biomarkers may not adequately reflect local tissue inflammation, and traditional cardiovascular therapies do not directly target local inflammatory signals from perivascular adipose tissue[117, 118].

With the previous findings in mind, many investigators have suggested that epicardial adipose tissue should be considered a risk factor for coronary atherosclerosis. Notably, recent clinical findings documented that patients with atherosclerotic lesions had significantly larger epicardial adipose tissue volumes than patients without atherosclerosis[116]. In comparison to the Framingham risk score (which accounts for age, systolic blood pressure, total cholesterol, and smoking status) epicardial adipose tissue volume was by far the strongest predictor of coronary atherosclerosis (odds ratios of 2.8 vs. 4.1). Importantly, no association was found between BMI and coronary atherosclerosis.

Together, the current research on perivascular adipose tissue is indeed promising. Both experimental and clinical findings have already increased our recognition of the paracrine activity of visceral adipose tissue, and its potential link to coronary artery disease (Figure 1-11). However, it is important to recognize that prior investigations are largely correlative in nature. Above all, no investigation has established a direct, causal link between perivascular adipose tissue and coronary artery disease.

Summary and Proposed Experimental Aims

The epidemic of obesity is an urgent health crisis. If unabated, the diseases and complications associated with obesity will undoubtedly stress healthcare systems, and ultimately result in decreased quality of living for millions of people worldwide. Particularly alarming is the increased incidence of the multifaceted MetS and associated risk for cardiovascular disease. While many studies have sought to identify indicators of cardiometabolic risk (i.e. BMI and/or waist-to-hip ratio), our ability to predict the

occurrence and severity of such complications remains limited. Hence, much work is still needed before we fully understand the link between obesity and cardiovascular disease.

To this end, many investigations have repeatedly demonstrated that adipose tissue is an active endocrine and paracrine signaling organ. Adipokines have been shown to influence metabolism, coagulation, inflammation, and vascular reactivity. These findings, in conjunction with altered adipokine production in the setting of obesity, have led to the proposed “Adipokine Hypothesis” linking obesity and vascular dysfunction. Importantly, visceral adiposity appears to confer unique risks towards the development of chronic inflammation and cardiovascular disease. In the end, adipokines are thought to contribute to vascular dysfunction within both the coronary micro- and macrovascular circulation.

Lastly, recent evidence strongly suggests that local perivascular adipose tissue directly contributes to coronary vascular disease. In particular, perivascular adipose tissue is increased with obesity, and has proven to be an abundant source of harmful adipokines (Figure 1-12). Notably, recent clinical findings demonstrate that pericardial adipose volume is by far the strongest independent predictor of coronary atherosclerosis[116]. While these observations are promising, no investigation has to date elucidated a direct, causative mechanism linking perivascular adipose tissue and coronary artery disease.

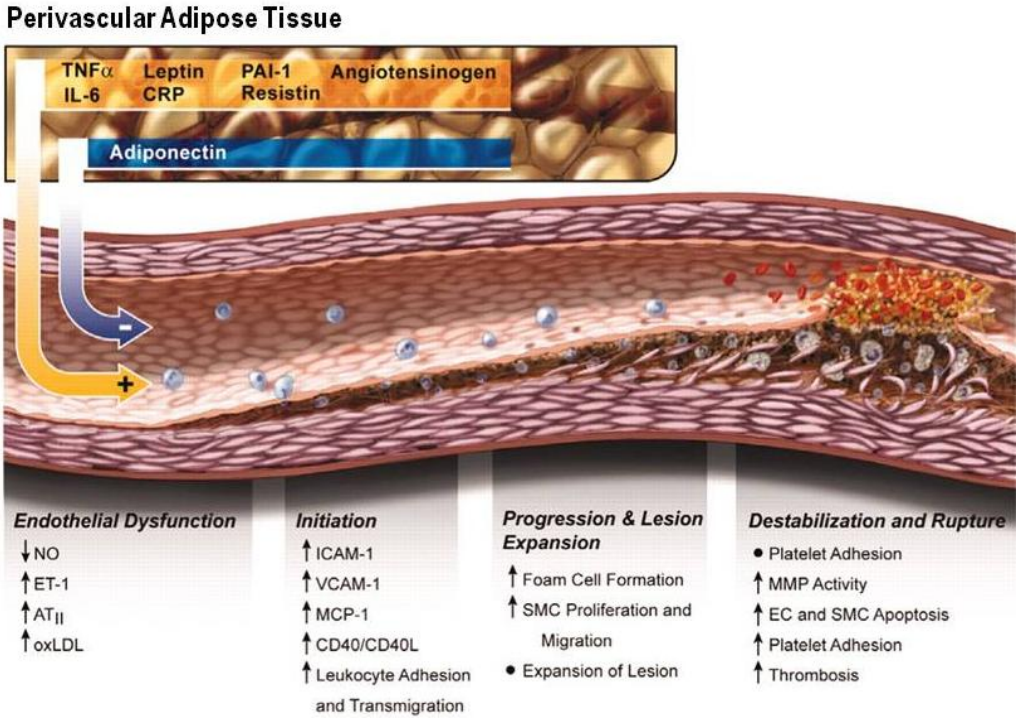

Figure 1-12 ***Perivascular adipose tissue and coronary atherosclerosis.*** *Schematic representation of the proposed contribution of perivascular adipose tissue to coronary artery dysfunction and disease. Perivascular adipose-derived factors may influence a number of stages involved in the development of atherosclerotic lesions. However, to date no investigation has successfully elucidated a specific mechanism linking perivascular adipose with coronary artery disease. Modified from Lau et al., AJP, 2005*[107]*.*

With these theories in mind, the central focus of this work is to investigate the potential role of coronary perivascular adipose tissue in the development of coronary endothelial dysfunction and atherosclerosis. Specifically, the goal of the following studies is to determine the cellular/molecular mechanisms by which local perivascular adipose tissue regulates vascular reactivity in health, and in the setting of the MetS. These objectives will be addressed in studies designed to examine the following Specific Aims (Figure 1-13):

1. **Test the hypothesis that perivascular adipose tissue significantly impairs normal coronary vascular reactivity.** Rationale for Aim 1 is based primarily on the previously mentioned studies suggesting that perivascular adipose tissue has an anti-contractile effect on vascular reactivity. We aim to specifically test the effect of perivascular adipose tissue from normal canines in the coronary circulation.
2. **Delineate the mechanisms by which perivascular adipose tissue impairs coronary endothelial-dependent vasodilation.** We hypothesize that perivascular adipose tissue impairs coronary endothelial function via a protein kinase C (PKC)-β dependent, site-specific phosphorylation of nitric oxide synthase at the amino acid residue Thr^{495}. Rationale for our hypothesis is based on the well documented role of PKC-β as a mediator of endothelial dysfunction[136-139].
3. **Identify the specific perivascular adipose-derived factor(s) that contribute to coronary vascular dysfunction in the MetS.** We hypothesize that the adipokine leptin significantly contributes to the coronary endothelial effects of perivascular adipose tissue via PKC-β. Rationale for this hypothesis is based in our laboratory's previous observation of acute leptin-induced coronary endothelial dysfunction[36].

In the end, findings from these proposed investigations will be among the first to provide a specific mechanism by which perivascular adipose tissue affects coronary endothelial function, and potentially contributes to vascular disease. Additionally, the proposed investigations would suggest that the adipokine leptin is critical in the initiation of obesity-associated atherosclerotic disease.

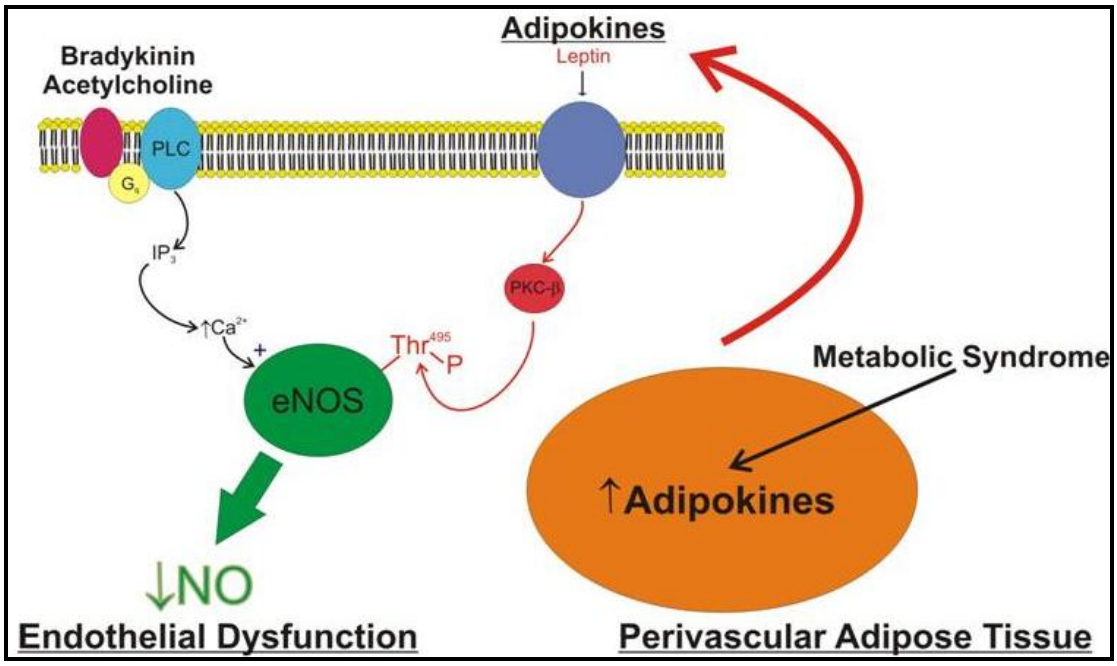

Figure 1-13 ***Proposed mechanism of how perivascular adipose contributes to coronary vascular disease.*** *We propose that inflammatory mediators and adipokines from perivascular adipose tissue directly impair coronary endothelial function through a PKC-β dependent mechanism.*

Chapter 2

Endogenous adipose-derived factors diminish coronary endothelial function via inhibition of nitric oxide synthase

Microcirculation

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Abstract

Adipocytokines may be the molecular link between obesity and vascular disease. However, the effects of these factors on coronary vascular function have not been discerned. Accordingly, the goal of this investigation was to delineate the mechanisms by which endogenous adipose-derived factors affect coronary vascular endothelial function. Both isolated canine coronary arteries and coronary blood flow in anesthetized dogs were studied with and without exposure to adipose tissue. Infusion of adipose-conditioned buffer directly into the coronary circulation did not change baseline hemodynamics; however, endothelial-dependent vasodilation to bradykinin was impaired both *in vitro* and *in vivo.* Coronary vasodilation to sodium nitroprusside was unaltered by adipose tissue. Oxygen radical formation did not cause the impairment because quantified dihydroethidium staining was decreased by adipose tissue and neither a superoxide dismutase mimetic nor catalase improved endothelial function. Inhibition of nitric oxide (NO) synthase with L-NAME diminished bradykinin-mediated relaxations and eliminated the subsequent vascular effects of adipose tissue. *In vitro* measurement of NO demonstrated that adipose tissue exposure quickly lowered baseline NO and abolished bradykinin-induced NO production. The results indicate that adipose tissue releases factor(s) that selectively impair endothelial-dependent dilation via inhibition of NO synthase-mediated NO production.

Keywords: coronary circulation, perivascular adipose tissue, vascular endothelium, adipocytokine; nitric oxide

Introduction

Adipose tissue is an active endocrine and paracrine organ that releases a variety of cytokines that influence many physiologic and pathophysiologic conditions [107]. Recent studies have implicated perivascular adipose tissue in the pathogenesis of vascular dysfunction and disease[140-143]. Adipocyte production of pathogenic adipocytokines and/or chemokines have been shown to stimulate chemotaxis [141], inflammation [44], smooth muscle proliferation [144] and activate key mediators of atherogenesis [107]. These potentially harmful adipocytokines have been speculated to promote coronary atherogenesis via local paracrine and vasocrine pathways [133]. In addition, perivascular adipose tissue has also been shown to significantly attenuate contractile responses of rat aorta [124-126, 145], rat mesenteric [127, 128] and human internal thoracic arteries [129] to a variety of vasoconstrictor compounds. However, there are also abnormalities of vasodilation associated with adipocytokines. Our laboratory recently demonstrated that the adipocytokines leptin and resistin significantly impair canine coronary endothelial-dependent vasodilation both *in vivo* and *in vitro* [36, 43] in normal animals. As might be expected, we have also demonstrated that obesity and insulin resistance alter the control of coronary blood flow and significantly impair the balance between oxygen delivery and myocardial metabolism [79]. This vascular dysfunction is related to sensitization of key coronary vasoconstrictor pathways [89, 143, 146], some of which could be influenced by factors released from adipose tissue.

The goal of the present investigation was to delineate the mechanisms by which endogenous adipose-derived factors affect coronary vascular endothelial production of nitric oxide (NO) at the level of both microvessels and conduit arteries. Potential mechanisms were examined by *in vitro* studies in isolated canine coronary arteries with or without perivascular adipose tissue as well as *in vivo* experiments in open-chest anesthetized dogs to evaluate microvascular resistance regulation by endothelial

vasodilators before and during treatment with adipose-conditioned buffer. This approach was used to document the effects of adipose tissue on coronary vascular reactivity in large arteries *in vitro* where coronary disease predominantly occurs as well as coronary flow responses *in vivo* which reflect alterations in function of microvascular resistance vessels.

Methods

This investigation was approved by the Institutional Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996). All dogs studied were lean mongrel dogs weighing between 20 and 30 kg.

Functional assessment of isolated epicardial coronary rings. Isolated coronary artery studies were performed as previously described [36, 43]. Briefly, left circumflex coronary arteries from lean dogs were dissected from the heart with or without the naturally occurring perivascular adipose tissue surrounding the conduit artery. Representative coronary arteries with or without perivascular adipose tissue were stained with Sudan IV and are shown in Figure 2-1. The arteries were cut into 3 mm rings and mounted in organ baths for isometric tension studies. Perivascular adipose tissue was either rigorously removed from the arterial rings or allowed to remain intact (approximately 0.25 g adipose per ring). Optimal length was found by assessing contraction to 60 mM KCl. Passive tension was increased in gram increments until there was < 10% change in active KCl contractions.

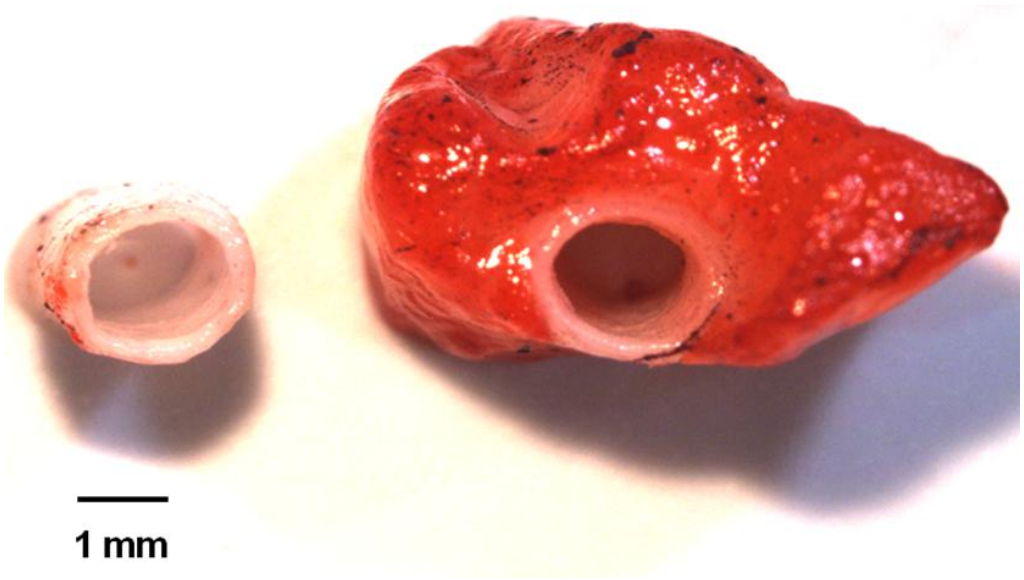

Figure 2-1 *Representative isolated left circumflex coronary arteries with or without perivascular adipose tissue stained with Sudan IV (adipose tissue staining red).*

Endothelial function was assessed by the addition of graded concentrations of bradykinin (0.1 nM/L - 10 µM/L, n = 5) or sodium nitroprusside (1.0 nM/L - 0.1mM/L, n = 3) to the tissue bath. In additional studies, bradykinin concentration responses were conducted in the presence of the NO synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME, 300 µM/L, n = 7), the superoxide dismutase mimetic tempol (10 µM/L, n = 3), and the H_2O_2 degrading enzyme catalase (1000 U/ml, n = 4). All results obtained during bradykinin and sodium nitroprusside dose response experiments are reported as the percent relaxation for each animal (Figures 2-3, 2-4, 2-5 and 2-6). 100 percent relaxation is defined as a return to the level of tension prior to U46619 contraction.

Coronary blood flow evaluation of microvascular performance. Dogs were initially sedated with morphine (3 mg/kg, sc) and anesthetized with α-chloralose (100 mg/kg, iv). The animals were then intubated and mechanically ventilated (Harvard respirator) with room air supplemented with oxygen. A catheter was placed in the right femoral vein for intravenous administration of supplemental anesthetic and sodium bicarbonate. The femoral artery was then cannulated to supply blood to an extracorporeal perfusion

system which subsequently perfused the left anterior descending coronary artery (LAD) at a controlled pressure. A left lateral thoractomy was performed to expose the heart, and the LAD was isolated distal to its first major diagonal branch. After the administration of heparin (500 U/kg, iv) the LAD was cannulated with a stainless steel cannula (3 mm external diameter, 2.2 mm internal diameter). Coronary perfusion pressure was measured through a saline filled catheter advanced to the orifice of the LAD cannula. The pressure of the perfusion system was held constant at 100 mmHg by a servo-controlled roller pump. Similarly, an in line Transonic Systems flow transducer (Ithaca, NY) was used in the perfusion system to measure coronary blood flow. Data were continuously recorded on IOX data acquisition software from Emka Technologies (Falls Church, VA). The preparation was allowed a ~30 minute recovery time before data were recorded for analysis.

In order to test the effects of endogenous adipose-derived factors on coronary microvascular endothelial function *in* vivo, bradykinin was infused (0.3 – 3.0 µg/min) in the absence and presence of adipose conditioned buffer (n = 6). The adipose-conditioned buffer was prepared in phosphate buffered saline that was allowed to shake and mix with parietal pericardial adipose tissue (3 g/ml) for 30 min at 37°C in a shaking water bath. The conditioned buffer was then filtered (0.2 µm) and infused directly into the coronary circulation via the perfusion system (0.3 ml/min). In additional studies, bradykinin was simultaneously infused with either L-NAME (150 µg/min, n = 5) or tempol (10 mg/min, n = 5). Lastly, studies were also conducted to determine if adipose-conditioned buffer had any direct effect on coronary hemodynamics. During these studies, adipose-conditioned buffer was infused at various rates (0.3 – 3.0 ml/min, n = 3) without the administration of bradykinin.

