EFFECT OF CORONARY PERIVASCULAR ADIPOSE TISSUE ON VASCULAR SMOOTH MUSCLE FUNCTION IN METABOLIC SYNDROME

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DEDICATION

This thesis is dedicated to my parents who inspired me to achieve my goals, and to my husband Joe, for his steadfast love and support throughout my graduate education.

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ABSTRACT

Meredith Kohr Owen

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Obesity increases cardiovascular disease risk and is associated with factors of the “metabolic syndrome” (MetS), a disorder including hypertension, hypercholesterolemia and/or impaired glucose tolerance. Expanding adipose and subsequent inflammation is implicated in vascular dysfunction in MetS. Perivascular adipose tissue (PVAT) surrounds virtually every artery and is capable of releasing factors that influence vascular reactivity, but the effects of PVAT in the coronary circulation are unknown. Accordingly, the goal of this investigation was to delineate mechanisms by which lean vs. MetS coronary PVAT influences vasomotor tone and the coronary PVAT proteome. We tested the hypothesis that MetS alters the functional expression and vascular contractile effects of coronary PVAT in an Ossabaw swine model of the MetS. Utilizing isometric tension measurements of coronary arteries in the absence and presence of PVAT, we revealed the vascular effects of PVAT vary according to anatomical location as coronary and mesenteric, but not subcutaneous adipose tissue augmented coronary artery contractions to KCl. Factors released from coronary PVAT increase baseline tension and potentiate constriction of isolated coronary arteries relative to the amount of adipose tissue present. The effects of coronary PVAT are elevated in the setting of MetS and occur independent of

endothelial function. MetS is also associated with substantial alterations in the coronary PVAT proteome and underlying increases in vascular smooth muscle Ca^{2+} handling via $Ca_V1.2$ channels, H_2O_2-sensitive K^+ channels and/or upstream mediators of these ion channels. Rho-kinase signaling participates in the increase in coronary artery contractions to PVAT in lean, but not MetS swine. These data provide novel evidence that the vascular effects of PVAT vary according to anatomic location and are influenced by the MetS phenotype.

Johnathan D. Tune, Ph.D., Chair

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Chapter 1: Introduction

The Pandemic of Obesity

Today there are more people in the world that are overweight than underweight (**Figure 1.1**)[2, 10]. In the last 50 years, humans have become an obese species. Expansive accumulation of fat depots enabled by "thrifty" genes was once a natural and advantageous adaptation of earlier human cultures to survive between periods of feast and famine[11]. However, as societies have evolved and modern agriculture developed, this "thrifty genotype" is destructive in an era of abundant food sources and increasingly sedentary lifestyle. Increased food availability has helped to mitigate world hunger, while overabundance of food and declining physical activity continues to fuel an obesity pandemic.

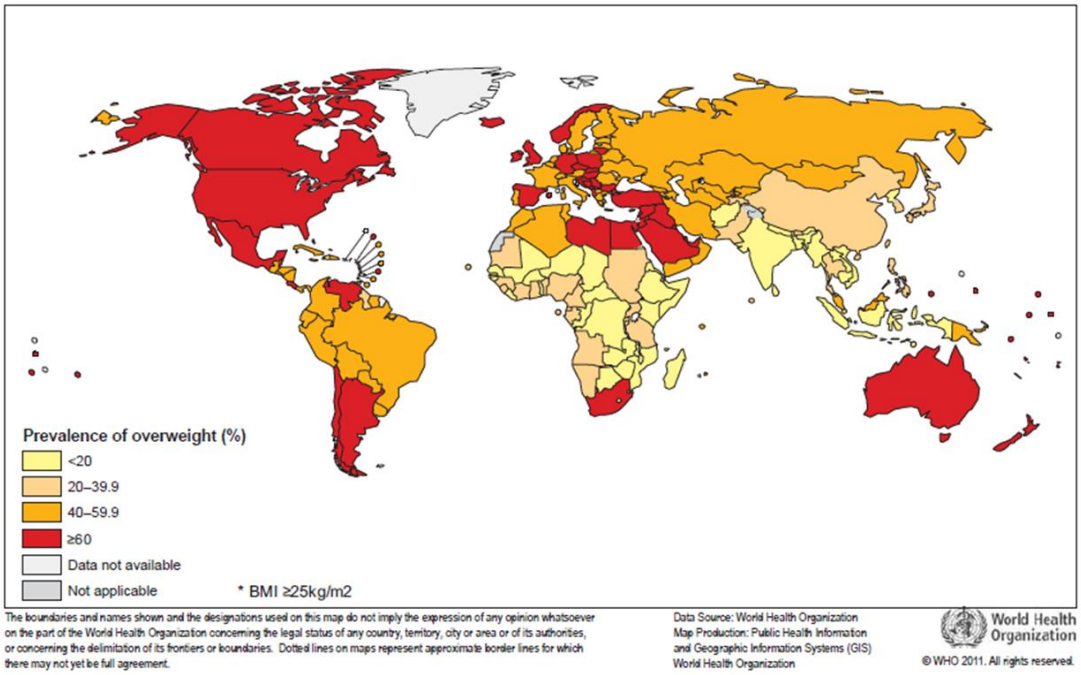


Figure 1.1 Pandemic of Obesity of Males, ages 20+. Worldwide, 2.8 million people die each year as a result of being overweight (BMI ≥ 25 kg/m^2) (including obesity (BMI ≥ 30 kg/m^2)).[2]

The most recent estimates reveal that ~36% of adults in the United States are obese (defined as a body mass index, BMI ≥ 30 kg/m^2)[12] and approximately 17% of children between the ages of 2-19 have already been classified as obese[13], implicating a perilous phenotype for the future[14]. Although America has acknowledged this growing problem, efforts to curb obesity in the United States have fallen short. Projections speculate that more than half of the US population will be obese by 2020[2]. Once considered a high income country problem, many low and middle-income countries are also experiencing widespread obesity, which is currently the fifth leading risk for death globally. These data illustrate the shocking predicament that plagues modern society[2].

Obesity, the Metabolic Syndrome and Cardiovascular Disease

Excess weight decreases mental concentration, productivity, can limit mobility, and even obstruct normal respiratory function, leading to sleep apnea[15]. While this growing pandemic is problematic in itself, the corresponding increase in obesity-associated cardiovascular diseases will wreak havoc on our healthcare system. Cardiovascular disease (CVD) remains the leading cause of death worldwide (**Figure 1.2**)[2]. Overweight individuals have an increased risk for heart diseases including heart attack, congestive heart failure, sudden cardiac death, angina, and dysrhythmia as well as other obesity associated morbidities that are placing additional pressure on healthcare providers[16]. The surgeon general suggests that even moderate excess weight (10 to 20 lbs.) can increase an individual's risk of cardiovascular-related death[2]. While modern medicine has

helped improve the outcome and even prevent some obesity-associated cardiovascular events, costly procedures and loss of productivity are contributing to growing financial burdens[17]. Recent estimates suggest the US spends between $147 and $210 billion dollars annually on diseases related to obesity[17]. Between 2010 and 2030, total direct medical costs related to cardiovascular disease are projected to triple, from $273 billion to $818 billion. Real indirect costs (due to lost productivity) are estimated to increase from $172 billion in 2010 to $276 billion in 2030[18]. Unless we can find ways to ameliorate the pathologic consequences of obesity, these projections ensure greater mortality and strain on our healthcare system and economy.

Figure 1.2 reveals the distribution of deaths due to non-communicable disease, including diabetes and cardiovascular disease. Although it is appreciated that obesity increases morbidity and mortality due to CVD, the mechanisms linking the two are poorly understood. Early intervention may be the most effective way of preventing CVD, but routine measurements such as body weight or BMI are not informative enough to assess CVD risk[19,20]. In addition to weight gain, factors

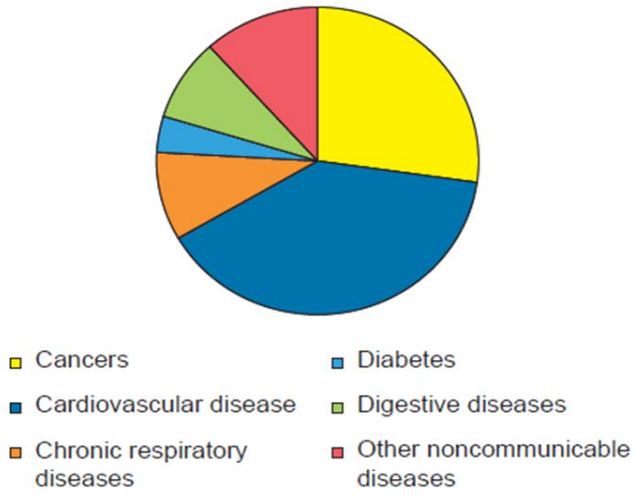
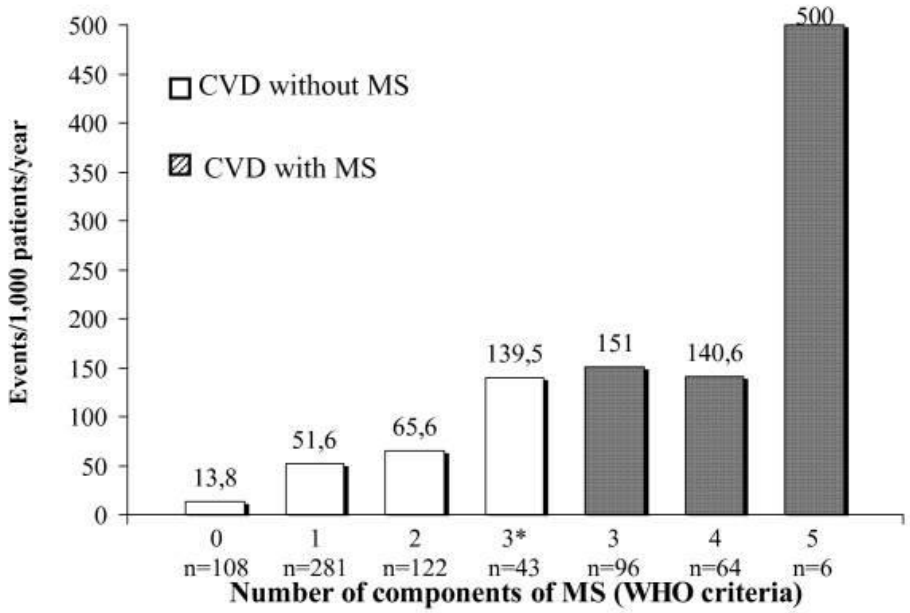


Figure 1.2 Proportion of global noncommunicable disease deaths under the age of 70, by cause of death. Cardiovascular disease remains the leading cause of death worldwide[2].

independent of body mass, such as genetic predisposition and inflammation also contribute to overall CVD susceptibility[6, 21]. Obesity alone increases CVD risk and all-cause mortality[22], but weight gain is typically accompanied by additional metabolic conditions including dyslipidemia, hyperglycemia, insulin resistance, impaired glucose tolerance and hypertension, each of which can exacerbate cardiovascular risk[23]. Collectively, three or more of these conditions render the diagnosis of the "Metabolic Syndrome" (MetS)[24] and each multiply the risk for a cardiovascular event, such as a fatal myocardial infarction or stroke[4, 25] (**Figure 1.3**).

Components of the MetS do not develop overnight. It is understood that early changes in metabolism can affect cardiovascular health long before the clinical diagnosis of disease. Physicians can test and identify each of these conditions and give a better risk assessment, but there are still many questions regarding the specific processes that link obesity/MetS to CVD. Abnormal weight gain during childhood or adolescence can have a large impact on diabetes and cardiovascular risk even at a normal BMI range[6], suggesting that methods to identify disease earlier are necessary to prevent the progression of cardiovascular disease.