Nitric oxide measurements. NO concentration was measured in isolated coronary arteries (n = 3) before and during exposure to 400 nM bradykinin with or without the

addition of perivascular adipose tissue. This concentration of bradykinin was a supramaximal concentration for NO production by isolated arterial segments. NO was evaluated by a polarographic technique, using a carbon fiber, recessed-tip glass microelectrodes as previously described [147]. The microelectrodes had a sharpened outer tip diameter of 7 – 10 µm and were polarized at +0.7 or +0.9 V relative to either a World Precision Instruments carbon fiber reference electrode (Sarasota, FL) or a simple silver-silver chloride electrode. The currents generated ranged from 0 – 20 pA. A calibration curve was established by measurement of the microelectrode current at NO concentrations of 0, 600, and 1,200 nM. These concentrations were based on the composition of the NO-N_2 precision calibration gases in saline at 37.5°C. The working resolution of the microelectrodes is typically <10 nM, allowing for random noise and current drift. The microelectrodes are completely insensitive to oxygen when positively polarized.

Rings of vessel were placed in a perfused bath (5 ml/min, 5 ml bath volume) of media similar to that used for tension studies and equilibrated with 95% oxygen and 5% carbon dioxide. The tip of the NO microelectrode was placed in the lumen of the vessel and touched an endothelial surface. The baseline concentration of NO was measured followed by the response to bradykinin (400 nM). Thereafter, the adipose tissue removed from the arterial ring was placed upstream of the ring in the flowing media for 30 minutes before repeating the NO measurements at rest and during bradykinin exposure.

Dihydroethidium staining. Dihydroethidium (DHE) staining for superoxide (O_2^-) was carried out as described previously [43]. Left circumflex coronary arteries with (n = 5) and without (n = 5) perivascular adipose tissue were incubated with 10 µM DHE at 37°C for 30 min with or without tempol (10 µM/L) administration. The arteries were embedded in OCT and flash frozen in liquid nitrogen. Tissue sections (10 µm) were then prepared

using a cryostat on thaw-mounted slides. Ethidium fluorescence was assessed with 508 nm excitation and 615 nm emissions. Scion Image for Windows was used to perform histogram analysis of brightness in images of DHE-stained arteries.

Adipokine content in adipose tissue extract. A multiplexed biomarker immunoassay from Linco Laboratories was used to measure the concentration of key adipokines in the adipose-conditioned buffer (Table 2-2). The assay was conducted according to the manufacturer's specifications. Briefly, the assay functions by using antibody coated beads, which are selective for specific human adipokines. Adipose-conditioned buffer samples used during the *in vivo* studies were collected, filtered (0.2 µm) and individually measured in triplicate on a 96 well plate. The antibody coated beads were incubated with each individual sample and allowed to bind overnight. Biotinylated detection antibodies were added to each well, and streptavidin-phycoerythrin was subsequently added in order to detect the fluorescence on each bead. A Luminex Instrument was used to both identify which adipokines were expressed and to quantify their relative concentrations.

Statistical analyses. Data are presented as mean ± standard error. For both *in vitro* and *in vivo* studies, a two-way repeated measures ANOVA was used to test the effects of the presence or absence of adipose tissue or conditioned buffer (Factor A) and various doses of sodium nitroprusside or bradykinin (Factor B) on coronary physical or chemical responses (Sigma Stat 3.0 Software). Identical statistics were performed for studies conducted in the presence of L-NAME, tempol and catalase. For the *in vitro* experiments, data were analyzed per animal. When statistical differences were found by ANOVA, a Student-Newman-Keuls multiple comparison test was performed. The criterion for statistical significance was $P < 0.05$ in all tests.

Results

Adipose tissue and baseline hemodynamics. Data shown in Figure 2-2 demonstrate that increases in the intracoronary infusion rate of the adipose-conditioned buffer (0.3 – 3.0 ml/min) did not significantly affect baseline coronary blood flow (*P* = 0.68). Effects of adipose-conditioned buffer, L-NAME and tempol on baseline coronary blood flow, mean aortic pressure and heart rate are given in Table 2-1. Intracoronary infusion of adipose-conditioned buffer (0.3 mL/min) did not significantly affect coronary blood flow (*P* = 0.58), mean aortic pressure (*P* = 0.96) or heart rate (*P* = 0.89). Baseline coronary hemodynamics were also unaffected by adipose-conditioned buffer in the presence of L-NAME (*P* = 0.58) or the superoxide dismutase mimetic tempol (*P* = 0.19). The average concentrations of adipokines in adipose-conditioned buffer are presented in Table 2-2. Notably, the concentration of resistin was substantially greater than the other adipokines.

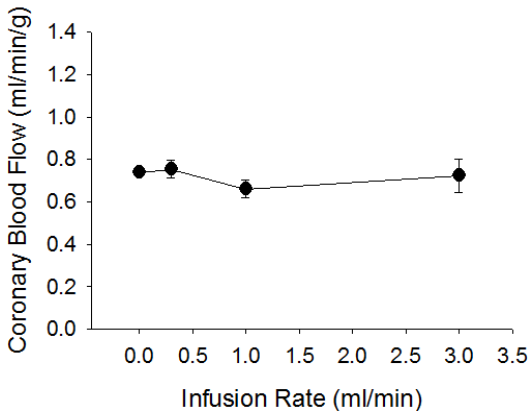

Figure 2-2 *Direct infusion of endogenous adipose-derived factors from adipose-conditioned buffer into canine coronary circulation has no effect on baseline coronary blood flow (n = 3).*

Table 2-1 Effects of adipose-conditioned buffer, L-NAME and tempol on baseline hemodynamic data in anesthetized, open-chest dogs.

	Coronary Blood Flow (ml/min/g)	Aortic Pressure (mmHg)	Heart Rate (beats/min)
Untreated			
Control (n=6)	0.50 ± 0.01	103 ± 6	87 ± 13
Buffer (n=6)	0.61 ± 0.05	96 ± 9	110 ± 24
L-NAME			
Control (n=5)	0.45 ± 0.05	105 ± 8	72 ± 9
Buffer (n=5)	0.50 ± 0.08	103 ± 9	90 ± 11
Tempol			
Control (n=5)	0.69 ± 0.03	102 ± 9	77 ± 10
Buffer (n=5)	0.94 ± 0.15	98 ± 12	94 ± 8

Values are mean ± SE. n = number of dogs.

Adipose tissue and coronary endothelial function. Adipose-conditioned buffer exposure significantly attenuated coronary endothelial-dependent vasodilation, as judged by blood flow responses, to the higher doses of bradykinin (1-3 μg/min; ~10 nM bradykinin) in open-chest anesthetized dogs (Figure 2-3A; $P < 0.01$). In isolated coronary arteries, arterial relaxation to bradykinin was also decreased by perivascular adipose tissue (Figure 2-3B; 1-100 nM; $P < 0.01$). In contrast, adipose tissue did not significantly affect endothelial-independent relaxation to sodium nitroprusside in isolated arteries (Figure 2-4).

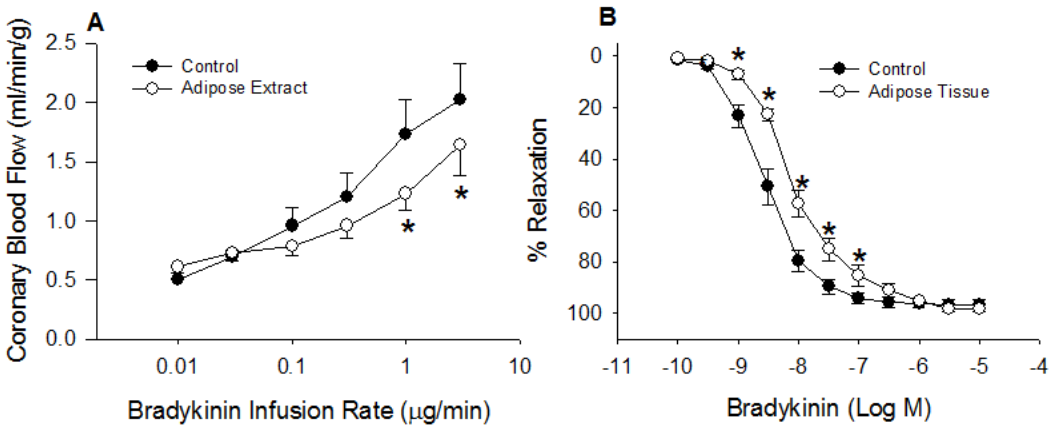

Figure 2-3 *Adipose tissue significantly attenuates coronary endothelial-dependent vasodilation to bradykinin in vivo (3A, n = 6) and in isolated coronary arteries (3B, n = 5). * = P < 0.01.*

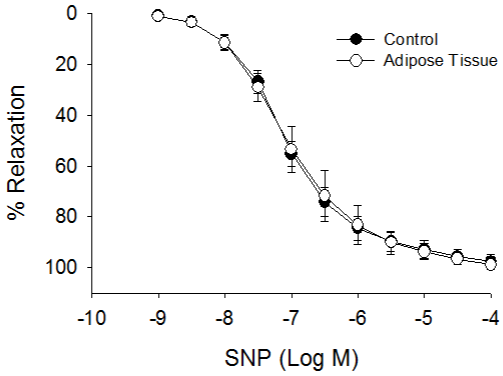

Figure 2-4 *Adipose tissue has no significant effect on coronary endothelial-independent vasodilation to sodium nitroprusside (SNP) n = 3.*

Adipose tissue and coronary reactive oxygen species. DHE staining for coronary O_2^- showed a significant decrease (67 ± 7% of control) in tempol-sensitive fluorescence between arteries with perivascular adipose tissue relative to arteries fully cleaned of adipose tissue (Figures 2-5A and 2-5B). Therefore, vessel exposure to adipose tissue did not increase O_2^- formation. Additional studies demonstrated that the administration of tempol (10 μM) to scavenge oxygen radicals did not improve reactivity of arteries with perivascular adipose tissue to bradykinin (Figure 2-5D). Instead, tempol significantly impaired relaxation of arteries, with or without adipose tissue, to bradykinin

(Figure 2-5C and 2-5D, $P < 0.001$). These findings were confirmed by additional *in vivo* studies in Figure 2-5E which found that tempol failed to reverse the adipose tissue-induced impairment of bradykinin-mediated coronary vasodilation in open-chest dogs (Figure 2-5E, $P < 0.001$). To test for H_2O_2 effects, catalase was applied prior to *in vitro* relaxation studies. Enzymatic degradation of H_2O_2 with catalase diminished the relaxation to bradykinin at 1 nM to 30 nM (Figure 2-5F) in comparison to the control relaxation shown in Figure 2-5C. However, arteries with perivascular adipose tissue continued to display further impairment ($P < 0.01$) to bradykinin.

Adipose tissue and nitric oxide production. Inhibition of NO synthase with L-NAME eliminated the relaxation differences between coronary artery rings with or without perivascular adipose tissue (Figure 2-6A, $P = 0.32$). Also note that compared to responses of untreated vessels in Figure 2-3B, L-NAME caused a right shift of the dose-relaxation curve indicating major suppression of endothelial dependent relaxation. These initial observations were further supported by *in vivo* studies demonstrating that pretreatment with L-NAME abolished the effect of adipose-conditioned buffer on bradykinin-mediated coronary vasodilation (Figure 2-6B; $P = 0.24$). Again, L-NAME caused a significant attenuation of the dose-blood flow response curve indicating major suppression of endothelial dependent relaxation in the *in vivo* coronary microvasculature (Figure 2-3A vs. Figure 2-6B). Importantly, measures of coronary NO concentration with an NO sensitive microelectrode showed that adipose tissue both markedly lowered basal NO production by 60% and eliminated coronary endothelial NO production in response to bradykinin (Figue 2-6C, $P < 0.05$).

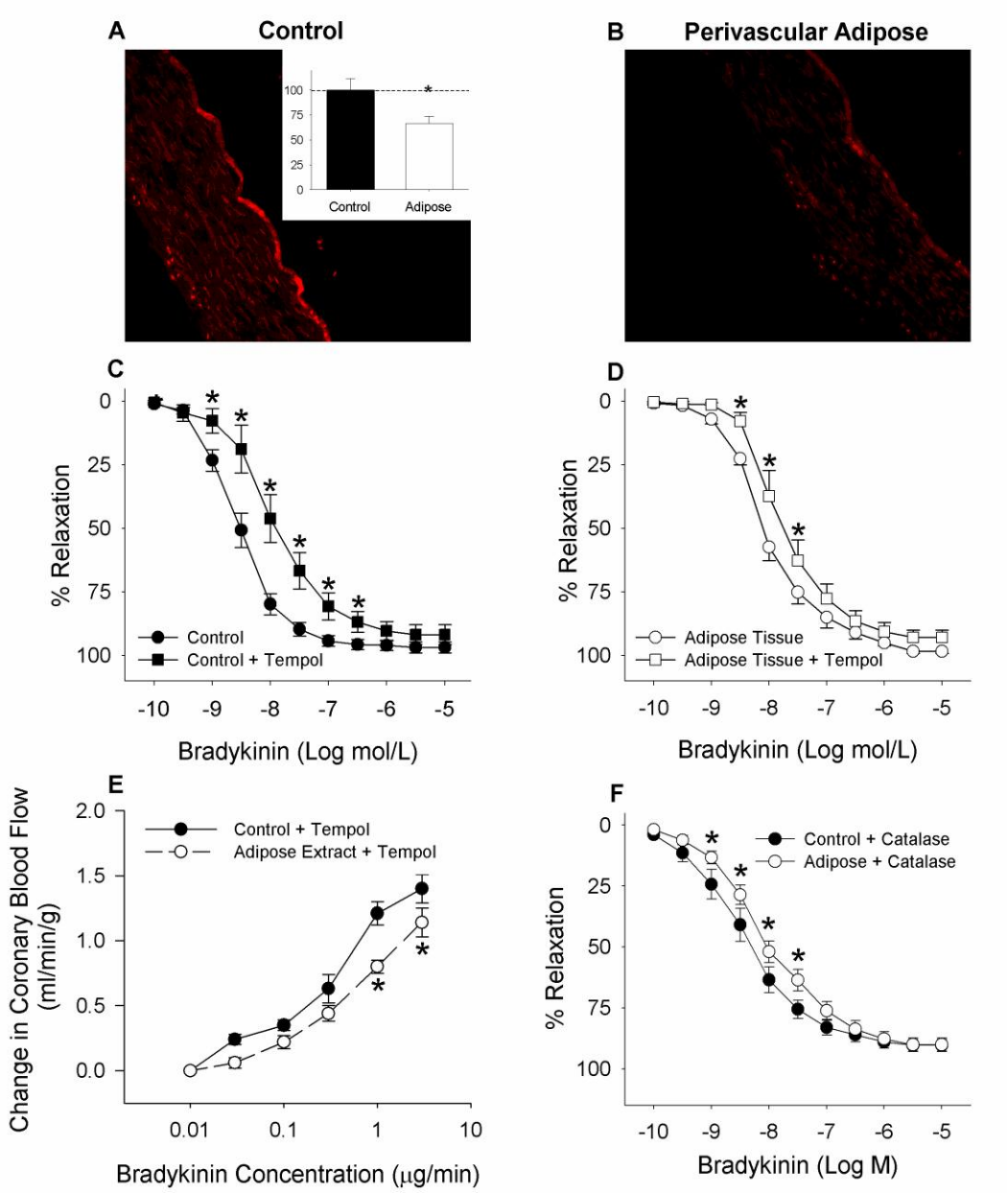

Figure 2-5 *DHE staining showed a significant decrease in fluorescence in coronary arteries with perivascular adipose tissue (Representative pictures A and B; inset shows average DHE fluorescence). The superoxide dismutase mimetic tempol did not improve reactivity of coronary arteries with perivascular adipose tissue (D); rather it significantly impaired reactivity of arteries without (C) and with (D) adipose tissue (n = 3). In addition, tempol also failed to improve reactivity in vivo (E, n = 5). * P < 0.001. Enzymatic degradation of peroxide (H_2O_2) with catalase also did not reverse the endothelial impairment *P < 0.01 (F, n = 4).*

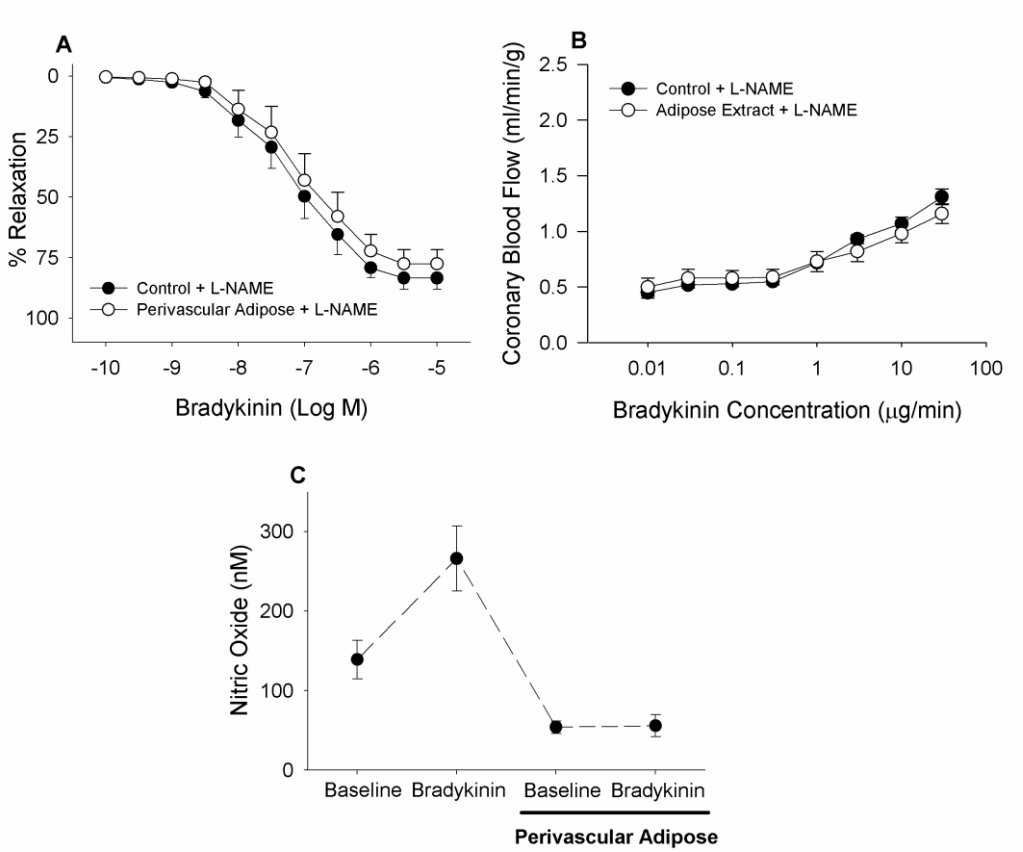

Figure 2-6 *Inhibition of nitric oxide synthase with L-NAME reversed the effect of adipose tissue on bradykinin-mediated vasodilation in isolated coronary arteries (A, n = 7) and in open-chest anesthetized dogs (B, n = 5). Perivascular adipose tissue markedly impaired bradykinin (400 nM)-mediated increases in NO production (C, n = 3).*

Table 2-2 *Adipokine expression in adipose-conditioned buffer. A multiplexed biomarker immunoassay was conducted in order to measure adipokine concentrations present within the adipose-conditioned buffer*

Adipokines	Concentration in adipose-conditioned buffer (pg/ml)
TNF-α	0.04 ± 0.01
IL-1β	0.24 ± 0.02
PAI-1(active)	0.42 ± 0.08
MCP-1	0.45 ± 0.01
HGF	1.64 ± 0.20
Leptin	2.40 ± 0.20
Resistin	5338.00 ± 1032.00

Values are mean ± SE for all measured samples (n = 10). IL-1 β – interleukin 1β, PAI-1 - plasminogen activator inhibitor 1, MCP-1 - monocyte chemoattractant protein-1, HGF - hepatocyte growth factor.

Discussion

The present investigation was designed to examine the mechanisms by which endogenous adipose-derived factors might impair coronary endothelial function within both the coronary microcirculation and conduit arteries. The major new findings from this study are that factor(s) released from adipose tissue: 1) do not affect baseline coronary blood flow or systemic hemodynamics; 2) significantly impair endothelial-dependent vasodilation to bradykinin in both *in vivo* preparations and isolated coronary arteries; 3) do not alter coronary endothelial-independent vasodilation to sodium nitroprusside, i.e. vascular smooth muscle response to NO; 4) do not significantly increase coronary O_2^- production or H_2O_2-mediated vasodilation; and 5) markedly attenuate coronary artery endothelial production of NO in isolated vessels. Taken together, these data indicate that adipose tissue releases factor(s) that selectively impair endothelial-dependent dilation via inhibition of NO synthase-mediated NO production. This impairment is independent of alterations in coronary vascular smooth muscle response to NO, O_2^- mediated decreases in NO-bioavailability or H_2O_2-mediated coronary vasodilation.

Adipose tissue and baseline coronary hemodynamics. Before trying to interpret any effect of adipose tissue, it was necessary to test the direct effect of adipose-derived factors on baseline coronary blood flow, arterial pressure and heart rate (Figure 2-2 and Table 2-1). Our results indicate that factors released by adipose tissue do not directly affect overall coronary microvascular resistance under normal resting conditions. Importantly, the present findings are consistent with earlier studies and the current L-NAME studies (Table 2-1) which demonstrated that blockade of NO synthase-mediated NO production has little effect on baseline coronary blood flow [72, 74].

Effects of adipose tissue on coronary endothelial function. Data from this study are the first to document that endogenous factors released from adipose tissue

selectively inhibit coronary endothelial-dependent dilation to bradykinin judged both by *in vivo* measures of coronary blood flow (regulated predominantly by microvessels; Figures 2-3, 2-5, and 2-6), by relaxation of isolated coronary arteries (where atherosclerotic disease predominantly occurs; Figure 2-3) and by *in vitro* measurement of NO (Figure 2-6C). Our findings indicate that the adipose-induced impairment of coronary endothelial function is mediated by a selective impairment of NO synthase mediated NO production because the effect of adipose tissue on bradykinin-mediated dilation was reversed by L-NAME (Figure 2-6A-B) and direct measures of NO demonstrated a decline in both basal NO and bradykinin stimulated NO production (Figure 2-6C). We propose that the impaired relaxation is specific to NO generation as we did not detect any effect of adipose tissue on relaxations to sodium nitroprusside (Figure 2-4) or to bradykinin in the presence of L-NAME (Figure 2-6), suggesting that adipose tissue does not affect dilation to prostacyclin or endothelial-derived hyperpolarizing factors.

The findings documented in this investigation are potentially marred by a few concerns. First, there was a potential that the measured endothelial impairment was simply an artifact of decreasing responses to bradykinin after repeated doses (i.e. tachyphylaxis). However, a recent study from our laboratory demonstrated no tachyphylaxis to repeated treatments of bradykinin both *in vivo* in open-chest anesthetized dogs and *in vitro* in isolated coronary arteries (4). Hence any measured experimental differences in bradykinin-mediated dilation are not an artifact of repeated bradykinin dose-response curves. Second, the isometric tension and NO production measurements were done under circumstances where there was at most a trivial amount of hemoglobin, oxyhemoglobin or myoglobin. Therefore, these iron containing organ compounds were highly unlikely to suppress NO bioavailability [142, 148, 149]. This conclusion is further supported by our *in vivo* coronary flow data.

Adipose tissue and coronary reactive oxygen species. Recently, many investigations have focused on the role of reactive oxygen species in regulating endothelial function [150, 151]. Specifically, O_2^- has been shown to decrease NO bioavailability by reacting to produce peroxynitrite [152]. Additional studies have also shown that H_2O_2 acts as an endothelium-derived hyperpolarizing factor and metabolic vasodilator of the coronary circulation [151, 153-155]. Together, both O_2^- and H_2O_2 are potential direct and/or indirect mediators of the observed endothelial dysfunction caused by adipose tissue. The fact that DHE staining of arteries with perivascular adipose tissue showed a significant decrease in fluorescence (Figure 2-5A and B) argues that adipose tissue does not impair coronary endothelial-dependent vasodilation via increases in O_2^- production. This is supported by additional *in vitro* and *in vivo* studies with tempol that showed this O_2^- dismutase mimetic did not significantly improve the adipose tissue induced impairment of bradykinin-mediated coronary vasodilation (Figure 2-5C, D, and E). In fact, administration of tempol significantly decreased bradykinin-mediated relaxation suggesting that O_2^- may function as a vasodilator, a hypothesis supported by other recent investigations [156-159]. Enzymatic degradation of H_2O_2 with catalase also failed to reverse adipose-induced endothelial impairment (Figure 2-5F). Taken together these important data argue against O_2^- or H_2O_2 as a mechanism of adipose-induced coronary endothelial dysfunction.

Identity of adipose tissue derived vasoactive factor(s). We performed a "targeted" immunoassay to measure the expression of key adipocytokines in adipose-conditioned buffer (Table 2-2). This assay was in no way exhaustive of all potential adipose-derived mediators; however this approach allowed us to identify candidate adipokines that could contribute to the adipose-induced endothelial impairment. It is important to point out that the use of anti-human antibodies for this canine adipose tissue assay was an unavoidable limitation of this assay. However, it should be pointed out that there was no

clear correlation between the % homology of human vs. canine adipokine protein sequences (ranged from 63 – 92%) and the measured adipokine concentrations (Table 2-2) which suggests that antibody specificity alone was not the reason for the low concentrations measured.

Using this targeted immunoassay we found that resistin levels were markedly high relative to the other measured adipokines (5.9 ± 1.4 ng/ml, Table 2-2). This resistin concentration results in an estimated average plasma concentration of 0.19 ± 0.09 ng/ml, which is significantly lower than concentrations reported in human plasma [160, 161] and the 10 ng/ml that our laboratory (4) as well as others (17; 33) previously demonstrated to impair coronary endothelial-dependent vasodilation. Additionally, the present study found that L-NAME reversed the effect of adipose tissue on bradykinin-mediated dilation (Figure 2-6A and B) but an early study from our laboratory showed that L-NAME failed to inhibit resistin-induced suppression of bradykinin dilation (4). These findings do not support resistin as the primary mechanism of adipose-induced coronary endothelial dysfunction.

Other adipokines could potentially contribute the observed coronary effects of adipose tissue. Recently, our laboratory found that leptin induces significant impairment of coronary endothelial-dependent vasodilation [36]. However, the measured concentration of leptin in the adipose-conditioned buffer (2.40 ± 0.20 pg/ml) is substantially lower than the ~10 ng/ml previously found to be necessary for leptin-induced coronary endothelial dysfunction [36]. Therefore, as was the case for resistin, the functional concentration for leptin was below the pathologic concentration that negatively influenced endothelial-dependent dilation. Alternatively, TNF-α and long chain fatty acids have both been shown to attenuate endothelial-dependent vasodilation, albeit via mechanisms that elevate oxidative stress/O_2^- production [57, 140, 162] which is inconsistent with our findings with adipose tissue (Figure 2-5). Therefore, at present it is difficult to attribute the

adipose-induced impairment to any known adipocytokine. However, it is plausible that several different adipose-derived factors could act in an additive and/or synergistic manner to diminish coronary NO production. Clearly future studies are needed to identify the exact adipose-derived factor(s) and cellular/molecular mechanisms involved.

In conclusion, results from this study are the first to demonstrate that endogenous adipose-derived factors diminish production of coronary endothelial NO as judged by pharmacological challenges and direct measurements of endothelial NO. The overall findings implicate that adipocytokines have the ability to rapidly depress coronary microvascular and arterial NO generation. It is important to note that during *in vivo* circumstances, perivascular adipose tissue has little or no access to the endothelial cells of either arteries or arterioles. We are only demonstrating that adipose tissue contains factors that when released and circulated throughout the heart cause potentially dire consequences for endothelial mediated dilation. These results provide us with a new potential explanation of how alterations in adipokine expression in obesity may contribute to the development of vascular dysfunction and coronary atherosclerosis.

Acknowledgements

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Chapter 3

Periadventitial adipose tissue impairs coronary endothelial function via PKC-β dependent phosphorylation of nitric oxide synthase

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Abstract

Endogenous perivascular adipose-derived factors have been shown to contribute to coronary vascular regulation by impairing endothelial function through direct inhibition of endothelial nitric oxide synthase (eNOS). However, our understanding of the underlying mechanisms remains uncertain. Accordingly, this study was designed to test the hypothesis that perivascular adipose tissue releases agents that attenuate coronary endothelial nitric oxide production via a protein kinase C (PKC)-β dependent mechanism. Isometric tension studies were conducted on isolated canine circumflex coronary arteries with and without natural amounts of perivascular adipose tissue. Adipose tissue significantly diminished coronary endothelial-dependent vasodilation and nitric oxide production in response to bradykinin and acetylcholine. Selective inhibition of endothelial PKC-β with ruboxistaurin (1 μM) abolished adipose-induced impairment of bradykinin-mediated coronary vasodilation and endothelial production of nitric oxide. Western blot analysis revealed a significant increase in eNOS phosphorylation at the inhibitory residue Thr^{495} in arteries exposed to perivascular adipose tissue. This site-specific phosphorylation of eNOS was prevented by inhibition of PKC-β. These data demonstrate that perivascular adipose-derived factors impair coronary endothelial nitric oxide production via a PKC-β dependent, site-specific phosphorylation of eNOS at Thr^{495}.

KEYWORDS: coronary circulation, perivascular adipose tissue, adipokine, endothelial nitric oxide synthase, protein kinase C-β

Introduction

In recent years, investigators have increasingly recognized adipose tissue as both an active endocrine and paracrine organ. As a signaling organ, the production of adipose-derived cytokines (adipokines) has been well documented to influence many physiologic and pathophysiologic conditions[107]. Specifically, adipokine production has been shown to influence a number of pathogenic pathways, including chemotaxis[141], inflammation[44], smooth muscle proliferation[144] and other key mediators of atherogenesis[107]. Although adipokines have been proposed to be the molecular link between obesity and cardiovascular disease[107], the exact relationship between adipose tissue and vascular function remains uncertain. Studies have implicated the surrounding perivascular adipose tissue as a local source of adipokines that contribute to both vascular function and disease[47, 118, 163, 164]. Coronary atherosclerotic disease frequently occurs in large arteries encased by adipose tissue[164], while the opposite is true of arterial segments located underneath myocardial bridges lacking perivascular adipose tissue[135]. Together, these observations suggest that vascular function and disease is partly dependent on the presence of perivascular adipose tissue.

Recent work from our laboratory documented that perivascular adipose tissue significantly impaired coronary endothelial function in response to bradykinin both *in vitro* and *in vivo*, further implicating local adipose tissue in the initiation and pathogenesis of coronary vascular disease[165]. In particular, we documented that adipose-derived factors diminished endothelial nitric oxide (NO) production through direct inhibition of NO synthase (NOS). This effect was independent of changes in oxidative stress, peroxide-mediated vasodilation and was not related to alterations in smooth muscle responsiveness to NO. These findings are important because dysfunction and injury of the vascular endothelium is widely accepted to be a critical precursor to the development

of atherosclerosis[101]. However, no investigation has successfully identified the mechanisms by which perivascular adipose-derived factor(s) impair coronary endothelial function.

Our current understanding of vascular disease would suggest that perivascular adipose tissue may serve as a paracrine source of harmful inflammatory adipokines. However, few studies have characterized how perivascular adipose tissue contributes to normal, healthy coronary endothelial function. Recent findings call attention to specific adipokines such as interleukins, TNF-α and leptin as potential perivascular adipose-derived factors altering endothelial function[47, 136, 165]. Although promising, these investigations have yet to clearly identify the exact paracrine mediators of coronary endothelial impairment. Furthermore, the task of identifying key mediators from perivascular adipose tissue without additional experimental evidence may prove to be arduous. Accordingly, the purpose of the present study was to delineate a common signaling pathway for perivascular adipose tissue-induced endothelial dysfunction.

We propose that perivascular adipose tissue attenuates coronary endothelial NO production via a protein kinase C (PKC)-β dependent, site-specific phosphorylation of endothelial NO synthase (eNOS) at Thr^{495}. PKC is a known negative regulator of eNOS activity and NO production[166]. Specifically, phosphorylation of eNOS at the inhibitory Thr^{495} site significantly diminishes enzymatic activity by disrupting the binding of Ca^{2+}/Calmodulin to eNOS[167, 168]. Further justification for our hypothesis is based on the well documented role of PKC-β as a mediator of endothelial dysfunction[136-139]. Two recent clinical trials observed that inhibition of this specific, predominately endothelial isoform of PKC markedly improved both macrovascular[139] and microvascular[138] endothelial function in type II diabetic patients. Similarly, additional investigations suggest that endothelial impairment as a result of increased adiposity and/or adipokine

release is potentially linked with PKC activity[136, 137]. However, to date no investigation has directly examined if PKC activity contributes to perivascular adipose-induced endothelial dysfunction.

Methods

This investigation was approved by the Institutional Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996). Seven lean, mongrel dogs weighing between 20 and 30 kg were used for all experiments. Dogs were euthanized with a lethal intravenous dose of pentobarbital (86 mg / Kg dog weight). After confirming cardiac arrest, a left lateral thoracotomy was performed to collect the heart.

Functional assessment of isolated epicardial coronary rings. Isolated coronary artery studies were performed as previously described[36, 43]. Briefly, left circumflex coronary arteries from lean dogs were dissected with or without the naturally surrounding perivascular adipose tissue (approximately 0.25 g adipose per ring). Arteries and perivascular adipose tissue were always taken from the same animal and collected at the same time. Care was taken to isolate the same 2-3 cm proximal portion of the circumflex artery that is naturally surrounded by perivascular adipose tissue. Arteries were cut into 3 mm rings and mounted in organ baths for isometric tension studies. To avoid any confounding differences between proximal and distal portions of the artery, the surrounding adipose was dissected off of every other arterial ring. Optimal length was found by assessing contraction to 60 mM KCl. Arteries were pre-contracted with the thromboxane A_2 mimetic U46619 (1 μM) in order to functionally assess endothelial function. Specifically, endothelial function was assessed by the addition of graded concentrations of bradykinin (0.1 nM/L-10 μM/L, n = 5) or acetylcholine (1 nM/L – 10 μM, n = 4) to the tissue bath. Some arteries without adipose tissue were also

incubated with the NO synthase inhibitor L-NAME (300 μM) prior to the dose response experiments. In additional studies, bradykinin concentration responses were conducted in the presence of the general PKC inhibitor Ro 31-8220 (1 μM, n = 7) or the PKC-β specific inhibitor ruboxistaurin (1 μM, n = 6). All results obtained during bradykinin dose response experiments are reported as the percent relaxation for arterial rings from individual animals (Figures 3-1 and 3-2). 100 percent relaxation was defined as a return to the level of tension prior to thromboxane A_2 mimetic (U46619) contraction.

Tissue collection and western blot analysis. Coronary arteries from lean dogs (n = 3 dogs) were isolated and incubated in 5 mL organ baths for approximately one hour at 4°C in Krebs buffered solution. All arteries used for protein analysis were cleaned of perivascular adipose tissue. Control arteries were allowed to incubate alone, while other arteries were incubated with either 3 g of perivascular adipose (floating in the bath) or both adipose and ruboxistaurin. Following incubation, arteries were immediately placed in liquid N_2 and stored at -80°C for western blot analysis as previously described[169-171]. Following protein isolation, equivalent amounts of protein were loaded onto 10% acrylamide gels for electrophoresis and blotting. After blocking for 1 h at ambient temperature, membranes were incubated overnight at 4°C with primary antibodies directed against eNOS and phosphorylated eNOS Thr^{495} (both 1:1000; Affinity BioReagents and BD Transduction Laboratories). Blots were washed and incubated with goat anti-rabbit or anti-mouse IgG-HRP secondary antibodies (1:5000; Santa Cruz Biotechnology) for 1.5 h at ambient temperature. Blots were then stripped and reprobed with β-actin antiserum (1:5000; Santa Cruz Biotechnology) as the internal control. Immunoreactivity was visualized using an ECL western blotting detection kit (GE Healthcare) and quantified by scanning densitometry (Bio-Rad Quantity One 1-D Analysis Software).

Nitric oxide measurements. NO concentration was measured in isolated coronary arteries (n = 3) before and during exposure to 400 nM bradykinin with or without the addition of perivascular adipose tissue as previously described[165]. NO was evaluated by a polarographic technique, using a carbon fiber, recessed-tip glass microelectrode[147]. This microelectrode has previously been demonstrated to not be sensitive to NO synthase inhibitors, including L-NAME and nitroarginine formed by L-NAME when properly constructed with a Nafion barrier. eNOS blockade dramatically reduces the NO signal, indicating measurement of endothelium derived NO[172, 173]. After placing arterial rings into a media perfused organ bath, the tip of the NO microelectrode was positioned within the vessel lumen and pushed against the endothelial surface to insure a stable, close contact. Baseline concentrations of NO were measured followed by the response to bradykinin and a washout. Arterial rings were then treated with ruboxistaurin (1 μM) for approximately 30 min before repeating NO measurements. Finally, perivascular adipose tissue was added to the flowing media upstream of the artery for an additional 30 minutes, and a similar experimental protocol was conducted.

Statistical analyses. Data are presented as mean ± standard error. For isometric tension studies, two-way ANOVA was used to test the effects of the perivascular adipose and various doses of bradykinin, while t-test analysis was used to compare half maximal effective concentration (EC_{50}) values (Sigma Stat 3.0 Software). A two-way repeated measures ANOVA was used to analyze western blot densities and NO measurements. All experiments were analyzed per animal. When statistical differences were found a Student-Newman-Keuls multiple comparison test was performed. The criterion for statistical significance was $P < 0.05$ in all tests.