Figure 1.3 Prevalence of Metabolic Syndrome (MS) and associated cardiovascular disease events. Diagnosis of Metabolic syndrome with World Health Organization (WHO) criteria. CVD risk factors include elevated lipids, obesity, diabetes, blood pressure and smoking and reductions in blood glucose tolerance. Subjects were followed for two years to evaluate the CVD events associated with metabolic syndrome. Events included complications from coronary artery disease, cerebrovascular disease, peripheral artery disease, retinopathy, nephropathy, neuropathy and death.[4]



Accordingly, **the long term goal of this research is to identify mechanisms by which obesity and MetS contribute to the initiation and progression of CVD**. Identifying these mechanisms will assist in providing novel therapeutic targets to reduce the cardiovascular complications in MetS.

Metabolic Syndrome and Coronary Artery Disease

As the coronary circulation is heterogeneous, coronary artery disease (CAD) encompasses both microvascular and macrovascular disease[26]. Microvascular dysfunction impairs the ability of the circulation to alter resistance, preventing alterations in blood flow to meet tissue demand. In contrast, atherosclerosis is a process where early diffuse CAD can change fluid dynamics across the length of the artery, but is more dangerous as it progresses, when artery stenosis can lead to plaque rupture, thrombosis, and tissue death[27].

Regulation of myocardial oxygen delivery is essential for normal cardiac function because the heart is constantly working and adapting to maintain cardiac

output. Myocardial oxygen demand varies depending on the relative energy expenditure of the organs (i.e. during periods of rest/exercise), therefore, the ability of the circulation to redirect blood flow from inactive to metabolically active organs is crucial for maintaining adequate energy supply. This is tightly regulated by vascular smooth muscle cells, which control tone with the integration of local hemodynamic, hormonal and nervous system signals.

Alterations in the control of coronary blood flow could underlie the dramatic risk of cardiovascular morbidity and mortality associated with the MetS. Growing evidence suggests that diffuse coronary vascular dysfunction is a powerful, independent risk factor for cardiac mortality among both diabetics and nondiabetics alike[28, 29]. Coronary flow reserve (CFR) is the maximum increase in blood flow through the coronary arteries above the normal resting volume. CFR is dependent on the extent of focal coronary artery stenosis, the fluid dynamic effect of diffuse atherosclerosis,[27] and the presence of microvascular dysfunction[28]. In diabetics, vascular dysfunction precedes overt atherosclerosis and is associated with greater cardiovascular mortality[29]. However, in both diabetic and non-diabetic patients, coronary vascular dysfunction as measured by impaired CFR was an independent correlate of cardiac and all-cause mortality. Although non-diabetic patients had lower cardiac mortality overall, diabetic patients that maintained CFR (>1.6) had similar cardiovascular mortality as non-diabetic patients with normal CFR, suggesting early alterations in vascular function underlie the adverse cardiovascular events in the MetS[28].

Coronary Microvascular Dysfunction in Metabolic Syndrome

Previous studies from our laboratory have established that obesity/MetS significantly impairs the ability of the coronary circulation to regulate microvascular resistance, which is required to balance myocardial oxygen delivery and metabolism[30, 31, 32, 33, 34]. Regulation of myocardial oxygen delivery is critical for maintaining overall cardiac function. The heart has limited anaerobic capacity and utilizes a high rate of oxygen extraction at rest (70-80%), requiring a continuous supply of oxygen to maintain normal cardiac output and blood pressure. Coronary microvascular dysfunction in the MetS is evidenced by reduced coronary venous PO_2 [31, 32, 33, 34], diminished vasodilation to endothelial-dependent and independent agonists (i.e. flow reserve)[35, 36, 28, 37, 38, 39], and altered functional and reactive hyperemia[31, 32, 33, 34, 40], all of which occur prior to overt CVD. Our findings indicate that this impairment is related to increased activation of vasoconstrictor neuro-humoral pathways (e.g. a_1 adrenoceptor[41], angiotensin/AT_1 signaling[30, 33] along with decreased function of vasodilatory K^+ channels (e.g. BK_{Ca} channels[42], K_V channels[34]). Recent evidence suggests that coronary microvascular dysfunction in MetS could also be related to increases in mineralocorticoid signaling which lead to marked alterations in the transcription, expression and activity of K^+ channels and L-type Ca^{2+} ($Ca_V1.2$) channels[43, 44, 45, 46, 47], which are central to electromechanical coupling in smooth muscle[48] and to the overall regulation of coronary vasomotor tone[48, 49]. The mechanism mediating the altered expression and/or function of these channels and contributing to microvascular dysfunction in MetS is currently under investigation.

Coronary Macrovascular Dysfunction in Metabolic Syndrome

In contrast to the microcirculation, the larger conduit vessels contribute very little to blood flow regulation[50, 51], but are more prone to atherosclerosis, a form of vascular dysfunction that develops over decades. CAD is one of the most common manifestations of atherosclerosis[52], which is a chronic disease characterized by the thickening of arteries. Atherosclerosis is caused by an innate immune response, involving the recruitment and activation of monocytes that respond to an excessive accumulation of modified lipids in the arterial wall.

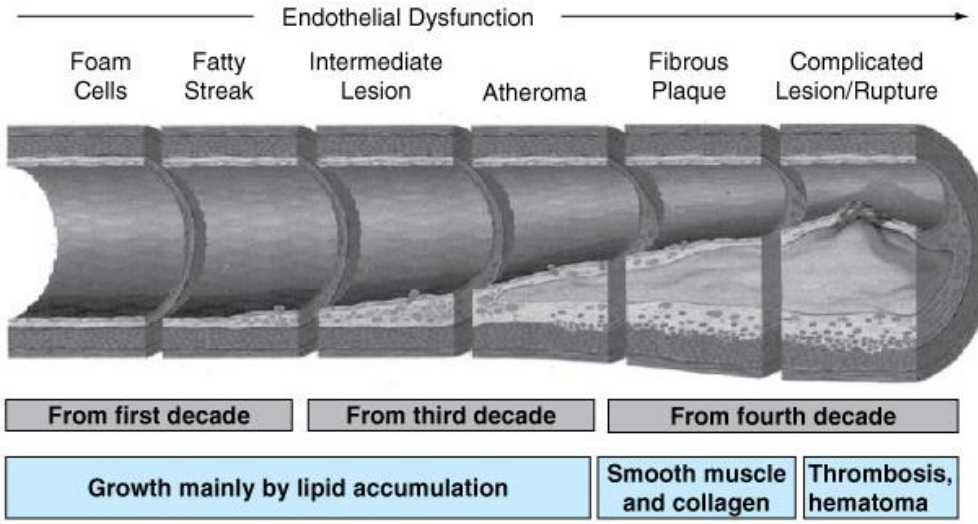


Figure 1.4 Atherosclerosis Timeline. As atherosclerosis develops, blunted responses to vasodilatory mediators and progressive endothelial dysfunction occur early, while smooth muscle proliferation and collagen production help to stabilize plaques as atherosclerosis progresses.[5]

The buildup of inflammatory cells within the arterial wall leads to local production of chemokines, interleukins, and proteases that enhance the influx of monocytes and lymphocytes, thereby promoting a vicious cycle of immune cell recruitment and the progression of lesions[53]. Individuals with atherosclerosis can remain asymptomatic for decades, but over time, inadequate removal of fats and

cholesterol from in and around the vasculature can lead to the development of plaques in the vessel wall (**Figure 1.4**). Overt plaque formation or ruptured plaques and subsequent thrombosis formation can impede blood flow to downstream tissues, often resulting in tissue death[26].

There are several potential mediators of atherosclerosis with increasing adiposity, including factors involved in blood pressure regulation, glucose tolerance, lipid metabolism, and chronic inflammation[16, 54, 7]. Systemic inflammation plays a pivotal role in the genesis and progression of atherosclerosis[55, 56, 53]. This inflammatory signaling is accompanied by endothelial and smooth muscle dysfunction as well as altered expression of angiogenic factors that result in structural remodeling and functional changes to the vessel[57]. The endothelium is an important paracrine organ that participates in regulating vascular tone, smooth muscle proliferation, and inflammation. Endothelial injury is thought to be an initiating event in atherosclerosis, causing adhesion of platelets and/or monocytes and release of growth factors, which leads to smooth muscle migration and proliferation[58]. Endothelial dysfunction is also characterized by impaired endothelial nitric oxide (NO) release and a subsequent decrease in blood flow to target tissues[59].

Smooth muscle dysfunction is another hallmark of atherosclerosis[60]. Healthy smooth muscle cells are fully differentiated and contractile, but in the face of cardiovascular risk factors they dedifferentiate to a more proliferative phenotype[61, 62]. During the progression of atherosclerosis, infiltration of lipid laden cells and inflammatory signaling lead to neointima formation, in which the vascular

media layer thickens as smooth muscle cells replicate to remodel the vascular wall[63]. Although endothelial dysfunction may initiate, contribute to, and exacerbate atherosclerosis[58], additional evidence suggests smooth muscle dysfunction could be the initiating event in atherosclerosis, organizing the angiogenic response that leads to accumulation and retention of lipids in the arterial wall[64]. Studies with adults at risk for atherosclerosis support the hypothesis that smooth muscle dysfunction may occur independently of impaired endothelial-dependent vasodilation. In these patients, vasodilation to exogenous NO with nitroglycerin (NTG) was impaired simultaneously with impaired endothelial-dependent vasodilation[65], suggesting smooth muscle and endothelial dysfunction occur concomitantly. Therapeutic interventions designed to prevent or revert the progression to these dysfunctional cell fates are critical for ameliorating cardiovascular disease in the metabolic syndrome.

In addition to what we understand about changes in blood flow and vessel remodeling that accompany MetS, knowledge of the specific cellular and molecular mechanisms that underlie changes in vascular smooth muscle function during the progression of atherosclerosis may elucidate targets for intervention. Intracellular calcium is a secondary messenger that is required for smooth muscle contraction. Alterations in intracellular calcium handling can cause changes in the function of these cells and is implicated in phenotypic modulation of smooth muscle cells, characterized by proliferation and migration[66]. Coronary smooth muscle cells of diabetic dyslipidemic swine exhibit impaired Ca^{2+} extrusion, down regulation of voltage-gated calcium channel ($Ca_V1.2$) expression, increases in Ca^{2+}

sequestration by the SR, increased nuclear localization of Ca^{2+}, and increased calcium-dependent K^+ channel activity[67, 68, 66] impairing the ability of these cells to properly regulate blood flow.

The elaborate cell signaling and heterogeneous nature of atherosclerosis often make it hard to distinguish between cause and effect in the pathogenesis of CVD, but it is clear that modulation of the coronary smooth muscle cell phenotype is required for overt atherosclerosis to occur. Both microvascular and macrovascular dysfunction contribute to cardiovascular morbidity and mortality[26], but it is still unclear what aspect of MetS is responsible for mediating these changes. In order to prevent obesity-induced CVD, we must understand how fat accumulation influences vascular cellular function. Together, these studies suggest that alterations in ion channel function and intracellular Ca^{2+} handling, indicative of changes in smooth muscle gene expression, are required for the progression of vessels into the diseased state, but the mechanisms by which weight gain and MetS increase CAD risk and contribute to micro/macrovascular smooth muscle dysfunction has yet to be determined.