Results

Perivascular adipose tissue and coronary endothelial function. To examine the effects of endogenous perivascular adipose-derived factors on coronary endothelial function, isometric tension studies were conducted in isolated coronary arteries with and without perivascular adipose tissue. Consistent with our recent data[165], we found that the presence of perivascular adipose tissue significantly decreased coronary endothelial-dependent vasodilation (Figure 3-1). Specifically, perivascular adipose attenuated arterial relaxation to both bradykinin in the concentration range from 1 nM to 320 nM ($P < 0.001$, Figure 3-1B), and acetylcholine in the concentration range from 32 nM to 100nM ($P < 0.001$, Figure 3-1A). Adipose tissue also increased the EC_{50} of bradykinin from 4.0 ± 1.2 nM to 14.7 ± 2.4 nM ($P < 0.01$), and of acetylcholine from 35.4 ± 10.5 nM to 89.7 ± 9.3 nM ($P < 0.05$). Perivascular adipose tissue had no effect on the maximal vasodilatory response to bradykinin (98 ± 2%) or acetylcholine (88.9 ± 3%). Additional experiments with clean arterial rings in the presence of the NO synthase inhibitor L-NAME (300 μM) demonstrated that pharmacologic inhibition of NO production was similar to the effect of perivascular adipose tissue alone. In particular, there was a modest difference between control arteries treated with L-NAME and arteries with perivascular adipose tissue in response to acetylcholine ($P < 0.05$, Figure 3-1A), while no difference was observed in response to bradykinin ($P = 0.81$, Figure 3-1B).

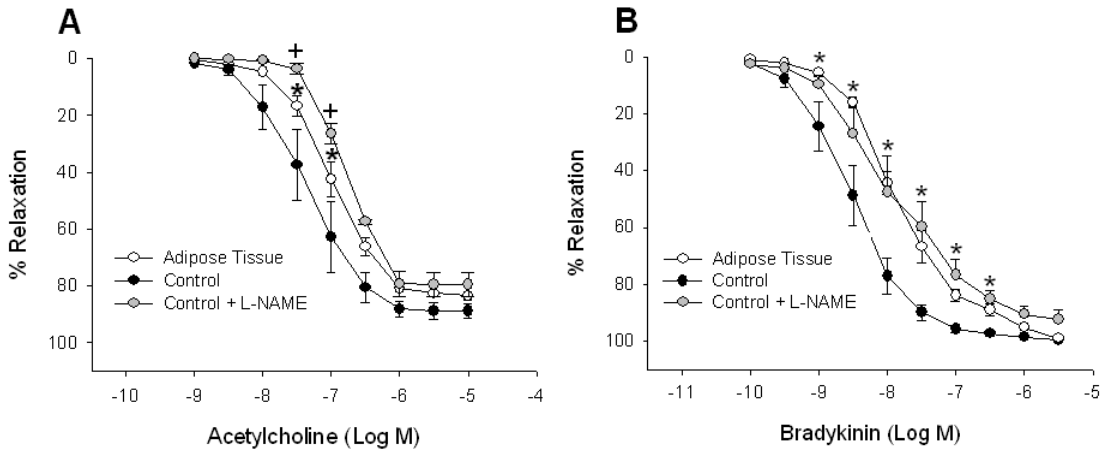

Figure 3-1 *A. Perivascular adipose tissue caused significant impairment of acetylcholine-mediated vasodilation in isolated coronary arteries (32 – 100 nM acetylcholine, n = 4). B. Likewise, perivascular adipose tissue also significantly impaired endothelial response to bradykinin (1 – 320 nM bradykinin, n = 5). Importantly, arteries with perivascular adipose tissue responded similarly to control arteries treated with the NO synthase inhibitor L-NAME (A and B). * P < 0.01 for adipose tissue vs. control.* $^{+}$ *P < 0.05 for adipose tissue vs. control +L-NAME.*

Protein Kinase C and adipose-induced impairment of coronary endothelial function. To test the hypothesis that PKC-β mediates perivascular adipose-induced endothelial impairment, we conducted additional isometric tension studies with different inhibitors of PKC. Administration of the general PKC inhibitor Ro-31-8220 (1 μM) or the PKC-β specific inhibitor ruboxistaurin (1 μM) eliminated the effect of perivascular adipose on bradykinin-induced coronary vasodilation (Figure 3-2; *P* = 0.49 vs. *P* = 0.86, respectively). Both Ro-31-8220 and ruboxistaurin had no effect on the vasodilation of control arteries without adipose tissue as the EC_{50} values were not statistically different (Ro-31-8220 EC_{50} = 2.5 ± 1.3 nM; ruboxistaurin EC_{50} = 2.7 ± 1.0 nM). Pretreatment with Ro-31-8220 and ruboxistaurin significantly enhanced endothelial-dependent vasodilation in arteries with perivascular adipose tissue, and maintained EC_{50} values at control levels (Ro-31-8220 EC_{50} = 2.4 ± 1.0 nM; ruboxistaurin EC_{50} = 2.2 ± 0.5 nM; *P* < 0.001 vs. untreated).

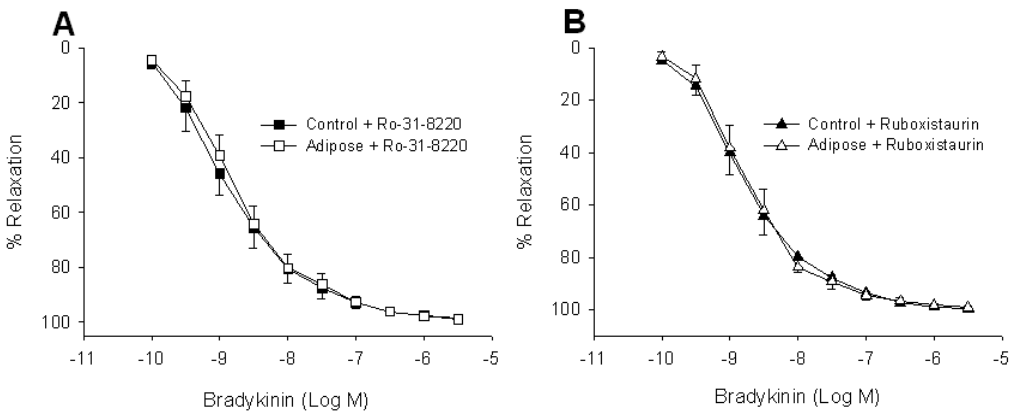

Figure 3-2 *A. Administration of the general PKC inhibitor Ro 31-8220 eliminated the difference between arteries with and without perivascular adipose (P = 0.49; n = 7). B. Similarly, the PKC-β specific inhibitor ruboxistaurin eliminated differences between both groups (P = 0.86; n = 6).*

Perivascular adipose and phosphorylation of eNOS. Compared to untreated-control arterial segments, incubation of coronary arteries with perivascular adipose tissue significantly increased the degree of site-specific eNOS phosphorylation at the inhibitory Thr^{495} residue (~140 kDal; Figure 3-3; $P < 0.01$). This increase in eNOS phosphorylation was prevented by pretreatment of arteries with ruboxistaurin ($P = 0.23$). No differences in expression of total eNOS or β–actin were noted between treatments (Figure 3-3; $P = 0.30$ and $P = 0.41$ respectively). In order to assess relative changes in the phosphorylation state of eNOS the ratio of phosphorylated eNOS-Thr^{495} to total eNOS (P-eNOS/eNOS) was calculated. We found that the P-eNOS/eNOS ratio was significantly elevated for arteries treated with perivascular adipose tissue (Figure 3-3; $P < 0.01$). This specific increase in the phosphorylation state of eNOS was prevented by pretreatment of arteries with ruboxistaurin.

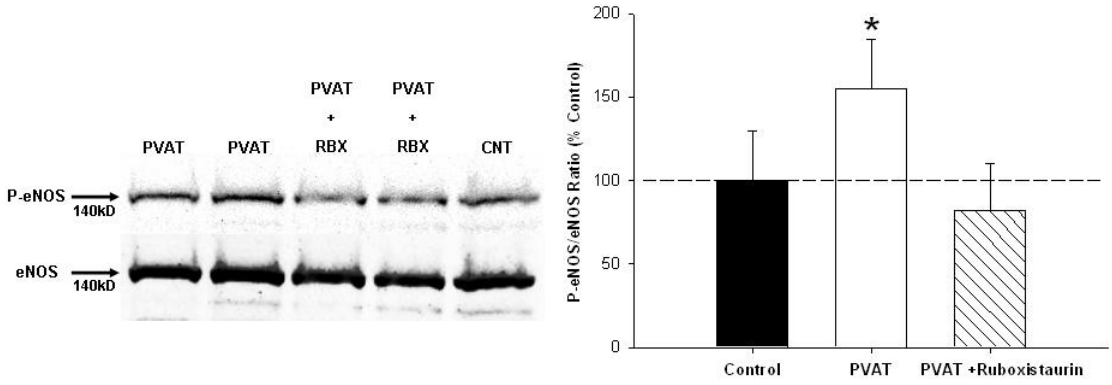

Figure 3-3 *Representative western blot staining for phosphorylated eNOS^{Thr495} (P-eNOS^{Thr495} and total eNOS ~140 kD) in coronary arteries. Arteries were either untreated (Control), treated with perivascular adipose (PVAT) or treated with both ruboxistaurin and perivascular adipose tissue (PVAT +Ruboxistaurin). Arteries treated with only perivascular adipose displayed increased fluorescence for P-eNOS^{Thr495} and had a significantly greater ratio of P-eNOS^{Thr495} to total eNOS (n=3). * P < 0.01.*

Protein kinase C, perivascular adipose and NO production. Additional experiments were also conducted to determine if inhibition of PKC-β improves coronary endothelial NO production in response to bradykinin in the presence of perivascular adipose tissue. Previously, we documented that perivascular adipose-derived factors significantly diminish both baseline and bradykinin-stimulated increases in coronary endothelial NO production ($P < 0.05$)[165]. In the present study, we found that baseline NO concentration averaged 292 ± 65 nM in untreated control arteries. Under control conditions, administration of bradykinin increased NO production by 193.3 ± 38 nM (Figure 3-4). Pretreatment of arteries with ruboxistaurin did not significantly affect either baseline or bradykinin-stimulated NO production. In contrast, the addition of perivascular adipose tissue dramatically decreased NO production, and bradykinin failed to significantly increase coronary NO production. Finally, pretreatment of arteries with ruboxistaurin prevented this effect of perivascular adipose tissue, as the increase in coronary NO production was similar to control conditions.

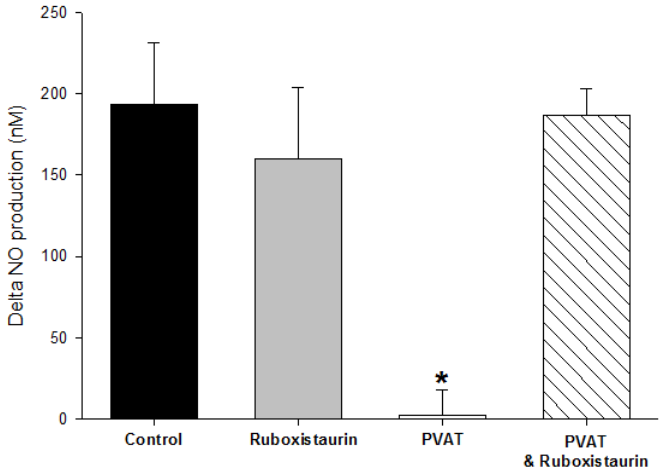

Figure 3-4 *Endothelial-derived NO was measured with a NO-sensitive microelectrode. The change in NO production (delta NO) in response to bradykinin (400 nM) is illustrated for each experimental condition. Pretreatment with ruboxistaurin protected endothelial function and maintained bradykinin-stimulated NO production in the presence of perivascular adipose tissue (n = 3). * P < 0.01 compared to all other conditions.*

Discussion

The present investigation was designed to elucidate the specific cellular/molecular mechanism by which perivascular adipose tissue impairs coronary endothelial function. We hypothesized that PKC, a known negative regulator of eNOS, was a key mediator of adipose-induced endothelial dysfunction. The major new findings of this study include: 1) Perivascular adipose-derived factors impair both bradykinin and acetylcholine-mediated vasodilation and NO production through direct inhibition of eNOS activity (Figure 3-1); 2) perivascular-adipose induced impairment of endothelium-dependent vasodilation is similar to pharmacologic inhibition of NO synthase (Figure 3-1); 3) selective inhibition of PKC-β prevents perivascular-adipose induced endothelial dysfunction in isolated coronary arteries (Figures 3-2 and 3-4); 4) factors released from perivascular adipose tissue increase site-specific phosphorylation of eNOS at the inhibitory Thr^{495} residue (Figure 3-3); 5) PKC-β inhibition significantly decreases the level of eNOS phosphorylation at Thr^{495} and 6) blockade of PKC-β prevents decreases in coronary endothelial NO production induced by perivascular adipose tissue (Figure 3-3).

Taken together, these results indicate that perivascular adipose tissue releases factor(s) that selectively impair endothelial-dependent dilation via a PKC-β dependent phosphorylation of eNOS at Thr[495].

In order to address the growing prevalence of cardiovascular disease, recent investigations have attempted to better understand both the natural and pathophysiologic relationship between perivascular adipose tissue and vascular function. Initial investigations suggested that perivascular adipose significantly attenuated the contractile responses of rat aorta[124-126, 145], rat mesenteric[127, 128], and human internal thoracic arteries[129] through the release of an adipose-derived relaxing factor (ADRF). While these initial findings suggest a role for local adipose in regulating arterial tone, it is hard to ignore the mounting evidence that perivascular adipose disrupts normal endothelial function and potentially contributes to atherogenesis[47, 118, 163, 164]. Previous studies have documented that coronary perivascular adipose tissue increases with obesity [132-134], and could serve as a visceral link between inflammatory adipokines released during obesity and cardiovascular disease[44, 49, 174]. In particular, recent work from our laboratory was the first to document that factor(s) released by coronary perivascular adipose tissue rapidly inhibit NO production and concurrently attenuate coronary endothelial-dependent vasodilation[165]. Importantly, we found that perivascular adipose tissue had no effect on coronary smooth muscle response to NO, superoxide (O_2^-) production or hydrogen peroxide (H_2O_2) mediated vasodilation.

The current findings confirm these previous observations (Figures 3-1 and 3-4), and demonstrate that perivascular adipose tissue has a generalized impairment of eNOS activity that is not limited to specific endothelium-dependent vasodilators (i.e. impairment of both bradykinin of acetylcholine). These observations implicate perivascular adipose-derived factors as a local regulator of endothelial function. More

specifically, local adipose-derived factors appear to “restrain” the normal endothelium derived NO produced by the underlying coronary vessels of lean animals (Figures 3-1 and 3-4). While our observation that naturally occurring adipose tissue impairs coronary endothelial function may seem troublesome, several key points must be considered. First, the effect of perivascular adipose tissue is unlikely to be systemic throughout the coronary circulation as only the conduit, non-resistance vessels have significant perivascular adipose. Second, the present findings demonstrate that impaired NO production is the primary cause of attenuated endothelial response (Figure 3-1A and B). Decreased NO production from these conduit, non-resistance arteries would minimally impair vascular regulation as blockade of NO synthesis alone produces little change in coronary blood flow[175-177]. Therefore, the present findings represent only a modest change in reactivity of the coronary vascular bed. However, these findings importantly suggest that perivascular adipose tissue could be a potential contributor to endothelial dysfunction and atherogenesis. However, future investigations are needed to more thoroughly examine this hypothesis in the setting of obesity.

Results from the present investigation indicate that perivascular adipose-induced impairment of coronary endothelial NO production is mediated via a PKC-β dependent pathway (Figures 3-2 through 3-4). Earlier studies have documented a potential relationship among obesity, PKC-β and endothelial dysfunction[136-139]. In particular, Tinsley *et. al.* found that interleukin-1beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α) can independently increase PKC activation, gap formation, and hyperpermeability across an endothelial monolayer[136]. Likewise, results from the Bohlen laboratory demonstrated that inhibition of PKC-β substantially reversed endothelial dysfunction in Zucker obese rats[137], while recent clinical trials have documented improved peripheral macrovascular endothelial function in type-II diabetic patients

treated with ruboxistaurin[139]. The present investigation extends these previous findings by implicating visceral coronary perivascular adipose tissue as an anatomic link between increased PKC activity and endothelial impairment. Present findings therefore connect a proposed mediator of cardiovascular disease with a local adipose depot, further suggesting that perivascular adipose-derived factors may contribute to the development of vascular dysfunction and disease.

Our data also indicate that perivascular adipose tissue impairs coronary endothelial NO production via site specific phosphorylation of eNOS by PKC-β at the key inhibitory residue Thr^{495}. This finding agrees with previous investigations documenting that phosphorylation of eNOS at Thr^{495} by PKC decreases NO production and disrupts protein-protein interaction between eNOS and calmodulin[168, 178, 179]. Hence, perivascular adipose-derived factors increase the basal phosphorylation state of eNOS-Thr^{495}, thereby impairing coronary endothelial increases in NO production. However, the current findings extend these previous studies by implicating a direct role for the β isoform of PKC in eNOS phosphorylation. It is important to note that eNOS is regulated by a very complex network of kinases (protein kinase A, B and C), phosphatases (Protein phosphatases 1 and 2), co-factors (i.e. tetrahydrobiopterin, flavin mononucleotide and nicotinamide adenine dinucleotide phosphate), protein-protein interactions and sub-cellular localization[166]. In general, many of these eNOS regulators have been shown to influence eNOS activity during disease states. In particular, phosphorylation of the Serine 1177 (Ser^{1177}) residue is thought to be among the most critical regulatory sites for eNOS activation[166]. Furthermore, disruption of Ser^{1177} residue[180] and altered availability of tetrahydrobiopterin[181-183] have both been shown to increase eNOS production of O_2^-. These observations are in direct contrast to our recent data which found no involvement of O_2^- in perivascular adipose-induced endothelial dysfunction[165]. Although still uncertain,

recent data also supports a role for Ser[116] as an inhibitory site of eNOS (when phosphorylated)[184]. Hence, alternative mechanisms for perivascular adipose-induced endothelial dysfunction exist. However, it is important to recognize that the PKC-β inhibitor ruboxistaurin prevented adipose-induced decreases in endothelial-dependent dilation and NO production (Figures 3-2 and 3-4), as well as increases in eNOS-Thr[495] phosphorylation (Figure 3-3). Therefore, our present findings argue against a significant role for these alternative pathways.

To date, no investigation has successfully identified the perivascular adipose-derived factor(s) that impair coronary endothelial function. However, the known association between PKC-β and endothelial dysfunction suggests that the active agent(s) released from perivascular adipose tissue are likely harmful inflammatory adipokines that are known to be elevated during obesity. Importantly, results from Tinsley *et al.* suggest a potential role for interleukins and TNF-α as perivascular adipose-derived factors mediating endothelial dysfunction[136]. These data are consistent with our recent findings which documented that perivascular adipose tissue expresses appreciable concentrations of resistin, leptin, IL-1β and TNF-α[165]. Importantly, each of these factors has been shown to independently produce endothelial dysfunction[36, 43, 57, 136]. In addition, other investigations have detected the presence of leptin, TNF-α and other adipokines in coronary perivascular adipose tissue[47, 165]. The present results importantly implicate a common PKC-β dependent pathway by which perivascular adipose-derived mediators induce coronary endothelial dysfunction. In addition, our findings support the "outside to inside" [40, 185, 186] signaling paradigm for perivascular adipose-derived factors in the pathogenesis of coronary vascular disease as well as PKC-β as a therapeutic target to improve endothelial function. Although our data establish a mechanistic link between perivascular adipose tissue and diminished

coronary endothelial NO production, additional studies are needed to examine the effects of perivascular adipose-derived adipokines in the setting of obesity and the MetS.

Acknowledgments

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Chapter 4

Epicardial perivascular adipose-derived leptin exacerbates coronary endothelial dysfunction in metabolic syndrome via a PKC-β dependent pathway

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Abstract

Factors released by perivascular adipose tissue (PVAT) have been documented to disrupt coronary endothelial function via phosphorylation of eNOS by PKC-β. However, our understanding of how PVAT contributes to coronary vascular disease as a complication of obesity/metabolic syndrome (MetS) remains limited. The current study investigated the mechanism by which adipose-derived factors impair coronary vascular function in MetS. Coronary arteries with and without PVAT were collected from lean or MetS Ossabaw miniature swine for isometric tension studies. Endothelial-dependent vasodilation to bradykinin was significantly reduced in MetS. PVAT did not affect bradykinin-mediated dilation in arteries from lean swine, but significantly exacerbated endothelial dysfunction in arteries from MetS swine. This PVAT-induced impairment was reversed by inhibition of either PKC-β with ruboxistaurin or leptin receptor signaling with a recombinant, pegylated leptin antagonist. Immunohistochemistry and western analyses revealed a marked increase in PVAT leptin expression in MetS swine. Additional studies found that coronary endothelial dysfunction induced by leptin alone was reversed by ruboxistaurin. Together, these findings indicate that increases in PVAT leptin expression exacerbate coronary endothelial dysfunction in MetS via a PKC-β dependent pathway. Our findings importantly implicate perivascular adipose-derived leptin as a pro-atherogenic mediator of coronary disease in MetS.

KEYWORDS**:** endothelial dysfunction, epicardial adipose tissue, perivascular adipose tissue, leptin, PKC-β, obesity

Introduction

The epidemic of obesity remains a daunting challenge for healthcare in the United States. Increased adiposity is associated with the metabolic syndrome (MetS); a known constellation of cardiovascular risk factors including insulin resistance, impaired glucose tolerance, hypertension, and dyslipidemia[4, 8]. Furthermore, as each component of the MetS is an independent risk factor for cardiovascular disease, it is not surprising that MetS exacerbates many cardiovascular diseases including stroke, coronary artery disease, and myocardial infarction[5, 6, 8]. Importantly, adipose tissue is now widely accepted as an active endocrine and paracrine organ. As a signaling organ, the production of adipose-derived cytokines (adipokines) has been well documented to influence many physiologic and pathophysiologic conditions[107]. Specifically, adipokine production has been shown to influence key pathogenic mediators of atherogenesis[107] including chemotaxis[141], inflammation[44], endothelial function[48] and smooth muscle proliferation[144]. Although adipokines have been proposed to be the molecular link between obesity and cardiovascular disease[107], the exact relationship between adipose tissue and vascular disease remains uncertain.

Recent studies have implicated adipose tissue that normally surrounds large coronary arteries, i.e. perivascular adipose tissue (PVAT), as a local source of adipokines that contribute to both vascular dysfunction and atherosclerotic disease[47, 116, 118, 163, 164]. This contention is supported by data indicating that coronary atherosclerotic plaques primarily occur in the larger epicardial arteries encased by PVAT[164] as well as other findings illustrating a positive association between epicardial PVAT volume and the severity of coronary artery disease[116, 187]. In particular, a recent investigation from Greif *et al.* documented that PVAT volume, hypoadiponectinemia, and inflammation represent the strongest risk factors for the presence of coronary atherosclerosis[116]. While

intriguing, evidence thus far is largely correlative, and no investigation has established a direct, causal link between PVAT and coronary artery disease.

Other investigations have recently documented that epicardial PVAT from patients with the MetS express significantly higher levels of harmful adipokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and leptin[47]. This increase in PVAT adipokine expression is consistent with the marked degree of endothelial dysfunction[71, 79] and atherosclerotic disease[188, 189] typically observed in the setting of the MetS. In particular, hyperleptinemia initiates several deleterious vascular events including proinflammatory cytokine production[190-192] and neointimal growth[193]. In particular, our laboratory has previously demonstrated that hyperleptinemia significantly impairs endothelial-dependent relaxation both *in vivo* and *in vitro*[36]. In spite of this, hyperleptinemia in the setting of obesity displays only a modest association with coronary heart disease[114]. These findings suggest that our understanding of MetS and adipokine pathology remains limited, and that additional mechanisms are likely responsible.

Our laboratory has demonstrated that factors released by PVAT in normal lean animals significantly impair coronary endothelial-dependent vasodilation and nitric oxide (NO) production via direct inhibition of NO synthase, both *in vitro* and *in vivo*[165]. Additional studies have shown that PVAT-induced endothelial impairment occurs via protein kinase C (PKC)-β dependent phosphorylation of endothelial NO synthase (eNOS) at the inhibitory amino acid residue Thr[495] [194]. These findings are important because they establish a mechanistic link between local cardiac PVAT and coronary vascular function. However, the degree to which alterations in PVAT adipokine expression in MetS affects coronary vascular dysfunction/disease (i.e. endothelial dysfunction[101]) has yet to be examined.

The purpose of the present investigation was to test the hypothesis that augmented PVAT-derived leptin exacerbates underlying coronary endothelial dysfunction in the MetS through a PKC-β dependent pathway. This hypothesis was tested in Ossabaw miniature swine fed either a normal maintenance diet (11% kcal from fat) or an excess calorie atherogenic diet (45% kcal from fat, 2% cholesterol, 20% kcal from fructose) that induces classic clinical features of the MetS[171, 195, 196]. Finally, vascular reactivity was assessed in isolated coronary arteries with or without the normally surrounding PVAT.

Methods

Swine model of metabolic syndrome. This investigation was approved by the Institutional Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996). Lean swine were fed ~2200 kcal/day of standard chow (5L80, Purina TestDiet, Richmond, IN) containing 18% kcal from protein, 71% kcal from complex carbohydrates, and 11% kcal from fat. MetS swine were fed an excess ~8000 kcal/day high fat/fructose, atherogenic diet containing 17% kcal from protein, 20% kcal from complex carbohydrates, 20% kcal from fructose, and 43% kcal from fat (lard and hydrogenated soybean and coconut oils), and supplemented with 2.0% cholesterol and 0.7% sodium cholate by weight (5B4L, Purina TestDiet, Richmond, IN). Prior to sacrifice, blood was drawn for glucose, insulin, and lipid assays [171, 195, 196].

Functional assessment of isolated epicardial coronary rings. Isolated coronary artery studies were performed for both experimental groups as previously described[43, 146, 165, 194]. Briefly, left anterior descending (LAD) coronary arteries were dissected with or without the naturally surrounding perivascular adipose tissue (approximately 0.25 - 0.50g adipose per ring). Arteries and perivascular adipose tissue were always taken from the

same animal and collected at the same time. Care was taken to isolate the same 2 - 3 cm proximal portion of the circumflex artery that is naturally surrounded by perivascular adipose tissue. Arteries were cut into 3 mm rings and mounted in organ baths for isometric tension studies (Figure 4-1). Optimal length was found by assessing contraction to 60 mM KCl, and the arteries were pre-contracted with the thromboxane A_2 mimetic U46619 (1μM). Vascular function was assessed by the addition of graded concentrations of the bradykinin (0.1 nM/L - 10 μM/L, n = 7 lean; n = 9 MetS) or sodium nitroprusside (1.0 nM – 0.1 mM, n = 4 lean; n = 6 MetS) to the tissue bath. In additional studies with arteries from MetS swine, bradykinin concentration responses were conducted in the presence of the PKC-β specific inhibitor ruboxistaurin (1 μM, n = 6) or a recombinant, pegylated leptin receptor antagonist (1 μM, n = 4, Protein Laboratories Rehovot) All results obtained during dose response experiments are reported as the percent relaxation for arterial rings from individual animals (Figures 4-2, 4-3 and 4-5). 100 percent relaxation was defined as the resulting tension following the administration of nitroglycerin (20 μM).

Immunohistochemical analysis. Segments of isolated coronary arteries with the surrounding PVAT were collected, immediately fixed by immersion in 10% formalin, and processed in paraffin for histological analysis according to manufacturer's specifications (Zymed Laboratories, Inc.). Briefly, paraffin-embedded tissue sections were initially rehydrated. Prior to blocking, antigen retrieval was performed in a citrate buffer (0.1 M). Tissue sections were then incubated overnight at 4°C with polyclonal antibodies directed against leptin (1:100; Abcam). Sections were then rinsed and incubated with an anti-rabbit biotinylated antibody followed by a tertiary streptavidin peroxidase conjugate (Zymed Laboratories, Inc.). Tissue sections were developed with a five minute exposure to 3-amino-9-ethyl-carbazole (AEC) and counter stained with hemotoxylin.

Representative photomicrographs were obtained using standard light microscopy (Nikon Spot camera system).

Western blot analysis. Western blotting was performed as previously described (n = 4)[146, 171, 194]. Tissues were isolated and immediately placed in liquid N_2 and stored at -80°C. PVAT was homogenized, centrifuged, and the resulting supernatants were collected for analysis. Equivalent amounts of protein were loaded onto 15% acrylamide gels for electrophoresis and blotting. After blocking for 1 h at ambient temperature with 5% nonfat milk, membranes were incubated overnight at 4°C with the same leptin polyclonal antibody that was used for immunohistochemical analysis (1:1000; Abcam). Blots were washed and incubated with donkey anti-rabbit IgG-HRP secondary antibody (1:4000; Santa Cruz Biotechnology) for 1 h at ambient temperature. The same blots were stripped and reblotted with β-actin antiserum (1:3000; Santa Cruz Biotechnology) as the internal control to confirm equal protein loading. Immunoreactivity was visualized using an ECL western blotting detection kit (GE Healthcare) and quantified by scanning densitometry (Bio-Rad Quantity One 1-D Analysis Software).

Statistical analyses. Data are presented as mean ± standard error. For isometric tension studies, a two-way ANOVA was used to test the effects of the perivascular adipose (Factor A) and various drugs (Factor B) on coronary dilator responsiveness. A t-test was used to compare half maximal effective concentration (EC_{50}) values (Sigma Stat 3.0 Software). All experiments were analyzed per animal. When statistical differences were found with ANOVA a Student-Newman-Keuls multiple comparison test was performed. The criterion for statistical significance was $P < 0.05$ in all tests.

Results

Representative phenotype of lean and MetS Ossabaw miniature swine. Phenotypic characteristics of lean and MetS swine are listed in Table 4-1. Compared to their lean counterparts, MetS swine exhibited a 49% increase in body weight (kg), 90% increase in fasting glucose, 662% increase in total cholesterol, and 140% increase in triglyceride levels. Figure 4-1 illustrates typical isolated arteries collected from lean (A) and MetS (B) swine. Note visible coronary atherosclerotic lesions typically observed in MetS swine (Figure 4-1B).

Table 4-1 Phenotypic characteristics of lean and metabolic syndrome Ossabaw swine

Phenotype	Lean	MetS
Body Weight (kg)	**45 ± 4**	**67 ± 3***
Fasting glucose (mg/dl)	**68 ± 4**	**129 ± 4***
Total cholesterol (mg/dl)	**66 ± 4**	**503 ± 41***
Triglycerides (mg/dl)	**20 ± 4**	**48 ± 5***

Values are mean ± SE for lean (n = 6) and MetS (n = 6) swine. * P < 0.05 vs. Lean.

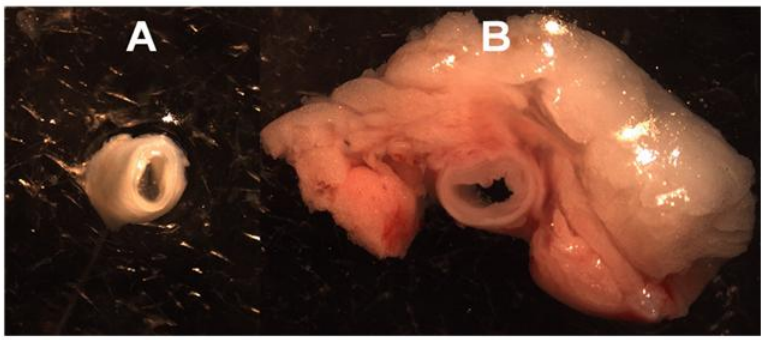

Figure 4-1 ***Representative coronary arteries from lean and MetS swine.*** *Arteries were cut into 3 mm rings with and without PVAT and mounted in organ baths for in vitro isometric tension studies. Arteries were brought to optimal length and pre-contracted with the thromboxane A2 mimetic U46619. Note visible neointimal formation in arteries from MetS swine (B).*

Effects of PVAT on coronary vascular function. To examine the effects of endogenous perivascular adipose-derived factors on coronary vascular function, isometric tension studies were conducted in isolated coronary arteries with and without PVAT in lean and MetS swine. In contrast to our previous investigations in canines [165, 194], lean PVAT had no significant effect on endothelial-dependent vasodilation to bradykinin (Figure 4-2A). Furthermore, endothelial-independent vasodilation in response to sodium nitroprusside was also unchanged by PVAT (Figure 4-2B).

On the contrary, the onset of MetS alone caused significant endothelial dysfunction that was markedly exacerbated by the presence of PVAT (Figure 4-2A and C). Specifically, the EC_{50} value of bradykinin in MetS coronary arteries without PVAT was 9.6 ± 4.3 nM (compared to 1.3 ± 0.5 nM for lean, $P < 0.05$). Perivascular adipose-derived factors clearly exacerbated underlying endothelial dysfunction as the EC_{50} value increased ~10-fold from 9.6 ± 4.3 nM to 92.3 ± 32.8 nM ($P < 0.05$), and bradykinin-mediated vasodilation was attenuated in the concentration range of 1 nM to 10 μM ($P < 0.001$). Importantly, the maximal dilator response to bradykinin (10 μM) was reduced in the presence of MetS PVAT from 87 ± 7% to 59± 6% relaxation ($P < 0.001$). Likewise, perivascular adipose-derived factors caused a modest attenuation of endothelial-independent relaxation to sodium nitroprusside in the concentration range of 100 to 320 nM ($P < 0.01$). The maximal response and EC_{50}, however, were not significantly altered by MetS PVAT.

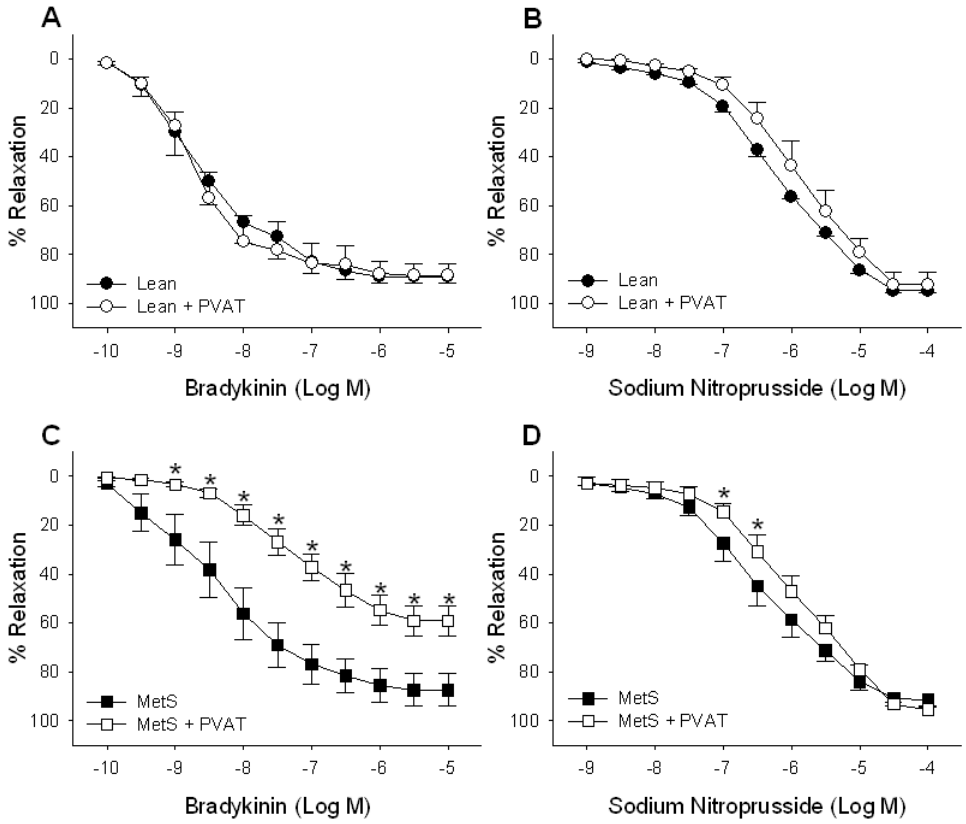

Figure 4-2 ***PVAT markedly impairs coronary endothelial-dependent vasodilation in MetS swine.*** *PVAT failed to attenuate bradykinin or sodium nitroprusside-induced vasodilation in arteries from lean-control animals (A and B). In contrast, arteries from MetS swine displayed significant endothelial dysfunction that was markedly exacerbated by PVAT (C). PVAT modestly reduced endothelial-independent vasodilation in MetS swine (D).*

Protein Kinase C-β and PVAT-induced coronary endothelial dysfunction. To test the hypothesis that PKC-β mediates perivascular adipose-induced endothelial impairment in MetS swine, we conducted additional isometric tension studies in the presence of the PKC-β specific inhibitor ruboxistaurin (1 μM). Consistent with our recent data[194], administration of ruboxistaurin significantly improved endothelial function in MetS swine (Figure 4-3, $P < 0.001$). Specifically, inhibition of PKC-β improved the EC_{50} value in response to bradykinin to 22.1± 11.9 nM ($P < 0.05$ vs. MetS with PVAT), and increased maximal vasodilatory response by ~ 23.5 ± 6.3% ($P < 0.05$).