Adipose Tissue, Distribution and Inflammation

To this cause, several investigators are actively researching adipose tissue and its dynamic endocrine and paracrine action in health and disease. White adipose tissue (WAT) is the fat that stores triglycerides and from which lipids are mobilized for systemic utilization when energy is required[69]. The discovery of leptin in 1994 encouraged scientists to reconsider the role of adipose tissue, and it is

now recognized as a metabolically active endocrine and paracrine organ[55, 70]. As developing preadipocytes differentiate to become mature adipocytes, they acquire the ability to synthesize hundreds of proteins, many of which are released as enzymes, cytokines, growth factors, and hormones involved in overall energy homeostasis (**Figure 1.5**). Moreover, adipose tissue does not just contain adipocytes (30-50%), but is also composed of stromavascular cells, including preadipocytes, fibroblasts, mesenchymal stem cells, endothelial progenitor cells, T cells, B cells, mast cells, and adipose tissue macrophages[71]. Each of these populations has its own chemical messenger arsenal that allows communication between cell types[72].

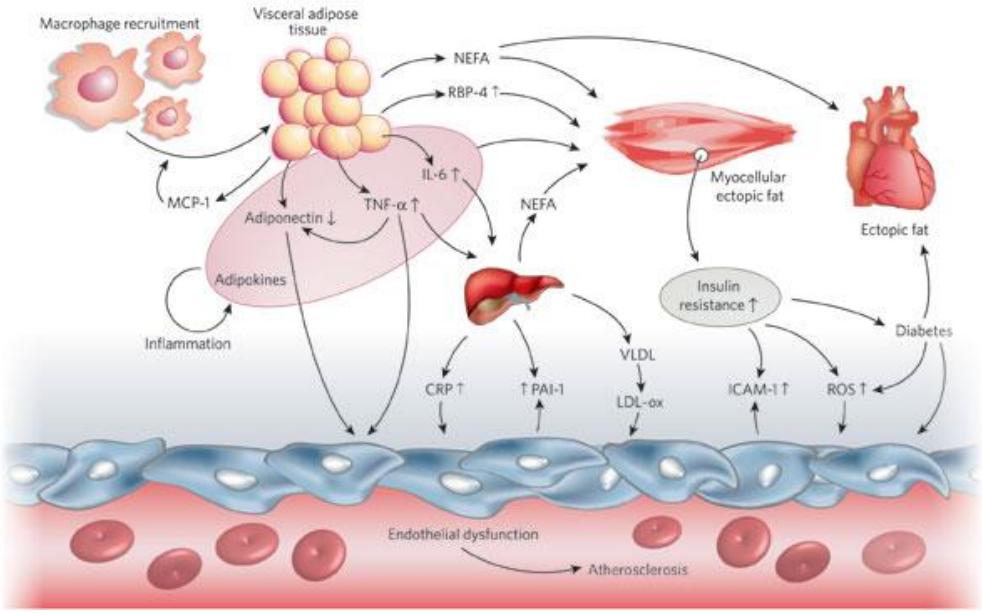


Figure 1.5 Factors derived from adipose tissue contribute to cardiovascular disease in obesity. Adipose contributes to endothelial dysfunction through the direct effect of adipokines, adiponectin and TNF-α, which are secreted by fat tissue after macrophage recruitment through MCP-1. Fat accumulation, insulin resistance, liver-induced inflammation and dyslipidemic features may all lead to the premature atherosclerotic process[6].

Originally, adipokines were defined as peptides secreted by adipocytes whereas cytokines referred to the peptides secreted from the stromavascular cells, but these terms often overlap as adipokines may be secreted from both[16]. These chemical messengers ("adipokines") allow adipose tissue to influence a breadth of physiological functions including energy and feeding regulation, glucose and lipid metabolism, thermogenesis, neuroendocrine function, reproduction, immunity, and most relevantly cardiovascular function[9, 73, 74, 75] (**Figure 4**). Adipokines can influence cardioprotection by promoting proper endothelial function and angiogenesis, as well as reducing hypertension, atherosclerosis, and inflammation[76, 77]. However, if this balance is disrupted, changes in adipokine signaling can lead to defective smooth muscle contractility, inflammation, and damage to blood vessels, resulting in conditions such as hypertension, atherosclerosis, as well as endothelial, smooth muscle, and myocardial dysfunction[16, 74]. Despite these associations, we still do not understand how specific adipokines may function in MetS to promote vascular dysfunction.

Adipose tissue can be found throughout the body. Following the onset of obesity, the secretory function of adipose is modified by changes in the cellular composition of the tissue, including alterations in the number, phenotype, and localization of immune, vascular, and structural cells. The function of adipose is dependent upon its anatomical location and the relative composition of the cells types present. There are several adipose tissue depots, including the visceral and subcutaneous, which are the two most abundant stores of fat in the body. These fats express unique profiles of adipokines[78] and individuals typically accumulate

excess fat in one or both of these depots. Accumulation of visceral adipose tissue, located inside the peritoneal cavity and on or around visceral organs plays a major role in the development of insulin resistance and is correlated with relative cardiometabolic risk[79], whereas subcutaneous adipose tissue, located just underneath the skin is a major source of leptin production and aids in energy homeostasis. Preferential distribution within this depot is associated with reduced risk of metabolic complications in obesity[80]. These differences suggest that body shape can be informative of CVD risk, but more importantly, highlight the heterogeneity of adipose tissue function dependent upon its location in the body. In addition to the major fat stores, adipose can be found on or surrounding organs such as the kidneys, liver, and the heart. These depots are thought to work individually, providing structural support and contributing to local organ function, as well as contributing systemically to overall energy homeostasis [7].

Excess quantities of fat on or around the heart may explain why apple-shaped individuals are more prone to cardiovascular complications, but studies also suggest that changes in adipokine expression can exacerbate disease risk in obesity[81, 82]. Adipokines have effects that may be beneficial and/or detrimental to cardiovascular physiology. For example, adiponectin is cardioprotective against myocardial ischaemia/reperfusion (I/R) injury, whereas leptin and tumor necrosis factor alpha (TNFα) may play a detrimental role in cardiac remodeling by limiting the extent of myocardial hypertrophy[16, 77]. Adipose tissue expansion in obesity and alterations in adipokine production have led to the proposed “Adipokine Hypothesis”, which implicates these signaling molecules as the causative link

between MetS and CVD[82]. It is now appreciated that adipokine expression changes in the disease state, but the mechanism for this change is unclear.

Obesity is characterized by a chronic, low-grade pro-inflammatory state in adipose tissue causing hyperplasia and hypertrophy of fat cells[83, 84]. As adipocytes hypertrophy with increasing weight gain, cells outstrip their blood supply which can lead to capillary rarefaction and localized hypoxia in adipose tissue[85]. This causes up-regulation of inflammatory adipokines, such as inflammation and causing alterations in insulin-mediated capillary recruitment[89, 90 57, 60, 69, 91].

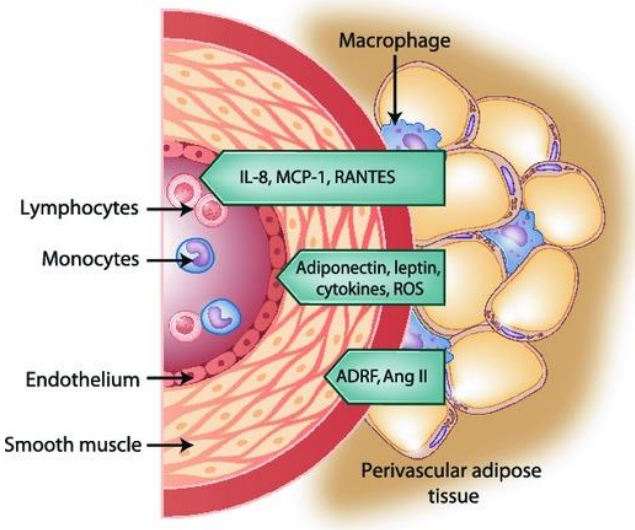


Figure 1.6 Perivascular adipose tissue. Interaction of perivascular adipose tissue with vascular endothelium, smooth muscle, and immune cells and several of the PVAT-derived mediators involved. PVAT is situated outside the adventitial layer of the vessel wall (a.k.a. periadventitial adipose tissue) with proximity allowing for paracrine signaling and regulation of vascular homeostasis[3].

A cohort study in Denmark revealed that arachidonic acid content in gluteal adipose tissue was positively associated with risk of myocardial infarction, regardless of diet[48], suggesting that changes to adipose tissue signaling may sustain CVD independent of obesity. Anti-inflammatory adipocyte products such as NO and adiponectin, which normally confer protection against inflammation and obesity-linked insulin resistance, are decreased in obese patients[8, 84, 89]. These

obesity-induced changes to adipose proximal to blood vessels can have direct vascular consequences on the underlying endothelium and vascular smooth muscle[89, 92] and can lead to vascular diseases such as hypertension, atherosclerosis, and vascular dysfunction. Adipose tissue itself is highly vascularized and surrounds virtually every vessel in the human body, providing mechanical support and making it capable of sending chemical messengers and vasoactive mediators into the bloodstream[93]. This fat that surrounds vessels, termed perivascular adipose tissue, or “PVAT”, is located outside the adventitial layer of the vessel wall (**Figure 1.6**).

Perivascular Adipose Tissue

Several studies propose that PVAT-derived factors traverse the vessel wall to directly influence local smooth muscle and/or endothelial cell function. This is supported by the fact that PVAT is contiguous with the adventitia and no fascia separates surrounding adipocytes from the vascular wall[94,] and adipocytes have been demonstrated to invade the outer region of the adventitia in the setting of obesity[95, 96], allowing this local tissue to mobilize near vessels with the potential for direct paracrine communication[9]. The vasa vasorum are small arteries that branch off conduit vessels, traverse the vessel wall, and return into the lumen of the conduit arteries[97, 98]. This extensive small artery network connects the adipose tissue to the vessel lumen and offers an additional route for PVAT action, limiting the necessary diffusion distance. The proximal location, active paracrine nature of

adipose tissue and clear association between obesity and cardiovascular disease implicate local PVAT and PVAT-derived factors in vascular dysfunction.

Although PVAT provides structural support and insulation to blood vessels which may be protective in its native setting[93], the specific changes to adipose tissue and the extent to which adipose-derived adipokines may influence vascular smooth muscle and endothelial function during disease progression are still unclear. In 1991, Soltis and Cassis compared the contractile responses of rat aortas cleaned of surrounding PVAT or with the natural PVAT left intact. Aortas with PVAT were less responsive to increasing concentrations of norepinephrine, suggesting that PVAT was producing a dilatory agent that buffered the degree of vasoconstriction[99].

Since this pivotal discovery, several groups have tried to characterize and identify the vascular effects of PVAT. This anti-contractile influence of PVAT led to the discovery of an adipocyte-derived relaxing factor (ADRF). Gollasch's group has shown that PVAT plays a major role in vasoregulation of visceral arteries, such as the aorta and mesenteric arteries (**Figure 1.7**),[100] and depending on the vascular bed and animal model, may cause endothelial dependent and/or independent vasorelaxation[101,102]. PVAT releases soluble factors that cause subsequent smooth muscle vasodilation by converging on a number of different K^+ channels[103, 101, 104, 105, 106]. PVAT-conditioned media (bath solution exchange) experiments revealed ADRF was transferable[107, 105], lost with heating (65°C), and

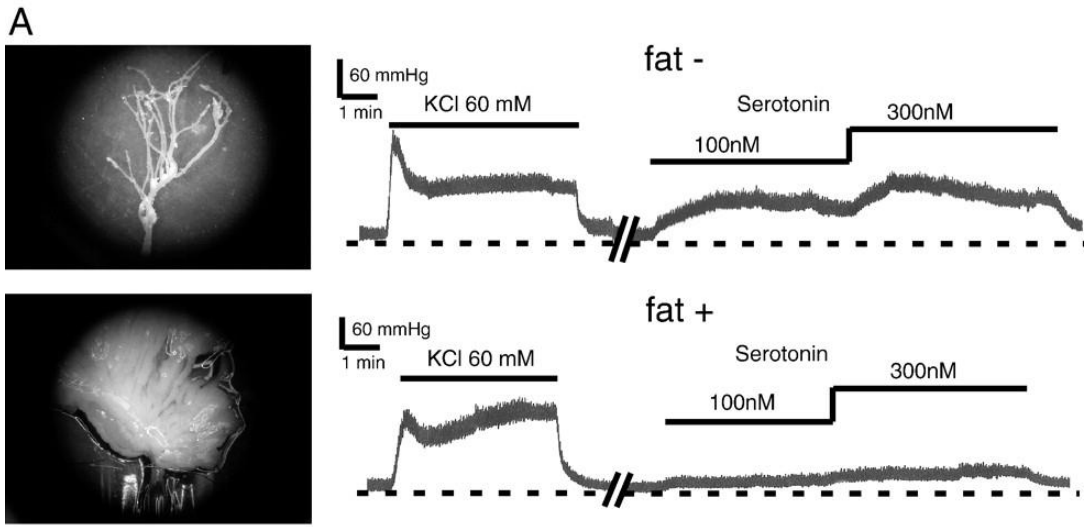


Figure 1.7. PVAT-derived factors limit vascular reactivity to serotonin in mouse mesenteric vascular beds via outside-to-inside paracrine signaling. Representative recording of perfusion pressure for perfused isolated mesenteric beds in the absence (fat-) and presence (fat+) of perivascular fat. Dashed lines represent 30 mmHg. Meticulous removal of PVAT from the mesenteric artery bed potentiated constriction to serotonin. This preparation of the entire mesenteric bed revealed the outside-to-inside paracrine signaling capability of local adipose tissue[1].

not adsorbed by fatty acid-free serum albumin[103], indicating the PVAT-derived factor is likely a peptide rather than lipid[103].

Only a handful of groups have been able to examine the vascular effects of human PVAT. In the internal thoracic arteries from patients undergoing elective coronary artery bypass grafting, Gao *et al*. found the presence of PVAT attenuated the maximal contraction to U-46619 and phenylephrine[105]. In this vascular bed, PVAT exerted its anti-contractile effects via endothelium-dependent relaxation through NO release and subsequent BK_{Ca} channel activation, and by an endothelium-independent mechanism involving H_2O_2 and subsequent activation of soluble guanylyl cyclase[105, 104]. In addition, healthy adipose tissue around human small arteries secretes factors that influence vasodilation by increasing NO bioavailability[8], also implicating endothelial NO production as a target of regulation of tone by PVAT.

The variety of cell types present in PVAT and the high degree of signaling complexity make it hard to distinguish which adipokine (or combination thereof) is the responsible ADRF(s). Fesus *et al.* showed that adiponectin could induce vasodilation of K_V channels in rat aorta and mouse mesenteric arteries, but was likely not the ADRF, as PVAT from adiponectin knockout mice maintained the anti-relaxing influence[1]. Gollasch found that inhibitors of the hydrogen sulfide producing enzyme, Cystathionin-γ-Lyase (CSE), inhibited the anti-contractile effects of PVAT, implicating H_2S as a candidate or potential modulator of ADRF[108, 100]. Other groups have implicated angiotensin 1-7[101], NO[109] and leptin[110] as possible endothelial-dependent ADRFs. Together, these studies implicate several ADRFs,

endothelial-dependent and independent signaling pathways, and various smooth muscle K^+ channels as potential mediators of PVAT's observed effect. Whether the variety of animal models and vascular beds, expression of adipokines, and/or underlying differences in endothelial and smooth muscle function are responsible for these differences will need to be determined.

In contrast to the well-documented ADRF, a limited amount of evidence suggests that PVAT may also have a constricting influence[111, 112, 112, 113]. In the mesenteric arteries of Wistar-kyoto rats, intact PVAT caused a greater contractile response to electrical field stimulation (EFS) than rings with PVAT removed. PVAT also potentiated contractions to KCl in rats. This was mediated by NAPDH-oxidase increases in superoxide production of PVAT[111, 112]. While potentiated vasoconstriction may actually represent attenuation of vasodilator influences by superoxide[112, 114], transfer experiments have demonstrated that the influence of PVAT is due to its function as a paracrine tissue rather than adipose merely obstructing or absorbing vasoactive mediators[103, 105, 115, 8, 16]. As PVAT has become respected as a local active paracrine influence on vascular function, several groups sought to examine whether PVAT is involved in the vascular dysfunction observed in obesity and MetS.

PVAT in obesity

Increased pro-inflammatory adipokines in PVAT after endovascular injury demonstrates that the vasculature has the capacity to communicate with the surrounding adipose tissue, allowing for cross-talk between endothelial cells,

vascular smooth muscle cells, and surrounding PVAT[116, 117]. Inflamed PVAT has particular ramifications for CVD, given the effects adipokines have on cardiovascular pathophysiology as well as obesity and diabetes[16]. During obesity, the architectural changes to PVAT from infiltrating inflammatory cells in addition to gene expression changes during disease progression have direct consequences on the normal vascular function of PVAT (**Figure 1.8**).

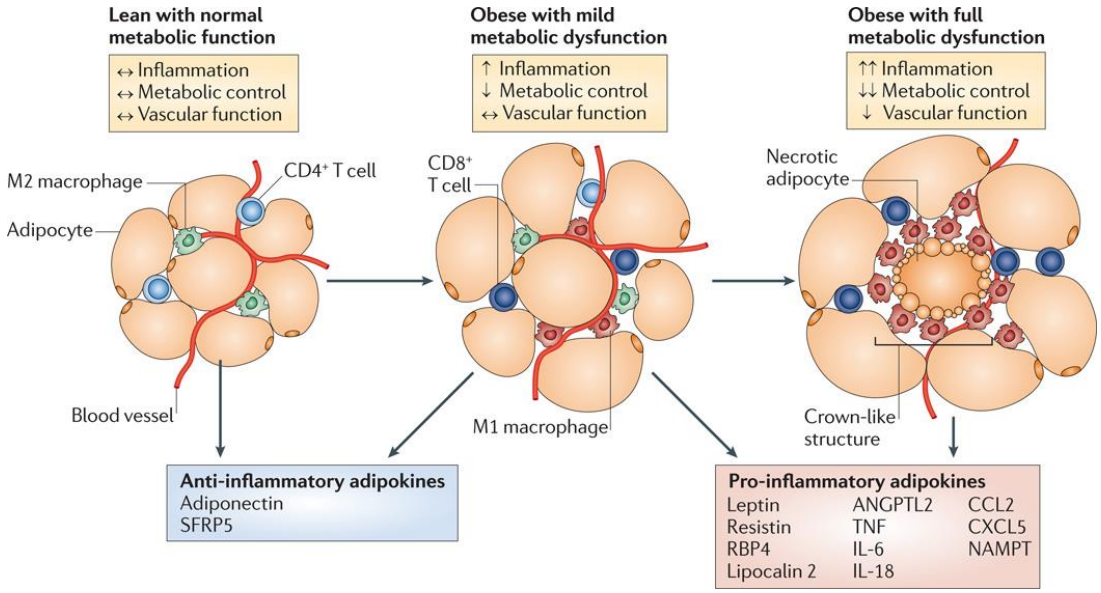


Figure 1.8 Phenotypic modulation of adipose tissue. With weight gain, adipocytes hypertrophy owing to increased triglyceride storage. With limited obesity, it is likely that the tissue retains relatively normal metabolic function and has low levels of immune cell activation and sufficient vascular function. However, qualitative changes in the expanding adipose tissue can promote the transition to a metabolically dysfunctional phenotype. Macrophages in lean adipose tissue express markers of an M2 or alternatively activated state, whereas obesity leads to the recruitment and accumulation of M1 or classically activated macrophages, as well as T cells, in adipose tissue[7].

In obesity, PVAT expansion causes oxidative stress, inhibiting NO production[105, 118] and abolishing the anti-contractile influence of the PVAT[8]. Endothelial dysfunction is characterized by a defect in the normal vasodilator response to agonists or changes in blood flow. Endothelial-derived NO causes vascular relaxation[119], but also suppresses atherosclerosis by reducing endothelial cell activation, smooth muscle proliferation, leukocyte and platelet activation, and reducing the number of monocyte-platelet aggregates in the circulation[120]. In hypoxia, macrophages and reactive oxygen species (ROS) also appear to attenuate anti-contractility in PVAT[121], while leptin, resistin, and visfatin may contribute to atherosclerosis, inflammation, and endothelial dysfunction[90, 110, 122,

[89]. Insulin affects vasoregulation by acting on different signaling pathways regulating NO and endothelin-1 release[123]. *In vitro*, inflammation induced with TNFα or hypoxia attenuated the anti-contractile effect of PVAT, suggesting alterations in the paracrine signaling of PVAT may directly influence insulin sensitivity of resistance vessels or tissue perfusion[124, 125]. The etiology of the vascular dysfunction in MetS is dependent on the vessel size and diffusion distance, organ localization, and underlying pathological status (inflammation, atherosclerosis, neovascularization from the intima) of the vessel itself[123].

In 2009, Greenstein *et al.* studied PVAT in human obesity. They isolated small arteries from human gluteal subcutaneous fat biopsies with and without fat and found the anti-contractile effect of PVAT was lost in arteries from obese patients[8] (**Figure 1.9**). This change in PVAT function in obesity was thought to be due to increased pro-inflammatory macrophages[121] and alterations in adipokine production[96, 94, 72]. This study on small arteries is an important indicator of how PVAT may contribute to blood flow regulation by altering the vascular response of small arteries, which ultimately drive blood flow.

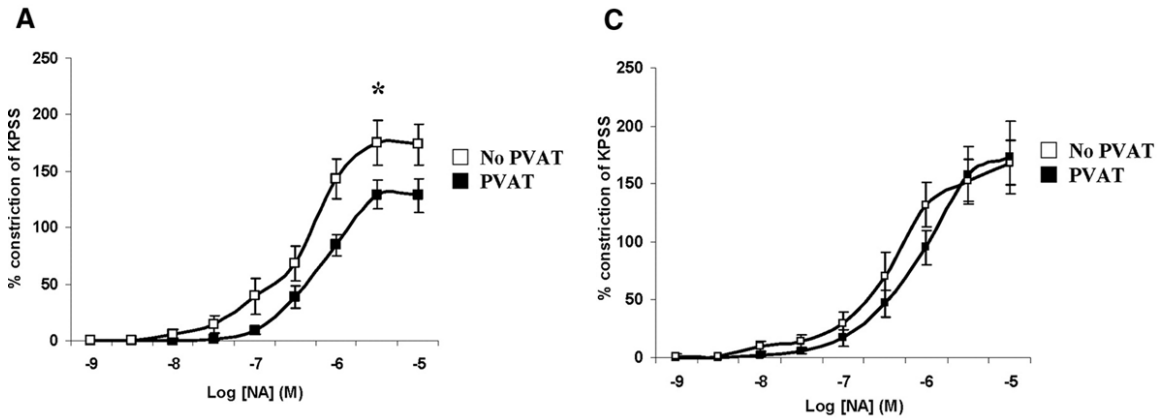


Figure 1.9 Effect of obesity and the metabolic syndrome on anti-contractile capacity of PVAT in small arteries from subcutaneous gluteal fat. A, In healthy control participants, PVAT exerted a significant anti-contractile effect compared with contractility of arteries without PVAT. C, In patients with obesity and metabolic syndrome, the presence of PVAT had no effect on contractility[8].