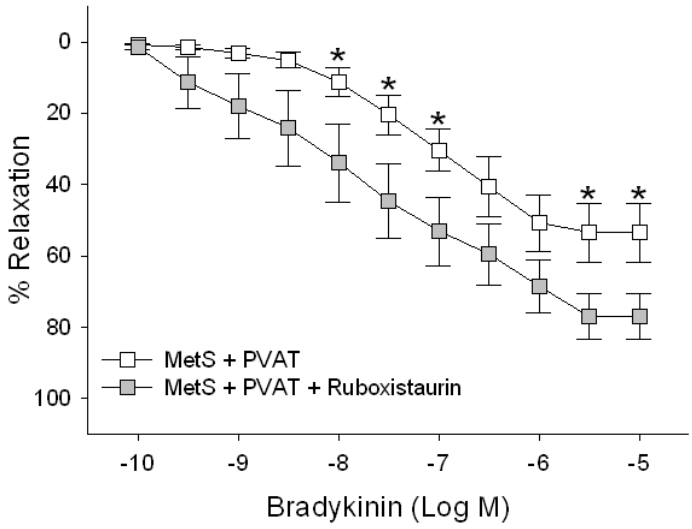

Figure 4-3 ***Inhibition of PKC-β improves endothelial function in MetS swine.*** *Administration of the PKC-β specific inhibitor ruboxistaurin (1 μM) significantly improved endothelial-dependent dilation to bradykinin. This observation supports our previous findings, and further suggests that PVAT-derived factors signal through a PKC-β dependent pathway.*

Leptin and leptin receptor expression within metabolic syndrome PVAT. To begin identifying potential mediating agents of PVAT-induced endothelial impairment, immunohistochemical experiments were performed on cross sections of coronary arteries with PVAT from both lean and MetS swine. Immunohistochemical analysis of lean arteries displayed no evidence of leptin expression in PVAT (data not shown). In contrast, positive leptin staining was detected in the surrounding PVAT layer (Figure 4-4A and B) of coronary arteries from MetS swine. Additional tissue sections were prepared to test for the presence endothelial leptin receptors. Importantly, leptin receptor staining was strongly detected along the endothelium of coronary vessels from MetS swine (Figure 4-4C). This observation is consistent with previous findings from our laboratory demonstrating that the long-form leptin receptor is expressed in canine coronary arterioles[36]. Quantitative western blot studies with the same antibody further confirmed the expression of leptin within MetS PVAT. In particular, MetS significantly

increased leptin protein expression in comparison to lean (Figure 4-5, $P < 0.05$), while β-actin expression was unchanged (Data not shown, $P = 0.6$).

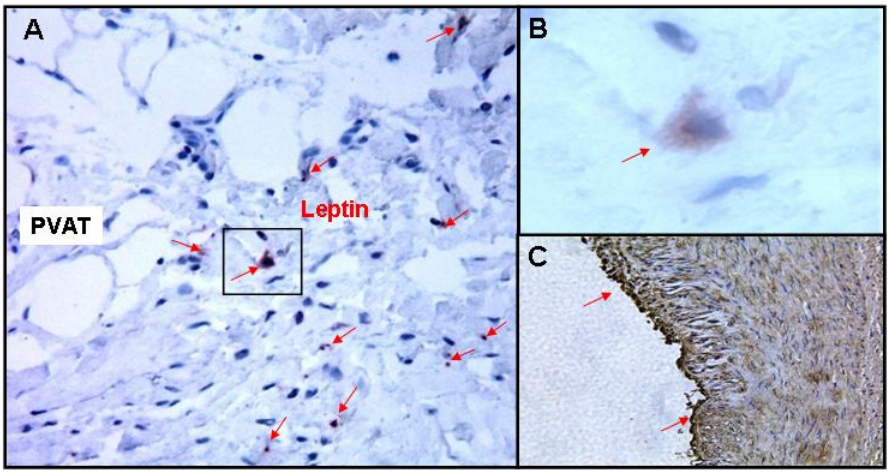

Figure 4-4 ***Leptin expression in the coronary PVAT of MetS Swine.*** *Immunohistochemistry of coronary artery cross sections displayed no evidence of leptin staining in lean swine (data not shown). In contrast, leptin staining was evident in PVAT from MetS swine (A). Magnified images further display leptin staining within adipocytes (B, 100X magnification). Positive leptin receptor staining was also detected along the vascular endothelium and neointima (C). Positive staining is denoted by red arrows.*

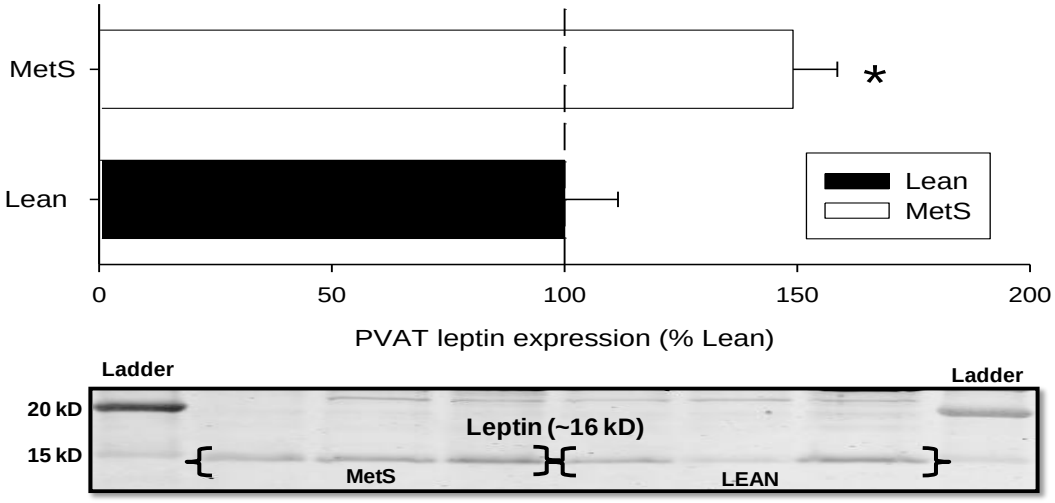

Figure 4-5 ***Leptin expression is significantly increased in the PVAT of MetS Swine.*** *Quantitative western blotting analysis was used to validate previous immunohistochemistry images. In particular, PVAT-derived leptin was detected in both lean and MetS Swine; however, MetS expression was significantly increased.*

***PVAT-derived leptin and coronary endothelial function*.** To test the hypothesis that local adipose-derived leptin mediates PVAT-induced endothelial impairment in MetS swine, we conducted isometric tension studies in the presence of a recombinant, pegylated leptin receptor antagonist (200 ng/ml). Administration of this leptin signaling antagonist improved endothelial response of MetS swine to bradykinin concentrations ranging from 10 nM to 10 μM (Figure 4-6A, $P < 0.001$). Furthermore, inhibition of leptin signaling significantly improved maximal response to bradykinin ($P < 0.001$), and tended to improve the EC_{50} response from 153 ± 60 nM to 29 ± 14 nM ($P < 0.09$).

Additional "proof of principle" studies were also conducted to determine the effects of exogenous leptin alone (30 ng/ml) on coronary arterial rings from lean swine without PVAT (Figure 4-6B). Consistent with previous studies[36, 93], acute administration of leptin attenuated endothelial-dependent dilation to bradykinin (0.32 to 10nM bradykinin, $P < 0.001$), and shifted EC_{50} response from 1.3 ± .5 to 5.2 ± 1.8 nM ($P < 0.05$). Co-administration of leptin and ruboxistaurin (1 μM) eliminated the leptin-induced endothelial impairment, and restored coronary vascular response to bradykinin.

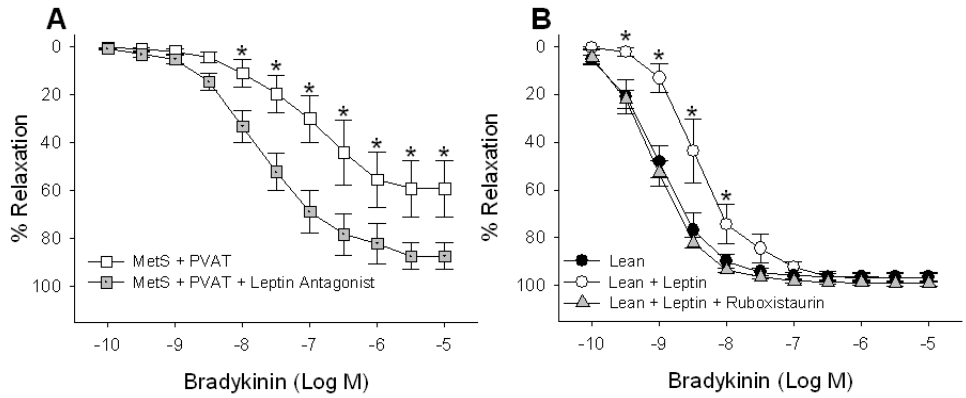

Figure 4-6 *PVAT-derived leptin exacerbates endothelial dysfunction in MetS swine through PKC-β.* *Inhibition of leptin receptor signaling with a recombinant, pegylated leptin antagonist (200 ng/ml) fully reversed the effect of PVAT in MetS swine (A). Furthermore, acute administration of leptin (30 ng/ml) caused significant endothelial impairment in response to bradykinin, which was reversed by the inhibition of PKC-β (B).*

Discussion

Recent investigations have implicated PVAT in the development of coronary atherosclerosis[47, 116, 118, 163-165, 194]. In order to describe a direct link between PVAT and coronary artery disease, the present investigation was designed to elucidate the mechanism by which perivascular adipose-derived factors diminish coronary endothelial function in the setting of MetS. In addition, this investigation sought to identify key perivascular adipose-derived agents likely contributing to endothelial impairment. We hypothesized that during MetS, perivascular adipose-derived leptin markedly exacerbates underlying endothelial dysfunction through a PKC-β dependent phosphorylation of eNOS. This purposed mechanism was based on previous findings from our laboratory demonstrating that perivascular adipose-derived factors from lean canines significantly diminish coronary NO production through PKC-β-dependent phosphorylation of NO synthase[165, 194].

The major new findings of this study include: 1) PVAT-derived factors from lean swine have no effect on coronary vascular function; 2) arteries from MetS swine display significant endothelial dysfunction that is markedly exacerbated by PVAT; 3) selective inhibition of PKC-β improves endothelial response in the presence of PVAT from MetS swine; 4) leptin is expressed in the surrounding perivascular adipose layer of MetS swine, while leptin receptor is expressed predominantly along the coronary vascular endothelium; 5) administration of a recombinant, pegylated leptin antagonist reverses the effect of PVAT from MetS swine; 6) acute administration of leptin causes significant endothelial impairment through a PKC-β dependent mechanism. Taken together, these results indicate that perivascular adipose-derived leptin markedly exacerbates coronary endothelial dysfunction in MetS via a PKC-β dependent mechanism.

Results from the present study are important because they are among the first to identify leptin as a causative, perivascular adipose-derived agent that impairs coronary endothelial-dependent dilation. Endothelial dysfunction has long been suggested to be an initiating event in the development of atherosclerosis[101, 104]; hence, current results provide direct evidence that surrounding PVAT contributes to atherogenesis in MetS. Present findings agree with other investigations documenting an attenuating effect of PVAT[165, 194], and are the first to document that PVAT from MetS swine causes a significantly greater impairment of coronary endothelial function. Taken together, these initial results underscore the need to consider the local production and paracrine release of harmful adipokines from PVAT as a critical link between obesity and coronary disease. Hence, the potential diagnostic and therapeutic benefit of monitoring the growth and adipokine expression pattern of PVAT should be further investigated for clinical use.

Above all, findings from this investigation illustrate the potentially critical contribution of leptin to the initiation of coronary atherogenesis. Both immunohistochemistry and western blot analysis clearly demonstrate that leptin production is elevated in the setting of MetS (Figures 4-4 and 4-5). Numerous investigations have documented that hyperleptinemia initiates several deleterious vascular events including proinflammatory cytokine production[190-192], and neointimal growth[193]. In particular, our laboratory has previously demonstrated that hyperleptinemia significantly impairs endothelial-dependent relaxation both *in vivo* and *in vitro*[36]. In addition, recent studies have demonstrated that leptin is a potent chemoattractant for monocytes and macrophages[197]. With these previous studies in mind, the current results further confirm the deleterious activity of leptin. Furthermore, the observation that a leptin antagonist can almost entirely mitigate the effect of PVAT illustrates the physiologic importance of perivascular adipose-derived leptin in mediating endothelial

dysfunction. These observations highlight the need to consider local anatomy as a critical factor in the pathogenesis of coronary artery disease, and suggest that leptin may function as a key mediator of “endothelial injury” and initiator of inflammation within the surrounding PVAT.

Aside from anatomic proximity, results depicted in Figures 4-2 through 4-6 suggest a potential mechanism of leptin signaling through endothelial receptors to ultimately modulate the activity of eNOS via PKC-β. This assertion is based on previous results from our laboratory demonstrating that perivascular adipose-derived factors significantly diminish endothelial NO production by increasing phosphorylation of eNOS by PKC-β at the inhibitory Thr^{495} residue[194]. Similarly, the present finding that leptin receptors are located along the vascular endothelium agrees with prior investigations which demonstrated that the long-form, signaling leptin receptor is expressed by human endothelial cells[36]. Importantly, the effect of acutely administered leptin on lean coronary arteries was completely reversed by inhibition of PKC-β (Figure 4-6B). Hence, this investigation is among the first to suggest that leptin receptor signaling is potentially coupled with PKC activation activity[198, 199]. Together, this investigation strongly suggests that leptin impairs vascular reactivity through PKC-β in a primarily endothelial-dependent (likely eNOS specific) fashion.

One concerning observation about the current findings was that perivascular adipose tissue from lean swine had no effect on endothelial-dependent relaxation. This is in direct contrast to previous investigations from our laboratory demonstrating the perivascular adipose tissue from lean, healthy mongrel canines significantly diminished endothelial production of NO[165, 194]. One likely explanation is differences in adipokine expression between canines and swine. It is also plausible that leptin is not the mediating agent responsible for perivascular adipose-induced endothelial impairment in

canines. Specifically, adipokines measured from lean, canine perivascular adipose tissue displayed significantly high levels of resistin[165]. In addition to phenotypic variation between species, an additional explanation is that normal (lean) levels of leptin are significantly higher in canines than Ossabaw miniature swine. In particular, there is approximately a fourfold difference in plasma leptin concentrations for lean canines (~6 ng/ml)[93] in comparison to lean Ossabaw swine (~1.5 ng/ml)[195]. In the end, the current use of a strong model of MetS provides more clinically relevant findings, and further promotes the present identification of leptin as a critical perivascular adipose-derived agent.

Interpretation of the present investigation, however, is limited by our understanding of how factors derived from outer adipose tissue effectively communicate with endothelial cells within the vascular lumen. Even with growing evidence suggesting that mediators originating outside of the coronary vasculature are capable of affecting vascular homeostasis, it remains unclear how these factors penetrate into the circulation. Importantly, recent investigations have documented that increased coronary vasa vasorum neovascularization could provide prompt delivery of perivascular-derived factors to the medial and intimal layers of the arterial wall[120, 121, 200]. Specifically, vasa vasorum neovascularization has been demonstrated to occur within the first weeks of experimental hypercholesterolemia and precede overt coronary endothelial dysfunction[120]. In context of the current findings, future investigations need to further clarify the importance of coronary vasa vasorum for transporting leptin and other potential adipokines from perivascular adipose tissue to the arterial wall.

The present results implicate leptin as a perivascular adipose-derived factor that significantly exacerbates coronary endothelial dysfunction in MetS. This attenuated vasodilatory response appears to function via a common PKC-β dependent pathway.

Together, this investigation highlights the potential therapeutic benefit of targeting PKC-β and epicardial adipose-derived leptin. Above all, this investigation supports the "outside to inside" [40, 185, 186] signaling paradigm for perivascular adipose-derived factors, and is the first to propose a direct, causative mechanism. Additional studies are needed to examine the physiologic contribution of coronary vasa vasorum to perivascular adipose-induced endothelial dysfunction and disease.

Acknowledgments

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Chapter 5

Discussion

The epidemic of obesity continues to intensify in developed nations throughout the world. As the prevalence of obesity and MetS increases, healthcare systems will undoubtedly be challenged to manage the expected increase of obesity-associated cardiovascular disease[2]. While research throughout the past decades has influenced many to acknowledge obesity as an inflammatory and pathologic condition, the exact mechanisms linking increased adipose and cardiovascular disease remain poorly understood.

There is mounting evidence that local visceral epicardial adipose tissue contributes to obesity-associated coronary disease. Specifically, perivascular adipose tissue surrounding the major conduit coronary arteries has been linked with unfavorable cardiometabolic complications[115-118]. Importantly, recent clinical findings suggest that perivascular adipose volume is by far the strongest predictor of coronary atherosclerosis[116]. These findings are novel because they propose an alternative, paracrine pathway in the development of vascular disease. However, no investigation has established a direct causal link between perivascular adipose tissue and coronary artery disease.

In an effort to address these developing theories, the central focus of this work was to investigate the potential role of coronary perivascular adipose tissue in the development of coronary endothelial dysfunction and atherosclerosis. Our goal was to determine the cellular/molecular mechanisms by which perivascular adipose tissue regulated vascular reactivity in health, and in the setting of the MetS. These objectives were addressed by the following Specific Aims:

1. **Test the hypothesis that perivascular adipose tissue significantly impairs normal coronary vascular reactivity.** Initial studies in normal, lean canines demonstrated that perivascular adipose-derived factors selectively impair coronary endothelial-dependent vasodilation via direct inhibition of NO synthase both *in vitro* and *in vivo*. This effect was independent of alterations in coronary vascular smooth muscle responsiveness to NO, O_2^- mediated decreases in NO-bioavailability, and/or altered H_2O_2-mediated vasodilation[165].
2. **Delineate the mechanisms by which perivascular adipose tissue impairs coronary endothelial-dependent vasodilation.** Additional experiments further documented that perivascular adipose-derived factors inhibit eNOS-mediated NO production in response to both bradykinin and acetylcholine. Importantly, these studies discovered that perivascular adipose-derived factors impair coronary endothelial NO production via a PKC-β dependent, site-specific phosphorylation of eNOS at Thr^{495}. Notably, administration of the PKC-β inhibitor ruboxistaurin protected endothelial function and maintained bradykinin-stimulated NO production in the presence of perivascular adipose tissue[194].
3. **Identify the specific perivascular adipose-derived factor(s) that contribute to coronary vascular dysfunction in the metabolic syndrome.** Concluding experiments established that perivascular adipose tissue in the setting of MetS considerably attenuates coronary endothelial function. Above all, these studies suggest that perivascular adipose-derived leptin markedly exacerbates underlying coronary endothelial dysfunction in MetS via a PKC-β dependent mechanism. Figure 5-1 summarizes these important findings.

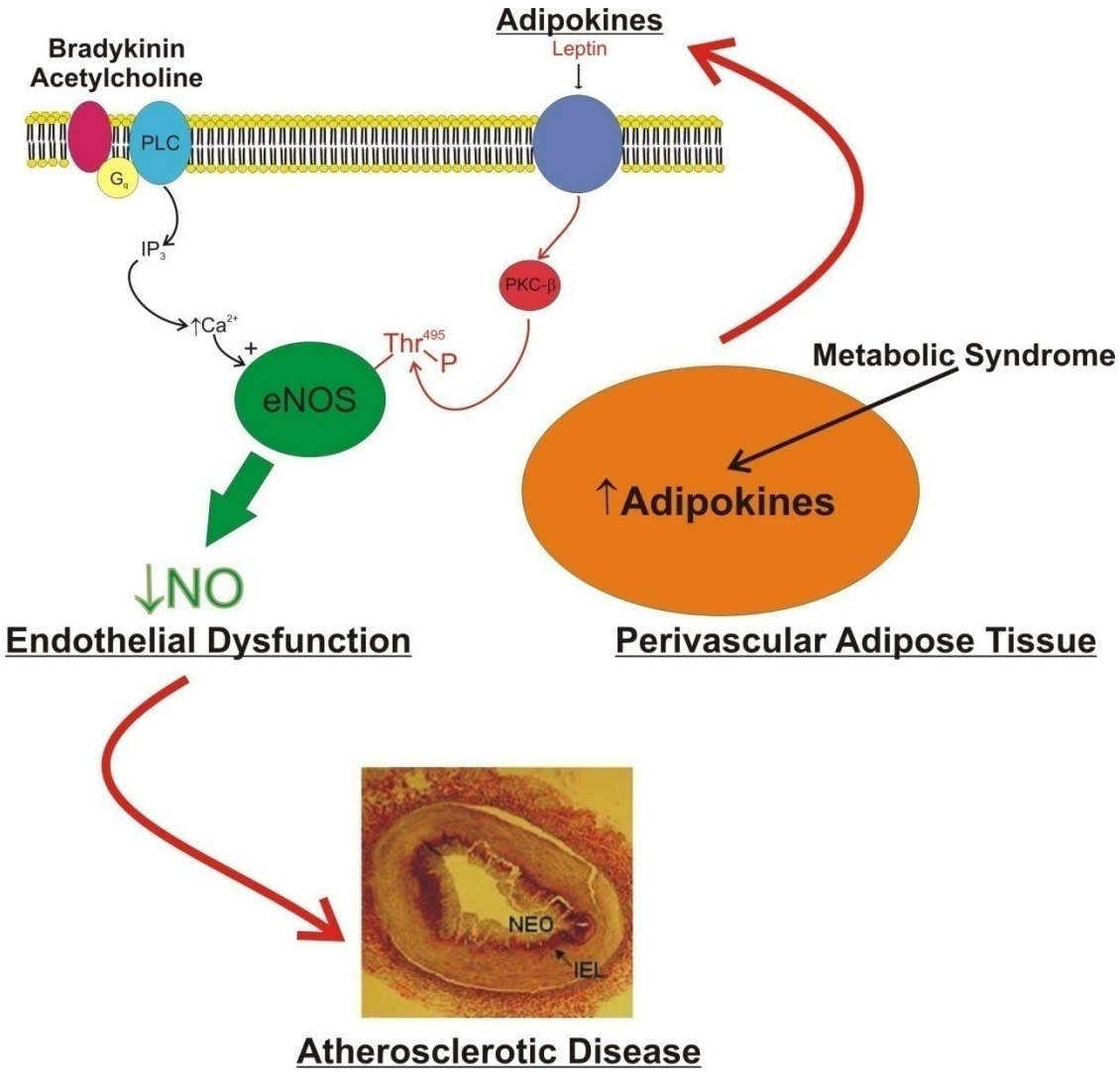

Figure 5-1 ***Proposed mechanism of how perivascular adipose contributes to coronary vascular disease.*** *Results from these investigations suggest that perivascular adipose-derived leptin (and potentially other inflammatory mediators and adipokines) directly impairs coronary endothelial function through a PKC-β dependent mechanism. This perivascular adipose-induced endothelial impairment may be the precipitating event in the development of coronary atherosclerosis.*

Implications

Findings from these investigations challenge many currently held theories and assumptions regarding coronary circulation and the pathogenesis of vascular disease. In particular, results from lean canines and MetS swine suggest that perivascular adipose tissue influences both normal and diseased coronary vascular function. These observations are compelling, and suggest that several issues central to cardiovascular physiology and disease must be reconsidered.

Contribution of Perivascular Adipose Tissue to Normal Coronary Circulation

Results from this work imply that perivascular adipose tissue is a significant regulator of normal, healthy coronary endothelial function. Hence, independent of any changes caused by MetS, perivascular adipose tissue appears to naturally restrain normal endothelial function of the underlying coronary vessel. While these observations may at first glance appear troublesome, several key points must be considered. First, the effect of perivascular adipose tissue appears to be limited to the relatively small portion of the vascular tree covered in adipose tissue. Any observed endothelial impairment is therefore not systemic throughout the entire vascular network.

Second, the present findings demonstrate that impaired NO production is the primary cause of the attenuated endothelial response (Figure 5-2). Results demonstrating that the effect of canine perivascular adipose tissue resembles pharmacologic inhibition of eNOS with L-NAME strongly suggest that other vasodilator pathways (i.e. cyclooxygenase and/or endothelium derived hyperpolarizing factors) are minimally altered. Notably, decreased NO production from these large, conduit arteries would modestly impair coronary blood flow regulation, and therefore represents a nominal change in the reactivity of the coronary vascular bed. This assertion is based on

numerous studies demonstrating the blockade of NO synthesis does not attenuate exercise-induced coronary vasodilation[175-177, 201]. Hence, under normal, lean conditions perivascular adipose tissue may minimally impair coronary function and blood supply to the heart.

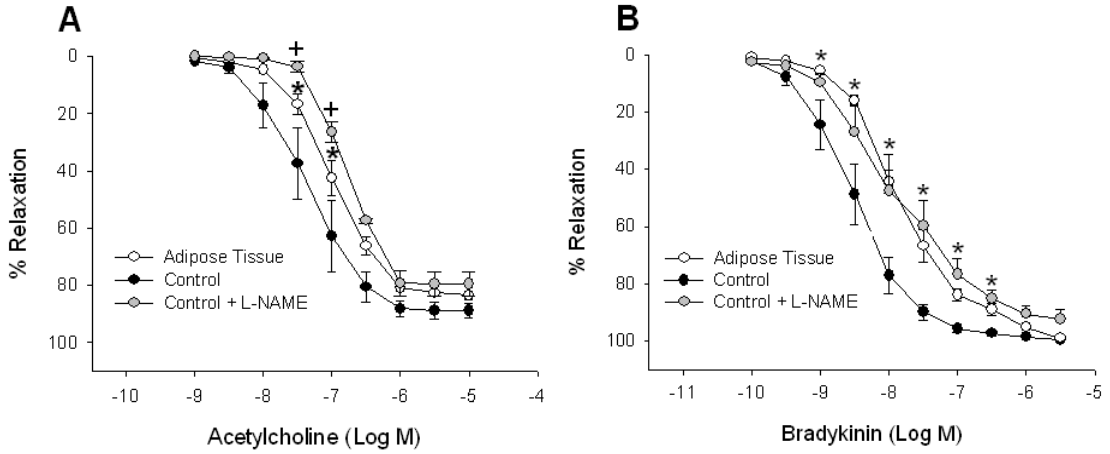

Figure 5-2 ***Perivascular adipose restrains normal endothelial function.*** *Present results suggest that normal, lean perivascular adipose tissue naturally restrains endothelial function within the underlying coronary vessel. This impairment appears to be predominantly due to attenuated NO production. Importantly, while endothelial response is significantly decreased, arteries are still capable of fully relaxing to endothelial-dependent vasodilators.*

With these implications in mind, few investigations have addressed whether vascular reactivity changes along the coronary artery with differing amounts of perivascular adipose tissue. However, our hypothesis would suggest that increasing the amount of perivascular adipose could result in a “dose-dependent” impairment of eNOS activity and vasodilation. This implication is important, as clinical studies suggest that perivascular adipose tissue is increased with obesity[132-134]. In addition, both the amount of perivascular adipose tissue and the arterial wall thickness decreases as conduit coronary arteries travel away from the ostia. As multiple agents potentially diffuse from perivascular adipose tissue, the more distal portions of the arteries may be more readily affected by surrounding adipose.

Together, the present results imply that coronary arteries covered in adipose may inadvertently be more susceptible for coronary dysfunction and atherosclerosis. This contention is supported by correlative studies reporting that the presence of a myocardial bridge suppresses atherosclerotic development within the underlying vascular intima[135]. These observations imply that naturally occurring perivascular adipose tissue is a significant regulator vascular reactivity. While the present studies focused solely on coronary function, our observations imply that other vascular networks commonly surrounded by adipose (i.e. renal or mesenteric arteries) may also be significantly affected by local perivascular adipose tissue.

Contribution of Perivascular Adipose Tissue to Coronary Artery Disease

In contrast to studies in lean animals, the final experiments directly address the issue of perivascular adipose tissue in the setting of MetS. Concluding experiments document that obese perivascular adipose tissue markedly exacerbates underlying coronary endothelial dysfunction. Given the numerous clinical observations correlating perivascular adipose tissue with coronary artery disease, these findings provide direct evidence of a paracrine link between epicardial adipose and vascular disease. Above all, the current data support our hypothesis that perivascular adipose-derived leptin directly mediates endothelial dysfunction through a PKC-β dependent mechanism.

These studies are the first to demonstrate that perivascular adipose-derived factors impair coronary endothelial function through a PKC-β dependent phosphorylation of eNOS. Importantly, experimental evidence clearly documents a specific biochemical pathway that when preemptively blocked, can effectively protect the endothelium from impairment (Figure 5-3A). While these initial experiments did not directly address perivascular adipose tissue in the context of obesity, subsequent work demonstrated

that PKC-β inhibition markedly improves endothelial response in MetS swine with perivascular adipose tissue (Figure 5-3B). Importantly, these observations connect epicardial adipose with a known mediator of obesity-associated endothelial dysfunction[137].

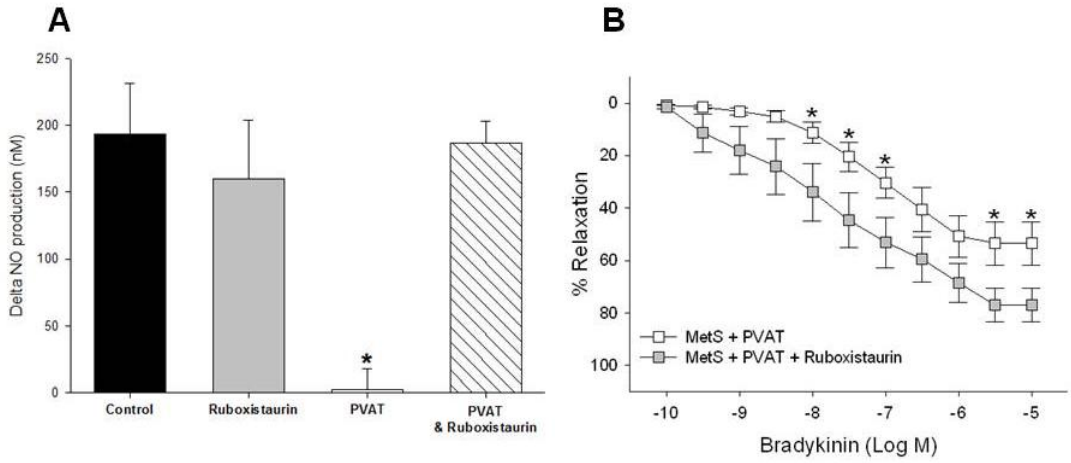

Figure 5-3 ***Blockade of PKC-β is beneficial to coronary endothelial function in the presence of perivascular adipose tissue.*** *In the presence of perivascular adipose tissue, the vascular endothelium is unable to produce NO in response to bradykinin. Pretreatment with the PKC-β inhibitor ruboxistaurin effectively protects the endothelium, and enables endothelial cells to significantly increase NO production in response eNOS activation (A)[194]. In the setting of MetS, administration of ruboxistaurin significantly improves endothelial response in the presence of perivascular adipose tissue (B).*

Subsequent experiments further suggest that perivascular adipose-derived leptin signals through endothelial leptin receptors to increase PKC-β activity. This assertion is consistent with previous investigations from our laboratory illustrating that the long-form (OB-Rb) leptin receptor is expressed by human coronary endothelial cells[36]. More importantly, the proposed involvement of PKC in the endothelial leptin signaling cascade is an interesting experimental correlation. While no investigation has to date clearly illustrated a specific mechanism linking leptin signaling with PKC, several investigations have observed similar effects. Specifically, leptin has been observed to regulate human platelet aggregation[202], insulin receptor signaling[199, 203], and colonic mucin production[199, 204] through PKC-dependent pathways. Interestingly, leptin has been shown to alter

intracellular calcium mobilization in response to the growth factor lysophosphatidic acid (LPA)[199, 205]. This specific effect was mediated by increased PKC activity coupled to the Box 3 motif of the OB-Rb through phosphatidylinositol 3-kinase (PI3K). Taking these findings into consideration, the present results imply that endothelial leptin receptors are capable of activating PKC at least in part through the PI3K signaling cascade (Figure 5-4).

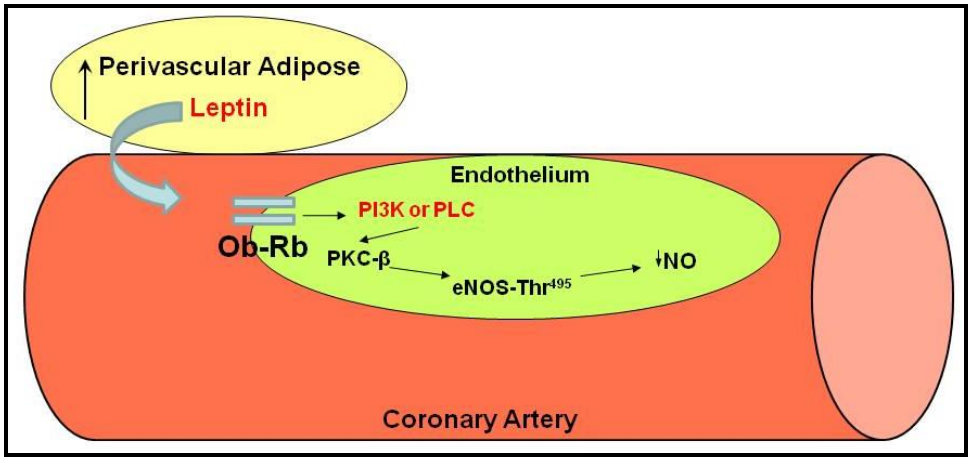

Figure 5-4 ***Potential signaling cascade for leptin-induced endothelial impairment.*** *Several investigations document that leptin signals in part through PKC dependent pathways. In particular, recent evidence suggests that leptin receptor (OB-Rb) activation results in phosphatidylinositol 3-kinase (PI3K) recruitment and PKC activation.*

Lastly, in light of the metabolic and inflammatory nature of perivascular adipose tissue[36, 41-45], current findings also underscore the influence adipokines may have on systemic cardiovascular function and vascular disease. Interestingly, leptin appears to be uniquely positioned as a critical mediator of endothelial dysfunction and arterial disease. Previous studies have demonstrated that leptin is a potent chemoattractant for circulating monocytes[197]. Consequently, while present studies clearly demonstrate that leptin directly impairs endothelial function, leptin may also promote the initial infiltration of inflammatory cells into the surrounding tissue.

This “dual” effect of leptin may render the endothelium susceptible to injury as well as promote the production of additional inflammatory cytokines. Notably, it is well documented that leptin is mainly produced and secreted by adipocytes in direct proportion to adipose mass[46, 199]. Hence, as the perivascular adipose mass increases with obesity[132-134], leptin may function as a “feed forward” initiator of vascular dysfunction leading ultimately to disease (Figure 5-5). This hypothesis is supported by experiments demonstrating that leptin has 4 to 5-fold increase in gene expression at the onset of an atherogenic diet and before the development of an advanced atherosclerotic lesion[206]. Moreover, MCP-1 (and potentially other chemokines) stimulates the further secretion of leptin[44].

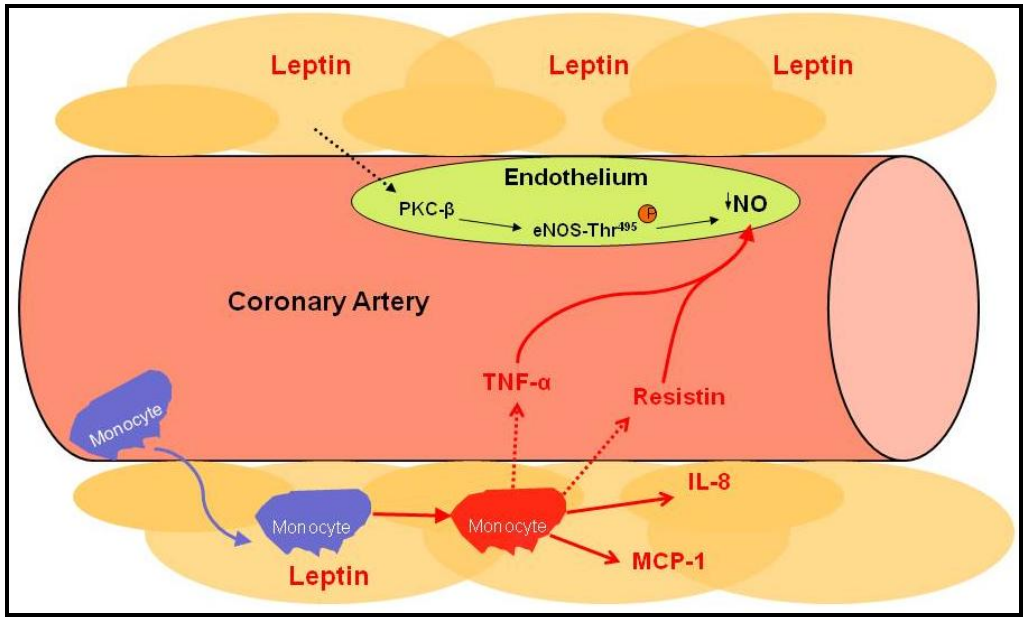

Figure 5-5 ***Proposed “feed forward” disease mechanism of perivascular adipose-derived leptin.*** *Results from this work suggest that perivascular adipose-derived leptin exacerbates coronary endothelial dysfunction through PKC-β. Previous studies have also shown leptin to be a potent monocyte chemoattractant[197]. Hence, as perivascular adipose mass increases with obesity a feed forward disease process involving increased leptin production begins. Therefore, leptin directly impairs endothelial function and initiates a local inflammatory response that may further damage the coronary artery.*

Future Directions & Proposed Studies

Findings from this body of work are numerous; however, several key issues need to be addressed in future studies. In particular, several questions surround the signaling cascade of leptin, the functional contribution of coronary vasa vasorum, and the link between leptin and adiponectin activity. Together, these proposed issues would help to advance our understanding of how perivascular adipose-derived adipokines contribute to coronary artery disease.

Leptin Signal Transduction

Future studies need to further characterize the link between leptin and eNOS activity. While findings from this work strongly implicate leptin to be a critical paracrine regulator of endothelial dysfunction, additional molecular studies with leptin and eNOS would provide further experimental evidence. Specifically, western blot "proof of principle" experiments similar to those illustrated in Chapter 3[194] would help demonstrate that leptin administration increases site-specific phosphorylation of eNOS at Thr^{495}. In addition, NO measurement studies in the presence of exogenous leptin and/or ruboxistaurin could further demonstrate the attenuating influence of leptin on NO production. Results from these experiments could unequivocally link endothelial leptin signaling with PKC-β mediated decreases in eNOS activity.

Subsequent experiments also need to elucidate the signaling cascade linking leptin and endothelial PKC activity. Unfortunately, the complexity of leptin signaling will make elucidating an unambiguous mechanism between leptin and PKC difficult. Particularly, leptin is associated with the JAK (Janus kinases)/STAT (signal transducers and activators of transcription), PI3K, and MAPK (mitogen-activated protein kinase) signaling cascades[199]. While leptin signaling remains poorly understood, repeating some *in vitro* isometric tension studies with lean Ossabaw swine arteries in the presence of

various inhibitors could quickly address the functional contribution of several kinases to the leptin signaling cascade. In particular, previous results suggest that inhibition of PI3K or phospholipase C (PLC) may effectively block leptin-induced endothelial dysfunction (Figure 5-4)[199, 205].