Atherosclerosis is a chronic condition that involves progressive cellular dysfunction. Studies in obese humans and animal models implicate both inflammation of PVAT and corresponding endothelial and smooth muscle dysfunction in the disease process. The nature of this disease process compounded with the variability of PVAT's influence between vascular beds and the variety of signaling molecules involved create more questions than answers regarding PVAT in disease. Studies are needed that better characterize particular vascular beds and PVAT depots in appropriate models of human disease, particularly CVD, which claims hundreds of thousands of lives annually. However, due to a limited number of large animal models, how coronary PVAT directly contributes to coronary vascular dysfunction, remains to be determined.

Coronary PVAT

The role of PVAT in the control of vasomotor tone and potential role in the pathogenesis of CAD is not well understood. Coronary PVAT is a visceral thoracic fat depot defined as the adipose tissue directly surrounding the coronary arteries[126]. Several groups implicate coronary PVAT in the initiation and progression of CAD. The Framingham heart study[127, 128] revealed that coronary PVAT volume was an independent risk marker for CVD. Echocardiography[129, 130], computed tomography[131, 132] and magnetic resonance imaging[127] have revealed the quantity of fat on the heart is correlated with parameters of the MetS, such as increased waist circumference, hypertriglyceridemia and hyperglycemia, and with CAD[92].

This naturally occurring adipose depot expands with obesity,[131, 133] and atherosclerotic plaques have been shown to occur predominately in epicardial coronary arteries that are encased in PVAT[134, 95, 131, 132]. Furthermore, autopsies revealed that patients with a myocardial muscle bridge spanning the epicardial surface of the heart had limited atherosclerosis within the portion of the vessel surrounded by muscle as opposed to PVAT[135]. Herrmann *et al.* demonstrated that increased coronary vasa vasorum neovascularization preceded overt coronary endothelial dysfunction and atherosclerotic disease in domestic swine fed a high fat diet, which could serve as a potential conduit that could traffic harmful adipokines between the PVAT and the vascular wall[98].

Adipokine	Species	Condition	Expression	References
Leptin	Swine	MetS + CAD	↑↑ protein	Payne *et al.* (2010)
	Human	CAD	↑ mRNA, ↑↑ protein secretion	Cheng *et al.* (2008)
				Langheim *et al.* (2010)
				Shibasaki *et al.* (2010)
Resistin	Human	CAD	↑↑ mRNA, ↑↑ protein secretion	Langheim *et al.* (2010)
Adiponectin	Human	Obese	↓protein secretion	Karastergiou *et al.* (2010)
		CAD	↓ mRNA, ↓↓ protein secretion	Langheim *et al.* (2010)
				Cheng *et al.* (2008)
				Karastergiou *et al.* (2010)
				Iacobellis *et al.* (2005)
				Eiras *et al.* (2008)
			↓protein	Spiroglou *et al.* (2010)
		Obese + CAD	↓protein secretion	Karastergiou *et al.* (2010)
TNF-α	Human	CAD	↔ mRNA, ↑↑ protein secretion	Cheng *et al.* (2008)
				Langheim *et al.* (2010)
		Obese + CAD	↑↑mRNA, ↑↑ protein secretion	Mazurek *et al.* (2003)
IL-6	Human	CAD	↑↑ mRNA, ↑↑ protein secretion	Cheng *et al.* (2008)
				Eiras *et al.* (2008)
				Shibasaki *et al.* (2010)
				Langheim *et al.* (2010)
		Obese + CAD	↑↑ mRNA, ↑↑ protein secretion	Mazurek *et al.* (2003)
IL-1β	Human	CAD	↑ mRNA	Shibasaki *et al.* (2010)
		Obese + CAD	↑↑ mRNA, ↑↑ protein secretion	Mazurek *et al.* (2003)
MCP-1	Human	CAD	↑↑ mRNA	Shibasaki *et al.* (2010)
				Langheim *et al.* (2010)
		Obese + CAD	↑↑ mRNA, ↑↑ protein secretion	Mazurek *et al.* (2003)
NPR-C	Human	CAD	↑ mRNA	Shibasaki *et al.* (2010)
Adrenomedullin	Human	CAD	↑ mRNA	Shibasaki *et al.* (2010)
			↓ mRNA, ↓ protein	Iacobellis *et al.* (2009)
Visfatin	Human	CAD	↑↑ protein secretion	Cheng *et al.* (2008)
			↑ protein	Spiroglou *et al.* (2010)
PAI-1	Human	CAD	↑↑ mRNA	Langheim *et al.* (2010)
MIF	Human	CAD	↓ mRNA	Langheim *et al.* (2010)
IL-1Rα	Human	Obese + CAD	↑ protein secretion	Karastergiou *et al.* (2010)
sICAM-1	Human	CAD	↑ protein secretion	Karastergiou *et al.* (2010)
		Obese + CAD	↑ protein secretion	
IL-16	Human	CAD	↑ protein secretion	Karastergiou *et al.* (2010)
		Obese + CAD	↑ protein secretion	
IL-13	Human	Obese	↑ protein secretion	Karastergiou *et al.* (2010)
		CAD	↑ protein secretion	
RANTES	Human	Obese	↑ protein secretion	Karastergiou *et al.* (2010)
		CAD	↑ protein secretion	
		Obese + CAD	↑ protein secretion	
Chemerin	Human	CAD	↑ protein	Spiroglou *et al.* (2010)
UCP-1	Human	MetS	↔ mRNA	Sacks *et al.* (2009)
		Diabetes	↔ mRNA	
PRDM16	Human	MetS	↔ mRNA	Sacks *et al.* (2009)
		Diabetes	↔ mRNA	
PGC-1α	Human	MetS	↑ mRNA	Sacks *et al.* (2009)
		Diabetes	↑ mRNA	
$sPLA_2$	Human	CAD	↑ mRNA	Dutour *et al.* (2010)
PPAR-γ	Human	CAD	↔ mRNA	Shibasaki *et al.* (2010)
NPR-A	Human	CAD	↔ mRNA	Shibasaki *et al.* (2010)

Table 1.1 Relationship between coronary PVAT expression, coronary artery disease and obesity/Metabolic Syndrome.[9]

In addition, several changes to PVAT expression occur with increasing obesity. Multiple groups have documented pathogenic adipokine profiles from human coronary PVAT with increasing macrophage infiltration compared to abdominal adipose[136, 137]. Our recent review highlights the known coronary adipose-derived factors that are altered with weight gain (**Table 1.1**)[9]. This is not a comprehensive list of adipokines secreted by coronary PVAT, but an introduction to the complex nature of adipose tissue and the dynamic changes that occur in paracrine signaling with the development of MetS. While there are clear associations between the volume, inflammatory state, and adipokine profile of PVAT and the severity of vascular dysfunction, we have yet to identify a mechanistic link.

Although numerous studies indicate that PVAT releases relaxing factors which attenuate vasoconstriction to a variety of compounds in peripheral vascular beds[103, 8], data on the vascular effects of coronary PVAT are equivocal[89, 9, 102, 138, 110]. Depending on the vessel size and diffusion distance, organ localization, and underlying pathological status (inflammation, atherosclerosis, neovascularization from the intima) the extent to which adipokines influence the vasculature may differ[123]. In particular, experiments in isolated arteries from lean and hypercholesterolemic swine show little/no effect of coronary PVAT on coronary artery contractions or endothelial-dependent vasodilation[102]. In contrast, PVAT has been found to impair coronary endothelial function *in vitro* and *in vivo* in normal-lean dogs[138] and significantly exacerbate underlying endothelial dysfunction in obese swine with MetS[110]. These differences in the paracrine effects of coronary

vs. other peripheral PVAT depots are likely related to alterations in adipocytokine expression profile between these beds, as well as the consequences of underlying state of micro/macrovascular dysfunction[113], [125]. While recent studies have begun to uncover pathophysiologic changes that occur in PVAT,[8, 56, 73, 88, 89, 96, 136, 139-141] the functional phenotypic effects of obesity on coronary PVAT remain poorly understood.

Proposed Experimental Aims

The current literature implicates PVAT in outside-to-inside coronary vascular dysfunction and our lab has shown that local PVAT aggravates endothelial dysfunction in MetS and that MetS is associated with altered $Ca_V1.2$ and K^+ channel function. However, the contribution of PVAT to vascular function in lean and obese states is still not well understood. With these theories in mind, the central focus of this work is to investigate the potential role of coronary PVAT in the development of smooth muscle dysfunction. Specifically, the goal of the following studies is to determine the mechanisms by which local PVAT regulates coronary vascular reactivity in lean and MetS swine. These objectives will be addressed in studies designed to examine the following Specific Aims:

1. **Test the hypothesis that coronary PVAT augments tension development of coronary arteries and is potentiated in MetS.** Rationale for AIM1 is based on the evidence that PVAT impairs coronary vasodilation in lean animals and coronary vascular dysfunction is related to increased activation of vasoconstrictor neuro-humoral pathways (e.g. α_1 adrenoceptors[41],

angiotensin/AT_1,[30, 33] mineralocorticoid signaling along with decreased function of vasodilatory K^+ channels (e.g. BK_{Ca} channels[31, 42], K_V channels[34, 40, 49]) in obesity/MetS. We aim to examine how PVAT specifically influences baseline and agonist-induced tension development in isolated lean and MetS coronary arteries.

2. **Identify and examine the potential coronary PVAT-derived factors that mediate the vascular effects in lean vs. MetS coronary arteries.** We hypothesize that marked changes in adipokine protein expression may underlie the difference in extent of vascular dysfunction between lean and MetS swine. We aim to perform a global proteomics assessment of the PVAT-derived factors that may influence vascular function in lean vs. MetS. Rationale for our hypothesis is based on the evidence that PVAT releases a transferable peptide that mediates its vascular effect which is functionally altered in several vascular beds in MetS.
3. **Identify the specific vascular smooth muscle signaling pathways and end effector ion channels that mediate the vascular consequences of PVAT in MetS.** Rationale for this aim is based on independent reports of PVAT and MetS altering ion channel function and Ca^{2+} handling. We hypothesize that local PVAT may mediate these changes in ion channel function and aim to examine the specific vascular smooth muscle ion channels involved in mediating the effects of PVAT.

Our findings provide novel evidence regarding the potential role for specific coronary PVAT-derived proteins in coronary vascular dysfunction in the setting of

obesity. Altogether, findings from the proposed investigations will be the first to specifically address the role coronary PVAT plays in smooth muscle function and possibly contributes to CVD. Proteins identified as dysregulated in MetS will provide potential targets for therapeutic intervention.

Chapter 2

Perivascular adipose tissue potentiates contraction of coronary vascular smooth muscle: Influence of obesity

Abstract

Background: This investigation examined the mechanisms by which coronary perivascular adipose tissue (PVAT)-derived factors influence vasomotor tone and the PVAT proteome in lean vs. obese swine.

Methods and Results: Coronary arteries from Ossabaw swine were isolated for isometric tension studies. We found that coronary (P = 0.03) and mesenteric (P = 0.04), but not subcutaneous adipose tissue, augmented coronary contractions to KCl (20 mM). Inhibition of $Ca_V1.2$ channels with nifedipine (0.1 μM) or diltiazem (10 μM) abolished this effect. Coronary PVAT increased baseline tension and potentiated constriction of isolated arteries to PGF2α in proportion to the amount of PVAT present (0.1-1.0 g). These effects were elevated in tissues obtained from obese swine and were observed in intact and endothelium denuded arteries. Coronary PVAT also diminished H_2O_2-mediated vasodilation in lean, and to a lesser extent in obese arteries. These effects were associated with alterations in the obese coronary PVAT proteome (detected 186 alterations) and elevated voltage-dependent increases in intracellular [Ca^{2+}] in obese smooth muscle cells. Further studies revealed that a Rho-kinase inhibitor fasudil (1 μM) significantly blunted artery contractions to KCl and PVAT in lean, but not obese swine. Calpastatin (10 μM) also augmented contractions to levels similar to that observed in the presence of PVAT.