Previous investigations have also documented that leptin is functionally similar to monocyte chemoattractant-1 (MCP-1)[197]. A study from Gruen *et al* observed that leptin receptors (OB-Rb) present on macrophages increase cellular motility to the same degree as MCP-1. These findings support our proposal that leptin is a "feed forward" initiator of vascular dysfunction (Figure 5-5). Future immunohistochemistry studies could provide experimental evidence of leptin functioning as a perivascular chemoattractant. Notably, experiments desgined to co-localize perivascular adipose leptin with macrophage infiltration may provide correlative evidence of leptin-induced monocyte infiltration.

Lastly, several investigations have described adiponectin as an anti-inflammatory adipokine that is commonly decreased in the setting of obesity[42]. Importantly, a recent clinical study documented that perivascular adipose volume and hypoadipnectinemia are the strongest indicators of coronary atherosclerosis[116]. Several other investigators have suggested that adiponectin may attenuate the effect of leptin and other inflammatory adipokines [41, 42, 44, 46, 58].

In consideration of these clinical findings, future experiments should investigate if hypoadiponectinemia directly contributes to the disease causing activity of hyperleptinemia. A study from Mohler *et al* illustrates that although adiponectin gene expression doubles during the first month of an atherogenic diet, leptin expression quadruples and is maintained for three to four months[206]. One potential experimental design includes using genetically engineered rodent models to overexpress adiponectin

and/or leptin. In addition, lentiviral vector overexpression of adiponectin in the perivascular adipose tissue of MetS swine offers a unique experimental approach that could show functional benefit in a large animal model of cardiovascular disease (Figure 5-6).

Vasa Vasorum: "Is there really a direct link"

The most noticeable uncertainty regarding our proposed mechanism is discerning whether perivascular adipose-derived factors are capable of traveling to the vascular wall and affecting the endothelium. While experimental evidence has demonstrated that increased coronary vasa vasorum neovascularization precedes overt coronary endothelial dysfunction and disease[120], no study has clearly demonstrated if vasa vasorum contribute to disease. Future studies should focus on developing experimental evidence that perivascular adipose-derived leptin contributes directly to disease. As Figure 5-6 illustrates, experimental techniques exist that could selectively over-express leptin in the coronary epicardial adipose tissue. This use of a lentiviral vector containing leptin would be an interesting "proof of principle" experimental approach to address this critical concern.

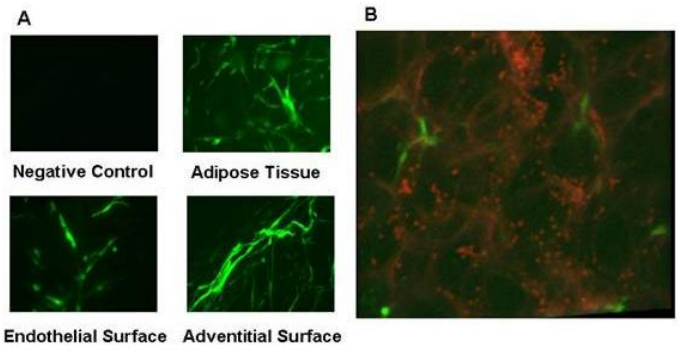

Figure 5-6 ***Lentiviral transduction incubation of Ossabaw coronary arteries.*** *Representative picture demonstrating in vitro lentiviral GFP (green) transduction of perivascular adipose tissue and left circumflex coronary artery (images of endothelial and adventitial surface, A). Confocal image of GFP expression in perivascular adipose tissue following in vitro lentiviral injection.*

In addition to leptin overexpression, future investigations could also attempt characterize a relationship between perivascular adipose growth, increased adipokine production, and increased vasa vasorum density. Specifically, it is important to address whether a particular cytokine and/or cellular event triggers the angiogenic response of vasa vasorum. Experiments monitoring the growth of perivascular adipose tissue and development of vasa vasorum density at various time points would permit investigators to correlate adipokine expression with various stages of disease.

Notably, hypoxia and/or vascular endothelial growth factor (VEGF) have both been associated with adipose growth[207]. Futhermore, VEGF and leptin genes are both hypoxia inducible, but potential links between VEGF and leptin gene expression have not been examined[207]. Together, vascular hypoxia, VEGF, and leptin may serve as an important link between perivascular adipose-induced dysfunction and vasa vasorum trafficking.

Identification of other perivascular adipose-derived agents

Finally, it is unlikely that leptin is solely responsible for perivascular adipose-induced endothelial dysfunction. Therefore, proteomic analysis of perivascular adipose tissue or conditioned buffer would likely help identify other mediating agents derived from perivascular adipose tissue. Employment of a multiplexed biomarker assay and targeted proteomics could enable future investigations to identify both known and unknown proteins that have significantly elevated perivascular adipose expression. In order to find functional significance, perivascular adipose-conditioned buffers could be prepared, fractioned by molecular weight, and systematically tested for any affect on vascular reactivity. Leptin receptor deficient rodents could also represent a unique opportunity to test the effect of perivascular adipose independent of leptin activity.

Clinical Implications

Since obesity-associated coronary artery disease is a leading cause of death and morbidity, implications from these investigations stand to substantially increase the clinical importance of epicardial adipose tissue. Increased perivascular adipose tissue surrounding conduit coronary arteries could be a new and potentially powerful diagnostic tool for predicting coronary atherosclerosis[116]. Diagnostic imaging and quantification of perivascular adipose volume, therefore, should be considered as a diagnostic indicator for patients who are particularly at risk of myocardial infarction. In addition, our observations underscore the clinical value of measuring leptin and other inflammatory adipokines in order to develop a personalized "pro-atherosclerotic" profile for each at risk patient. These imaging techniques would be most applicable to the general at risk population, given the relative difficulty (and unwarranted harm) of directly measuring perivascular adipose expression. However, direct sampling and measurement of epicardial adipokine production following coronary artery bypass graft procedures potentially represents an effective determinant of when (and where) vascular disease may reoccur.

Findings from these investigations also suggest that preemptive blockade of PKC-β may help to ameliorate endothelial function and prevent early stages of vascular disease. With the incredibly rise of childhood obesity, therapies directed towards blocking the development of cardiovascular disease are more important than ever. Early therapeutic targeting of PKC-β may provide beneficial health outcomes, and decrease the severity/onset of vascular disease.

Lastly, perivascular adipose may also contribute to the pathogenesis of other obesity-associated cardiovascular diseases. Notably, hypertension has been associated with endothelial dysfunction[208]. The current findings suggest that local perivascular

adipose surrounding other vascular networks may contribute to endothelial dysfunction and potentially hypertension[40]. Given our observations and the proposed link between leptin and hypertension[209, 210], perivascular adipose tissue appears to be conveniently positioned to influence vascular resistance and local blood pressure regulation.

Concluding Remarks

Despite the growing obesity epidemic, the pathophysiologic mechanisms underlying the link between obesity and cardiovascular disease remain poorly understood. Obesity and MetS are associated with endothelial dysfunction, which is proposed to initiate coronary artery disease. The central goal of the previous studies was to demonstrate that local perivascular adipose tissue was a significant regulator of coronary vascular function and contributor to the development of coronary artery disease. Results from these investigations clearly illustrate that perivascular adipose-derived factors impair coronary endothelial function through direct inhibition of NO synthase. This impairment was mediated via a PKC-β dependent, site-specific phosphorylation of eNOS at Thr^{495}. Importantly, perivascular adipose-derived leptin markedly exacerbated endothelial dysfunction through a PKC-β dependent mechanism in the setting of MetS. These investigations are among the first to characterize a specific mechanism linking perivascular adipose with vascular disease. Above all, the present findings suggest that perivascular adipose tissue contributes to coronary disease, and highlight the potential diagnostic benefit of monitoring epicardial adipose growth and expression.

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Curriculum Vitae

Gregory Allen Payne

EDUCATION

Indiana Academy for Science, Mathematics, and Humanities

2001, Academic Honors Diploma

Yale University, New Haven, Connecticut

2005, Bachelor of Science, Chemistry

Indiana University, Indianapolis, Indiana

2010, Doctor of Philosophy, Cellular and Integrative Physiology

2011, Medical Degree, Medical Scientist Training Program

RESEARCH EXPERIENCE

2000 Molecular Genomics Laboratory, Research Assistant, Ball State University – Mentor: Caroline Vann, Ph.D. (2000 to 2001)

2002 Clinical Psychiatric Electrophysiology, Undergraduate Research Assistant, Yale University School of Medicine – Mentor: Nashaat Boutros, M.D. (2002)

2003 Medicinal Chemistry Laboratory, Undergraduate Student Research, Yale University Department of Chemistry – Mentor: Andrew Hamilton, Ph.D. (2003 to 2005)

2007 Coronary Physiology Laboratory, Graduate Student Research, Indiana University School of Medicine, Department of Cellular and Integrative Physiology – Doctoral Thesis – Mentor: Johnathan D. Tune, Ph.D. (2007 to 2010)

FUNDING AWARDS

2007 Indiana Medical Scientist Training Program Grant, Indiana University School of Medicine – Honored Recipient

2007 Edwin T. Harper Scholarship Award, Indiana University School of Medicine – Honored Recipient

2010 Ruth L. Kirschstein National Research Service Award for Individual Predoctoral Fellows (F31), Department of Health and Human Services, National Institutes of Health, NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

PEER REVIEWED PUBLICATIONS

1. Yin H, Lee GI, Park HS, **Payne GA**, Rodriguez JM, Sebti SM, Hamilton AD. Terphenyl-Based Helical Mimetics That Disrupt The p53/HDM2 interaction. *Angewandte Chemie Intl. Ed.* 2005 Apr 29; 44(18):2704 - 2707

2. **Payne GA**, Borbouse L, Bratz IN, Roell WC, Bohlen HG, Dick GM and Tune JD. Endogenous adipocyte-derived factors diminish coronary endothelial function via inhibition of nitric oxide synthase. *Microcirculation*. 2008 Jul;15(5):417-26

3. Dick GM, Bratz IN, Borbouse L, **Payne GA**, Dincer UD, Knudson JD, Rogers PA, Tune JD. Voltage-dependent K^+ channels regulate the duration of reactive hyperemia in the canine coronary circulation. *Am J Physiol Heart Circ Physiol*. 2008 May; 294(5):H2371-81

4. Knudson JD, **Payne GA**, Borbouse L, Tune JD. Leptin and mechanisms of endothelial dysfunction and cardiovascular disease. *Current Hypertension Reports*. 2008 Dec; 10(6):434-439. Review

5. **Payne GA**, Bohlen HG, Dincer UD, Borbouse L, Tune JD. Periadventitial adipose tissue impairs coronary endothelial function via PKC-β dependent phosphorylation of nitric oxide synthase. *Am J Physiol heart Circ Physiol.* 2009 Jul;297(1):H460-5

6. Borbouse L, Dick GM, Bender SB, Dincer UD, **Payne GA**, Neeb ZP, Bratz IN, Sturek M, Tune JD. Impaired functional expression of coronary BK_{Ca} channels in metabolic syndrome. *Am J Physiol Heart Circ Physiol*. 2009 Nov;297(5):H1629-37

7. Borbouse L, Dick GM, **Payne GA**, Payne BD, Svendsen MC, Neeb ZP, Alloosh M, Bratz IN, Sturek M, Tune JD. Contribution of BK_{Ca} channels to local metabolic coronary vasodilation: Effects of metabolic syndrome. *Am J Physiol Heart Circ Physiol.* 2010 Mar; 298(3):H966-73

8. Borbouse L, Dick GM, **Payne GA**, Berwick ZC, Neeb ZP, Alloosh M, Bratz IN, Sturek M, and Tune JD. Metabolic syndrome reduces the contribution of K^+ channels to ischemic coronary vasodilation. *Am J Physiol Heart Circ Physiol.* 2010 Apr;298(4):H1182-9

9. **Payne GA**, Borbouse L, Kumar S, Alloosh M, Sturek M, Tune JD. Epicardial perivascular adipose-derived leptin exacerbates endothelial dysfunction in the metabolic syndrome via a PKC-β dependent pathway. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2010 Sep;30(9):1711-7. Epub 2010 Jun 24

10. Berwick ZC, **Payne GA**, Lynch B, Dick GM, Sturek M, Tune JD. Contribution of adenosine A_{2A} and A_{2B} receptors to ischemic coronary dilation: Role of K_V and K_{ATP} channels. *Microcirculation (In Press)*

ABSTRACTS

1. **Payne GA.** DNA fingerprinting project: “UniverCity”. *Proceedings from the Indiana Academy of Science* 2000

2. **Payne GA,** Boutros N. Sensory Gating of Cocaine-Addicted Individuals. *Annual Biomedical Research Conference for Minority Students (ABRCMS)* 2002

3. **Payne GA,** Hamilton AD. Development of an Antagonist to Disrupt Bcl-x_L/Bak Complex Based on α-Helix Mimicry. *ABRCMS* 2003

4. **Payne GA**, Borbouse L, Dick GM, Tune JD. Endogenous adipocyte-derived factors diminish coronary endothelial-dependent vasodilation via inhibition of nitric oxide synthase. *Circulation* 2007

5. Borbouse L, **Payne GA**, Dick GM, Sturek M, Tune JD. Impaired contribution of voltage-dependent K^+ channels to ischemic coronary vasodilation in Ossabaw swine with metabolic syndrome. *FASEB J* 2008

6. Borbouse L., **Payne GA**, Dick GM, Alloosh M, M. Sturek, J. D. Tune. Role of large conductance Ca^{2+} - activated K^+ (BK_{Ca}) channels in loval metabolic coronary vasodilation in Ossabaw swine with metabolic syndrome. *FASEB J* 2008

7. **Payne GA**, Borbouse L, Dincer UD, Bohlen HG, Dick GM, and Tune JD. Perivascular adipose tissue impairs coronary endothelial function via protein kinase C-β dependent phosphorylation of nitric oxide synthase. *FASEB J* 2008

8. **Payne GA,** Borbouse L, Sturek M, and Tune JD. Perivascular adipose-derived leptin exacerbates coronary endothelial dysfunction in the metabolic syndrome via a PKC-β dependent pathway. *Keystone Symposium,* 2009

9. **Payne GA,** Borbouse L, Kumar S, Neeb Z, Alloosh M, Sturek M and Tune JD. Epicardial perivascular adipose tissue exacerbates coronary endothelial dysfunction in metabolic syndrome via leptin-induced activation of PKC-β. *FASEB J 2010*

INVITED ORAL PRESENTATIONS

2002 *Sensory gating in cocaine-addicted individuals.* Annual Biomedical Research Conference for Minority Students

2003 *Terphenyl helical mimetics that disrupt Bcl-x_L / Bak complex.* Annual Biomedical Research Conference for Minority Students – "Outstanding Oral Presentation Award"

2007 *Endogenous adipocyte-derived factors diminish coronary endothelial-dependent vasodilation via inhibition of nitric oxide synthase*. American Heart Association Scientific Sessions

2008 *Perivascular adipose tissue impairs coronary endothelial function via protein kinase C-β dependent phosphorylation of nitric oxide synthase.* Experimental Biology 2008

2008 *Perivascular adipose tissue impairs coronary endothelial function via protein kinase C-β dependent phosphorylation of nitric oxide synthase.* Medical Scientist Training Program Annual MD/PhD National Student Conference

2010 *Epicardial perivascular adipose tissue exacerbates coronary endothelial dysfunction in metabolic syndrome via leptin-induced activation of PKC-β.* Experimental Biology 2010

INVITED POSTER PRESENTATIONS

2006 *Perivascular adipose tissue alters coronary arterial smooth muscle and endothelial function.* Indiana Center for Vascular Biology and Medicine (ICVBM) Annual Retreat

2008 *Perivascular adipose tissue impairs coronary endothelial function via protein kinase C-β dependent phosphorylation of nitric oxide synthase.* Experimental Biology 2008

2009 *Perivascular adipose-derived leptin exacerbates coronary endothelial dysfunction in metabolic syndrome via a PKC-β dependent pathway*. Keystone Symposia: Complications of Diabetes and Obesity, Vancouver, British Columbia

2010 *Epicardial perivascular adipose tissue exacerbates coronary endothelial dysfunction in metabolic syndrome via leptin-induced activation of PKC-β.* Experimental Biology 2010

ACADEMIC HONORS AND AWARDS

2002 BioSTEP: Biomedical Science Training and Enrichment Program, Yale University School of Medicine – Student Participant

2003 BioSTEP: Biomedical Science Training and Enrichment Program, Yale University School of Medicine – Student Participant

2004 Science, Technology, And Research Scholar (S.T.A.R.S.) II, Yale University – Research Fellow

2005 Science, Technology, And Research Scholar (S.T.A.R.S.) II, Yale University – Research Fellow

2006 Student Summer Research Program in Academic Medicine, Indiana University School of Medicine – Research Participant

2006 Indiana Center for Vascular Biology & Medicine, Indiana University School of Medicine – Second Place Abstract Award

2007 Edwin T. Harper Scholarship Award, Indiana University School of Medicine – Honored Recipient

2008 Sigma Xi Research Competition, Indiana University School of Medicine – Honorable Mention

2008 Indiana Medical Scientist Training Program Grant, Indiana University School of Medicine – Honored Recipient

2008 Melvin Denis Memorial Travel Award for “Dedicated Passion to Science”, M.D. / Ph.D. National Conference, Keystone, Colorado – Annual Award Recipient

2008 Theodore J. B. Steir Fellowship for “Excellence in Research”, Indiana University School of Medicine, Department of Cellular and Integrative Physiology – Annual Award Recipient

2009 Theodore J. B. Steir Fellowship for “Excellence in Research”, Indiana University School of Medicine, Department of Cellular and Integrative Physiology – Annual Award Recipient

TEACHING EXPERIENCE

2005 Undergraduate Chemistry Tutor, Yale University

2006 Medical School Year I Curriculum Tutor, Student National Medical Association Local Chapter, Indianapolis, Indiana

2007 Medical and Graduate School Tutor for Human Physiology, Indiana University School of Medicine

2008 Graduate Course Lecturer for *Human Physiology* (F503), Indiana University School of Medicine Department of Cellular & Integrative Physiology (One Hour)

2009 Graduate Course Lecturer for *Animal Models of Human Disease* (G727), Indiana BioMedical Gateway Program Department of Cellular & Integrative Physiology (One Hour)

PROFESSIONAL ORGANIZATIONS

American Medical Association
American Physiological Society
Sigma Xi- The Scientific Research Society
Student National Medical Association

EXTRACURRICULAR ACTIVITIES

2005 IUSM Westside Family Health Fair – Student volunteer

2005-2007 IUSM Curriculum Council Component I and II – Student Representative

2005-2007 SNMA: “*Male Rights of Passage.”* – Mentor and tutor

2006-2008 IUSM Graduate and Medical School Physiology Tutor

2007-2008 IUSM Curriculum Council Steering Committee – Student Representative

2008-2009 Liaison Committee on Medical Education Student Review Coordinator

2008-2009 Howard Hughes Medical Institute Mentoring Program: Crispus Attucks High School, Indianapolis, IN