Conclusions: Vascular effects of PVAT vary according to anatomic location and are influenced by an obese phenotype. Augmented contractile effects of obese coronary PVAT are related to alterations in the PVAT proteome (e.g. calpastatin),

Rho-dependent signaling, and the functional contribution of K^+ and $Ca_V1.2$ channels to smooth muscle tone.

Introduction

Adipose tissue normally surrounds the major conduit coronary arteries on the surface of the heart. The volume of this perivascular adipose tissue (PVAT) expands with obesity[130, 142] and has been shown to be a strong, independent predictor of coronary atherosclerosis[132]. Recent studies implicate PVAT as a ready source of vasoactive factors and inflammatory mediators capable of influencing vasomotor function[143]. Thus, there is growing evidence that adipocyte-derived factors originating outside of the coronary vasculature are capable of affecting vascular homeostasis[1, 100, 139, 144]. This “outside-to-inside” signaling paradigm is supported by several studies indicating that adventitial factors significantly diminish vascular function[1] and influence compositional changes in the inner intimal layer[91]. Thus, local adipose in the heart could be an important regulator of vascular function and disease progression.

Although numerous studies indicate that PVAT releases relaxing factors which attenuate vasoconstriction to a variety of compounds in peripheral vascular beds[8, 103], data on the vascular effects of coronary PVAT are equivocal[9, 102, 110, 138]. In particular, experiments in isolated arteries from lean and hypercholesterolemic swine show little/no effect of coronary PVAT on coronary artery contractions or endothelial-dependent vasodilation[102]. In contrast, PVAT has been found to impair coronary endothelial function *in vitro* and *in vivo* in normal-lean dogs[138] and significantly exacerbate underlying endothelial dysfunction in obese swine with the metabolic syndrome (MetS)[110]. These differences in the paracrine effects of coronary vs. other peripheral PVAT depots are likely related to alterations in

adipocytokine expression profile between these beds, as well as the consequences of underlying co-morbidities (e.g. obesity) on these profiles[113, 125]. While recent studies have begun to uncover pathophysiologic changes that occur in PVAT[9, 145], the functional phenotypic effects of obesity on coronary PVAT remain poorly understood.

Accordingly, the goal of this investigation was to dissect the mechanisms by which lean and obese PVAT-derived factors influence vasomotor tone and the coronary PVAT proteome. In particular, we tested the hypothesis that obesity markedly alters the functional expression and vascular effects of coronary PVAT in favor of an augmented vasoconstriction. This hypothesis is supported by earlier data demonstrating that obesity increases the intracellular Ca^{2+} concentration in isolated smooth muscle cells[42, 66] and enhances coronary vasoconstriction to neurohumoral modulators both *in vitro* and *in vivo* [33, 41, 146]. Our findings provide novel evidence regarding the potential role for specific coronary PVAT-derived proteins in coronary vascular dysfunction in the setting of obesity.

Methods

Ossabaw swine model of obesity

Lean animals (n = 37) were fed ~2200kcal/day standard chow containing 18% kcal from protein, 71% kcal from complex carbohydrates, and 11% kcal from fat. Obese animals (n = 23) were fed an atherogenic diet containing ≥ 8000 kcal/day, 16% kcal from protein, 41% kcal from complex carbohydrates, 19% kcal from fructose, 43% kcal from fat and supplemented with 2.0% cholesterol and

0.7% sodium cholate by weight (5B4L and KT-324, Purina Test Diet, Richmond, IN). Swine of either sex were fed their respective diets for 6-12 months. Lean male Sprague-Dawley Rats (n = 4, 250-300 g) were also utilized for aortic ring experiments.

Functional assessment of isolated coronary rings

Studies on isolated coronary arteries in the presence and absence of coronary PVAT were performed as previously described[110] (see Supplement for detailed methodology and protocols). Briefly, coronary arteries from lean and obese swine were dissected and cleaned of adventitial adipose tissue. Adjacent adipose was cleaned of myocardium and stored in ice-cold Ca^{2+}-free Krebs for later use. Arteries were cut into 3 mm rings and mounted in organ baths for isometric tension studies (**Figure 2.1**). Once an optimal level of baseline passive tension was obtained (~4 g), arteries were stimulated with either KCl (10-60 mM) or prostaglandin F2α (PGF2α; 10 μM) to obtain control responses in the absence of PVAT. Known quantities of adipose tissue (0.1-1.0 g) were then added to the organ baths and arteries allowed to incubate for 30 min at 37°C. Arteries were then stimulated again with KCl or PGF2α in the presence of PVAT; i.e. paired studies in the absence and presence of PVAT were performed on the same arteries. Time control studies were also performed in response to these agonists following a 30 min incubation period in the absence of PVAT. Identical studies were also performed in rat aortic rings exposed to rat peri-aortic adipose tissue. Equimolar replacement experiments of K^+ for Na^+ revealed no differences in the amount of

tension developed in isolated coronary arteries when compared to paired responses without equimolar substitution (**Supplemental Figure 2.1**).

For bioassay experiments, organ baths were drained after control responses and filled with a filtered (0.2 µm) PVAT conditioned supernatant (0.3 g PVAT in 5 ml of Ca^{2+} containing Krebs buffer). Arteries were incubated with this supernatant for 30 min prior to repeat administration of 20 mM KCl. Experiments to examine the effects of lean and obese PVAT on coronary vasodilatory responses to H_2O_2 (1 µM-1 mM) were performed in isolated lean and obese coronary arteries pre-constricted with either KCl (60 mM) or U46619 (1 µM).

Additional "crossover" experiments were performed in lean coronary arteries incubated for 30 min with coronary PVAT obtained from a lean or obese animal sacrificed on the same day. Effects of coronary PVAT-derived factors on sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) were examined by inhibition of SERCA with cyclopiazonic acid (CPA, 10 µM). The role of Rho kinase in PVAT-mediated coronary vasoconstriction was assessed by incubation of lean and obese arteries with the Rho kinase inhibitor fasudil (1 µM).[147] Further studies to examine the effects of calpastatin (1-10 µM) or negative (scrambled) calpastatin peptide (10 µM) on coronary artery contractions were also performed.

Proteomic analyses

Upon sacrifice, hearts were extracted and the aorta immediately perfused with 1L ice-cold Ca^{2+}-free Krebs to remove the blood proteins. Coronary PVAT was excised from the heart, rinsed with PBS and minced. PVAT (0.3 g) was incubated

in 5 mL Ca^{2+}-free Krebs in a 37°C shaking bath and filtered (0.2 μm) before flash freezing the filtrate in liquid N_2. Filtered protein concentration was determined by the Bradford method (Bio-Rad)[148]. Samples were reduced and alkylated by triethylphosphine and iodoethanol and subjected to trypsin digestion as described[149]. Digested samples were analyzed using a Thermo-Finnigan linear ion-trap (LTQ) mass spectrometer coupled with a Surveyor autosampler and MS HPLC system (Thermo-Finnigan). Tryptic peptides were analyzed using a C18 RP column as described[149]. Data were searched against the most recent UniProt protein sequence database of Eutheria using SEQUEST (v. 28 rev. 12) algorithms in Bioworks (v3.3). General parameters were set to: peptide tolerance 2.0 amu, fragment ion tolerance 1.0 amu, enzyme limits set as “fully enzymatic - cleaves at both ends”, and missed cleavage sites set at 2. Searched peptides and proteins were validated by PeptideProphet[150] and ProteinProphet[151] in the Trans-Proteomic Pipeline (TPP, v3.3.0) (http:// tools.proteomecenter.org/software.php). Only proteins with probability ≥ 0.90 and peptides with probability ≥ 0.80 were reported. Protein quantification by label-free quantitative mass spectrometry (LFQMS) was performed using IdentiQuantXLTM software as described[152]. Protein quantities are based on the sum of all corresponding peptide ion intensities derived from the area-under-the-curve of their extracted ion chromatograms and thus have no units.

Fluorescence Imaging

Whole-cell intracellular Ca^{2+} levels were measured at room temperature (22 to 25°C) using the fluorescent Ca^{2+} indicator fura-2, AM and the InCa++ Ca^{2+}

Imaging System (Intracellular Imaging, Cincinnati, OH) as previously described[43]. Briefly, freshly dispersed cells were incubated with 2.5 µM fura-2, AM (Molecular Probes) at 37°C for 30 min before being washed in low Ca^{2+} buffer without fura-2 for 20 min. Fura-2 loaded cells were switched to a 2 mM Ca^{2+} physiologic salt solution before being placed on a coverslip contained within a constant-flow superfusion chamber that was mounted on an inverted epifluorescence microscope (model TMS-F, Nikon). Data are expressed as area under the curve (AUC) of the $F_{360/380}$ ratio during stimulation with KCl (80 mM).

Statistical analyses

Data are presented as mean ± standard error. Phenotypic data for lean vs. obese swine were assessed by t-test. For isometric tension studies, two-way ANOVA was used to test the effects of the PVAT (Factor A) relative to doses of specific agonists (Factor B). When statistical differences were found with ANOVA ($P < 0.05$) a Student-Newman-Keuls multiple comparison test was performed. Comparison of individual protein quantity via experimental group means generated by LFQMS was performed within the IdentiQuantXL platform using a student's t-test. All *P*-values were transformed into q-values to estimate the False Discovery Rate (FDR)[153]. To interpret the biological relevance of the differential protein expression data, protein lists and their corresponding expression values (fold change) were uploaded onto the Ingenuity Pathway Analysis (IPA) software server (http://www.ingenuity.com) and analyzed using the Core Analysis module to rank the proteins into top molecular and cellular functions and canonical pathways.

Results

Vascular effect of PVAT from different anatomical depots

The representative tracing in **Figure 2.2A** outlines the protocol utilized to examine the vascular effects of coronary, subcutaneous and mesenteric PVAT on tone of isolated coronary arteries from lean swine at baseline and in response to 20 mM KCl. In these paired studies, arteries were contracted with KCl before and after 30 min incubation with 0.3 g of PVAT. Data are expressed as delta active tension, which reflects the difference in tension generated in response to KCl, independent of modest increases in baseline tension that tended to occur during the incubation period (average 0.67 ± 0.16 g; $P = 0.06$). Thus, changes in delta active tension do not take into account changes in baseline tension. In lean coronary arteries, coronary ($P = 0.03$) and mesenteric ($P = 0.04$) PVAT significantly increased the tension generated in response to 20 mM KCl (**Figure 2.2B**) relative to time control KCl responses in the absence of PVAT (dashed line). These coronary contractions were completely reversed by inhibition of $Ca_V1.2$ channels with 10 μM diltiazem (**Figure 2.2A**) or 0.1 μM nifedipine. In contrast, incubation with subcutaneous PVAT did not significantly alter coronary artery contractions to KCl ($P = 0.67$), with values similar to time controls. Further studies in tissues obtained from Sprague-Dawley rats also revealed no effect of rat aortic PVAT on KCl-induced contractions of thoracic aortic rings (**Figure 2.2B**).

Additional bioassay experiments which involved the transfer of swine coronary PVAT-conditioned media, instead of the addition of whole 0.3 g pieces of

PVAT to the tissue bath, also revealed similar increases in tension development in response to KCl administration relative to paired, non-PVAT treated control responses (P = 0.04; data not shown).

Vascular effects of lean vs. obese coronary PVAT

Phenotypic data on a subset of lean (n = 6) and obese (n = 10) swine utilized for this investigation are outlined in Table 1. Swine listed were fed high-calorie atherogenic diet for ~6 months, which resulted in significant increases in body weight, heart weight, total cholesterol and the LDL/HDL ratio. To initially examine the effects of lean vs. obese coronary PVAT on baseline tension, coronary artery rings were incubated with known quantities of PVAT (0.1-1.0g) for 30 min. For these studies, PVAT from lean and obese swine were added to tissue baths containing coronary arteries obtained from the same lean or obese heart; i.e. lean PVAT paired with lean coronary artery and obese PVAT paired with obese coronary artery. We found that coronary PVAT increased baseline tension of both lean and obese coronary arteries (**Figure 2.3A**) and noted that this effect was dependent on the amount of PVAT added to the bath (**Figure 2.3B**). Importantly, the increase in basal tone was markedly augmented in tissues obtained from obese vs. lean swine (P < 0.001). Administration of coronary PVAT also increased tension of isolated coronary arteries pre-contracted with 10 μM PGF2α (**Figure 2.3C**). This increase in tension generated with PVAT was also related to the amount of PVAT present in the bath (P < 0.001) and was significantly augmented in obese vs. lean swine (P < 0.001) (**Figure 2.3D**). Coronary contractions to PGF2α

+ PVAT were only partially reversed by the administration of 10 μM diltiazem (82 ± 7%, **Figure 2.3C**), but were completely reversed by 1.0 μM nifedipine (99 ± 0.4%).

Cumulative responses of endothelium intact and denuded coronary arteries to increasing concentrations of KCl (10-60 mM) before and during incubation with coronary PVAT are shown in **Figure 2.4**. Addition of 0.3 g of PVAT increased active tension generated by endothelium intact coronary arteries in both lean (*P* = 0.005, **Figure 2.4A**) and obese swine (*P* = 0.009, **Figure 2.4B**). Similar responses in the presence of PVAT were also observed in endothelium denuded coronary arteries from lean (**Figure 2.4C**) and obese swine (**Figure 2.4D**) (denudation confirmed by < 30% relaxation to 1 μM bradykinin). Further studies also revealed that H_2O_2-mediated vasodilation was markedly attenuated by the presence of coronary PVAT (**Figure 2.5A**) and that this inhibitory effect was much more prominent in tissues obtained from lean (**Figure 2.5B**) vs. obese (**Figure 2.5C**) swine. Importantly, H_2O_2-induced dilation was completely abolished by pre-contracting lean and obese coronary artery rings with 60 mM KCl (**Figure 2.5**).

To evaluate the vasoactive properties of lean vs. obese coronary PVAT independent of differences in coronary artery responsiveness, we performed "crossover" experiments in which KCl-induced contractions of coronary arteries from lean-control swine were assessed before and after incubation with PVAT from either lean or obese swine (**Figure 2.6A**). For these studies, tissues were obtained from lean and obese swine sacrificed on the same day. We found that lean and obese coronary PVAT augmented contractions of lean coronary arteries to 20 mM

KCl to a similar degree (**Figure 2.6B**). Further studies also showed that increases in KCl-induced coronary artery contractions to 20 mM KCl in the presence of PVAT were not significantly altered by inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase with cyclopiazonic acid (CPA, 10 μM, **Figure 2.6C**). However, consistent with earlier studies[31], fura-2 imaging experiments revealed that increases in intracellular Ca^{2+} concentration in response to 80mM KCl were significantly elevated in isolated coronary artery smooth muscle cells not exposed to PVAT from obese vs. lean swine (**Figure 2.6D**).

Obesity markedly alters the protein expression profile of coronary PVAT

To examine whether differences in vascular responses to coronary PVAT are associated with changes in the expression profile of PVAT, a global proteomic assessment was performed on supernatants obtained from lean and obese coronary PVAT. Data revealed substantial alterations in the proteome in the setting of obesity. Overall, we detected alterations in 186 proteins ($P \leq 0.05$) in obese vs. lean PVAT (complete listing of 1,472 quantified non-redundant proteins provided in Supplemental Table 1). A listing of the top up-regulated and down-regulated proteins is provided in Table 2. Ingenuity Pathway Analysis software revealed several proteins involved in cellular growth and proliferation (51 molecules) and cellular movement (39 molecules). In particular, increases in RhoA (2.9-fold) and calpastatin (1.6-fold) are of interest as these pathways are directly linked to smooth muscle contraction, Ca^{2+} sensitization, and both are implicated in the progression of hypertension[154, 155].

To examine whether Rho kinase signaling participated in the augmented effects of coronary PVAT in coronary arteries, we performed studies on isolated arteries in the presence of the Rho kinase inhibitor fasudil (1 μM). We found that fasudil alone dramatically decreased maximal active tension development in response to KCl in lean coronary arteries (**Figure 2.7A vs. Figure 2.4A**, $P < 0.001$) and diminished the effect of PVAT on KCl contractions (**Figure 2.7C**). This effect of fasudil was less apparent in obese arteries where maximal contractions to KCl were similar to that of untreated-controls (**Figure 2.7B vs. Figure 2.4B**, $P = 0.108$). Despite blunting lean coronary KCl contractions, in the presence of fasudil, PVAT still elevated contractions relative to control in both lean and obese arteries ($P = 0.003$ and $P = 0.037$, respectively).

Additional proof-of-principle studies were performed to investigate the effects of calpastatin on coronary artery contractions. In these experiments, lean coronary arteries were incubated with increasing concentrations of calpastatin peptide for 30 min without PVAT (1-10 μM, **Figure 2.7D**). We found that calpastatin dose-dependently increased tension development of lean coronary arteries to 20 mM KCl ($P = 0.008$) and that 10 μM calpastatin augmented contractions to a similar extent as PVAT itself (1.82 ± 0.45 vs. 1.49 ± 0.19). Negative control experiments revealed no effect of a scrambled calpastatin peptide (10 μM) on coronary artery contractions to 20 mM KCl (**Figure 2.7D**).

Discussion

The present investigation was designed to elucidate the mechanisms by which lean and obese PVAT-derived factors influence vasomotor tone and the coronary PVAT proteome. The studies were designed to test the hypothesis that obesity markedly alters the functional expression and vascular effects of coronary PVAT in favor of an augmented contractile phenotype. The major new findings of this study include: 1) vascular effects of PVAT vary according to anatomical location as coronary and mesenteric, but not subcutaneous adipose tissue augmented coronary artery contractions to KCl; 2) factors derived from coronary PVAT increase baseline tension and potentiate constriction of isolated coronary arteries to PGF2α relative to the amount of adipose tissue present; 3) vascular effects of coronary PVAT are markedly elevated in the setting of obesity and occur independent of effects on, or alterations in coronary endothelial function; 4) augmented effects of obese coronary PVAT are associated with substantial alterations in the PVAT proteome and underlying increases in vascular smooth muscle Ca^{2+} handling via $Ca_V1.2$ channels, H_2O_2-sensitive K^+ channels or upstream mediators that converge on these channels; 5) factors converging on Rho-kinase are largely responsible for the increase in coronary artery contractions to PVAT in lean, but not obese swine. These findings provide the first evidence that factors released from coronary PVAT initiate/potentiate coronary vasoconstriction and that this effect is augmented in the setting of obesity.

Differential effects of coronary PVAT in lean vs. obese swine

Currently, data on the vascular effects of coronary PVAT are rather limited and conflicting, as PVAT has been shown to either decrease endothelial-dependent dilation in lean-healthy dogs[118] or to have limited/no effect on endothelial function in lean[110, 156] or hypercholesteromic[102, 156] swine. In contrast, findings from Payne *et al.* indicate that coronary PVAT significantly exacerbates underlying endothelial dysfunction in the setting of obesity and the MetS[110]. Additional findings suggest that coronary PVAT has relatively modest "anti-contractile" effects in lean and hypercholesterolemic swine[102], which differs from numerous other studies which found evidence of adipose derived relaxing factors (ADRFs) in PVAT surrounding vessels such as the aorta, mesenteric arteries, and internal thoracic arteries (see [100] for review). Taken together, these earlier studies suggest that the expression and effects of PVAT-derived factors may differ substantially between vascular beds and can be influenced by underlying disease states.

Our present data support differential effects of PVAT from different anatomical depots in that coronary and mesenteric, but not subcutaneous adipose tissue augmented coronary artery contractions in response to smooth muscle depolarization with 20 mM KCl (**Figure 2.2B**). Consistent with previous studies[107], this effect was not observed in aortic tissues obtained from rats. Further studies supporting the presence of coronary adipose-derived constricting factors showed that the addition of coronary PVAT to PGF2α-constricted arteries actually increased coronary vasomotor tone (**Figure 2.3C**), as opposed to decreasing

tension, which would be expected if ADRFs predominated in coronary PVAT. Bioassay experiments involving the transfer of PVAT-conditioned media to isolated coronary arteries support the release of transferable (paracrine) constricting factors from coronary PVAT (P = 0.04). Importantly, these effects were dependent on the amount of PVAT added to the baths (**Figure 2.3**) and were significantly greater than responses observed in parallel time-control studies. Our findings are consistent with studies that found PVAT attenuated vasodilator influences in obese mice[114] and potentiated vasoconstriction to electrical field stimulation in rat mesenteric arteries[111].

A novel finding of this investigation is that the effects of coronary PVAT on baseline and agonist-mediated contractions were markedly elevated in tissue obtained from obese vs. lean swine (**Figure 2.3B and 2.3D**). To address whether this augmentation was related to inherent mechanistic differences in vascular smooth muscle, crossover studies were performed in which coronary PVAT from either lean or obese swine was added to clean, lean coronary arteries. We found that PVAT from lean and obese swine increased coronary contractions to 20 mM KCl to a similar extent (**Figure 2.6B**). Additional experiments in clean, obese arteries yielded similar results (data not shown). Consistent with earlier data from our laboratory and others which indicate that obesity augments $Ca_V1.2$ current and vasoconstriction[42, 157, 34], the present fura-2 Ca^{2+} imaging studies support that voltage-dependent increases in intracellular Ca^{2+} concentration to KCl are significantly greater in isolated smooth muscle cells from obese vs. lean swine (**Figure 2.6D**). Based on these findings we propose that the augmented contractile

effects of obese coronary PVAT are related to inherent differences in smooth muscle responsiveness between obese and lean coronary arteries, and that this effect is mediated by elevated activity/ expression of $Ca_V1.2$ channels and/or alterations in the role of K^+ channels in obese arteries[34]. Importantly, inhibitory effects of PVAT derived-factors on K^+ channels (**Figure 2.5**) would also serve to activate $Ca_V1.2$ channels and augment coronary artery contractions[49]. However, the exact K^+ channel subtypes on smooth muscle and/or the endothelium affected by coronary PVAT warrants further investigation.

We postulate that these effects of PVAT derived factors occur independent of influences on coronary endothelium or decreased Ca^{2+} buffering by the sarcoplasmic reticulum as endothelial denudation (**Figure 2.4C and 2.4D**) or inhibition of the SERCA pump with CPA (**Figure 2.6C**) had little/no effect on the contractile effects of PVAT. However, we cannot rule out the possibility that the observed effects of coronary PVAT are mediated by factors released from endothelial cells within the PVAT vasculature and/or by factors released from other cell types within the PVAT.

Effects of obesity on coronary PVAT proteome and mechanisms of coronary contraction

Although recent evidence indicates that hypercholesterolemia, obesity and diabetes alter phenotypic expression patterns of specific adipokines in coronary PVAT at the protein and mRNA level[9, 141, 156], no study has performed global proteomic screening of the coronary PVAT proteome in lean vs. obese subjects. Data from our LC-MS/MS revealed significant dysregulation in the abundance of numerous proteins in obese PVAT supernatant, many of which were not previously reported[9]. In general, Ingenuity Pathway Analysis indicated that a significant number of altered proteins corresponded with pathways associated with cellular growth-proliferation and movement. In particular, it was intriguing that expression of RhoA was significantly elevated in samples obtained from obese vs. lean coronary PVAT (**Table 2.2**), which suggests that the enhanced effects of obese coronary PVAT could be mediated via increases in Rho-kinase-mediated constriction. Interestingly, we documented that inhibition of Rho-kinase with fasudil markedly reduced the maximal contractions to KCl in lean arteries (**Figure 2.4A versus 2.7A**), but had little effect on maximal KCl contractions in obese arteries (**Figure 2.4B versus 2.7B**). In addition, fasudil significantly diminished PVAT-mediated increases in coronary artery contractions to 20 mM KCl in lean but not obese arteries (**Figure 2.7C**). These data indicate that PVAT-derived factors increase coronary artery contractions in lean swine via a Rho-kinase dependent mechanism, whereas increases in contraction in obese swine occur via Rho-

independent pathways; i.e. alteration in the contribution of K^+ channels (**Figure 2.5**) or augmented function of $Ca_V1.2$ channels (**Figure 2.6D**).

Additional proof of principle studies were performed based on our global proteomics data which detected significant up-regulation of calpastatin fragments in PVAT supernatant of obese vs. lean swine. This protein, encoded by the CAST gene, was recently shown to be a partial agonist for the intracellular domain of $Ca_V1.2$ channels in smooth muscle[158]. Our experiments documented, for the first time, that calpastatin dose-dependently augments coronary artery contractions, at levels similar to that observed in the presence coronary PVAT (**Figure 2.7D**). Although these data suggest that calpastatin is a coronary adipose-derived constricting factor, further studies are needed to more directly address the vascular effects of calpastatin.

Limitations of the study

It is important to recognize that these studies were conducted using coronary samples obtained from lean and obese swine hearts, thus it is presently unclear to what extent these findings translate to the human clinical setting. However, our data provide direct evidence of differential effects of PVAT from different anatomic depots in the same species as well as the influence of underlying phenotype (obesity) that should be further explored. Coronary artery tension in this study was measured after relatively short term exposure to PVAT *in vitro* (30 min). Thus, whether such effects of PVAT would manifest *in vivo* has yet to be established. LFQMS analysis revealed only the most abundant proteins

present in the PVAT supernatants and those identifiable/quantifiable by trypic digestion. This approach may not detect potentially relevant proteins present in low concentration (e.g. specific adipokines such as leptin (18kDa) or resistin (11 kDa) or those that were either not effectively proteolyzed or are so small as to yield a few detectable peptides). We acknowledge that the focus on the coronary PVAT proteome eliminates examination of bioactive lipids or other macromolecules that may contribute to the effects of PVAT. However, while we cannot rule out this possibility, our initial studies have demonstrated no effect of lysophosphatidic acid on baseline coronary artery tension or KCl-induced contractions. Furthermore, we submit that it is unlikely that vasoactive factors responsible for the effects of coronary PVAT in this investigation are hydrophobic lipids, as these molecules are insoluble in the Krebs buffer utilized in the present experiments. This point is supported by the recent study of Lee *et al.* which required the use of a PVAT superfusion bioassay cascade system in order to discern vascular effects of PVAT-derived palmitic acid methyl ester[159]. Clearly, further investigation regarding the identity of the coronary PVAT-derived factors, the specific cell types involved and the influence obesity has on these cells and factors is warranted.

Conclusions and implications

Data from this investigation provide novel evidence that coronary PVAT is capable of releasing factors that initiate/potentiate contraction of coronary arteries, independent of effects on coronary endothelium. Importantly, the vascular influence of PVAT is specific to anatomic location and is augmented in the setting

of obesity. We propose this augmented effect of PVAT is related to alterations in Rho-dependent signaling, increased functional expression of $Ca_V1.2$ channels and/or diminished/altered activity of K^+ channels in obese coronary arteries. In addition, marked alterations in the expression profile of the coronary PVAT proteome in obese swine uncovers new potential therapeutic target proteins (e.g. calpastatin) and signaling pathways that may not only contribute to the regulation of vascular smooth muscle tone, but to the initiation of smooth muscle differentiation and proliferation observed in obesity-induced cardiovascular disease[9, 141, 160, 161].

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Disclosures

The authors have no conflicts to disclose.

Tables and Figures

	Lean	Obese
Body Weight (kg)	52 ± 3	82 ± 5*
Heart wt. (g)	183 ± 13	278 ± 14*
Glucose (mg/dl)	75 ± 2	86 ± 5
Insulin (μU/ml)	21 ± 5	27 ± 7
Total cholesterol (mg/dl)	88 ± 5	567 ± 60*
LDL/HDL ratio	1.6 ± 0.1	12 ± 4
Triglycerides (mg/dl)	43 ± 5	98 ± 25

Table 2.1 Phenotypic characteristics of lean and obese Ossabaw swine. Values are mean ± SE for 12-month old lean (n = 6) and obese (n = 10) swine. *P < 0.05 t-test, lean vs. obese swine.

Up-Regulated Proteins

Gene Name	Protein Name	Fold Change	P-value
RHOA	Transforming protein RhoA	2.9	0.04
LAMC1	Laminin, gamma 1	1.7	0.004
CS	Citrate synthase	1.7	0.03
HSPA2	Heat shock-related 70 kDa protein 2	1.7	0.05
HSPD1	60 kDa heat shock protein, mitochondrial	1.6	0.005
CAST	Calpastatin	1.6	0.006
ALDH7A1	Alpha-aminoadipic semialdehyde dehydrogenase	1.6	0.04
PROSC	Proline synthase co-transcribed bacterial homolog protein	1.6	0.05
PGM3	Phosphoacetylglucosamine mutase	1.5	0.0002
CSTB	Cystatin-B	1.5	0.0006

Down -Regulated Proteins

Gene Name	Protein Name	Fold Change	P-value
DDAH2	Dimethylarginine dimethylaminohydrolase 2 (Fragment)	5.5	0.02
VBP1	Prefoldin subunit 3	3.8	0.03
CAPG	Macrophage-capping protein	3.1	0.02
PSMA3	Isoform 2 of Proteasome subunit alpha type-3	2.7	0.02
HNRNPAB	Heterogeneous nuclear ribonucleoprotein A/B	2.7	0.003
ZNF439	Zinc finger protein 439	2.6	0.03
F9	Coagulation Factor IX	2.6	0.002
ME1	Malic Enzyme	2.5	0.0009
PGK	Phosphoglycerate kinase	2.4	0.02
BLMH	Bleomycin hydrolase	2.4	0.006

Table 2.2 Secreted protein expression profile of coronary PVAT in obese versus lean swine. Values for fold change in expression of obese (n = 5) vs. lean (n = 5) coronary PVAT supernatants.

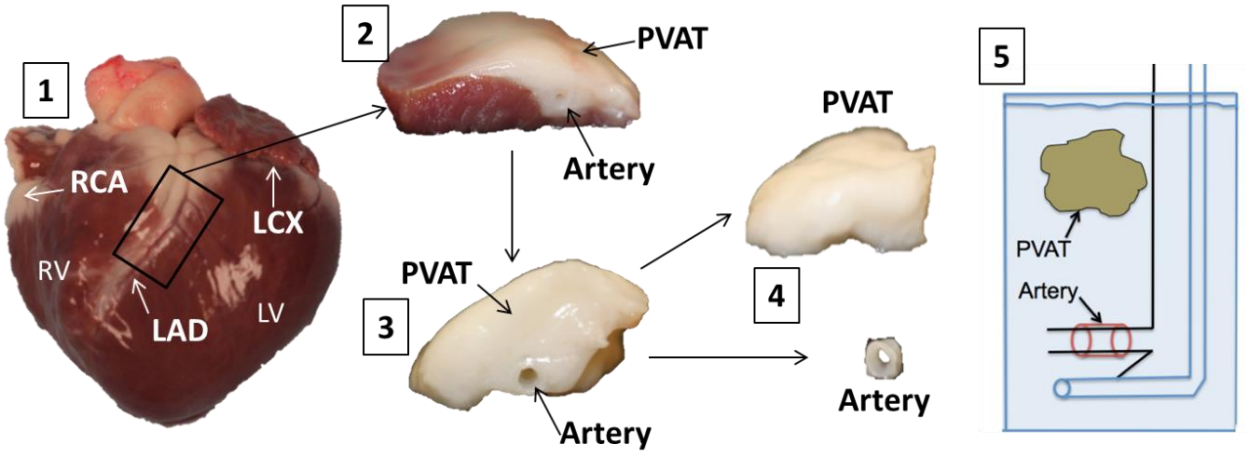

Figure 2.1 Representative picture illustrating isolation of coronary artery PVAT and isometric tension methodology. RV (right ventricle), LV (left ventricle), RCA (right coronary artery), LCX (left circumflex artery), LAD (left anterior descending artery), PVAT (perivascular adipose tissue). 1) Lean and obese hearts were excised upon sacrifice and perfused with Ca^{2+}-free Krebs to remove excess blood; 2) Arteries and PVAT were grossly isolated from the heart; 3) the myocardium was removed; 4) arteries were further isolated and surrounding PVAT dissected away; 5) 3 mm lean and obese arteries were mounted in organ baths at 37°C.

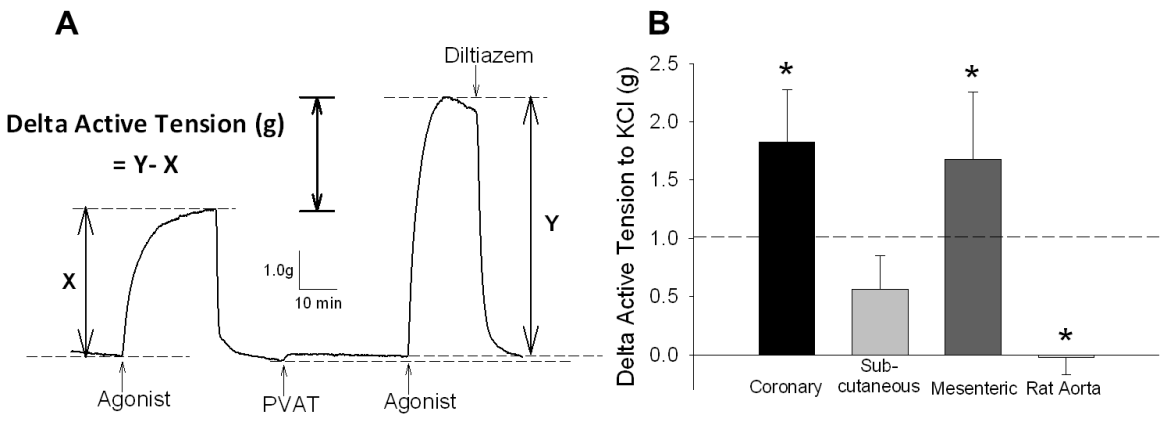

Figure 2.2 Representative tracing of paired experiments to assess the vascular effects of PVAT from different anatomical depots. A, Representative wire myograph tracing of tension generated by arteries before (x) and after (y) the addition of PVAT to the organ bath. Upward deflections indicate an increase in tension (constriction). The difference in tension generated by each artery before (x) and after (y) PVAT is expressed as Delta Active Tension (g) and is independent of changes in baseline with PVAT. B, Delta active tension (g) of coronary arteries before and after exposure to coronary PVAT, subcutaneous adipose or mesenteric PVAT (0.3 g each). **P* < 0.05 vs. average of paired time controls (represented by dashed line; 1.01 ± 0.21 g).

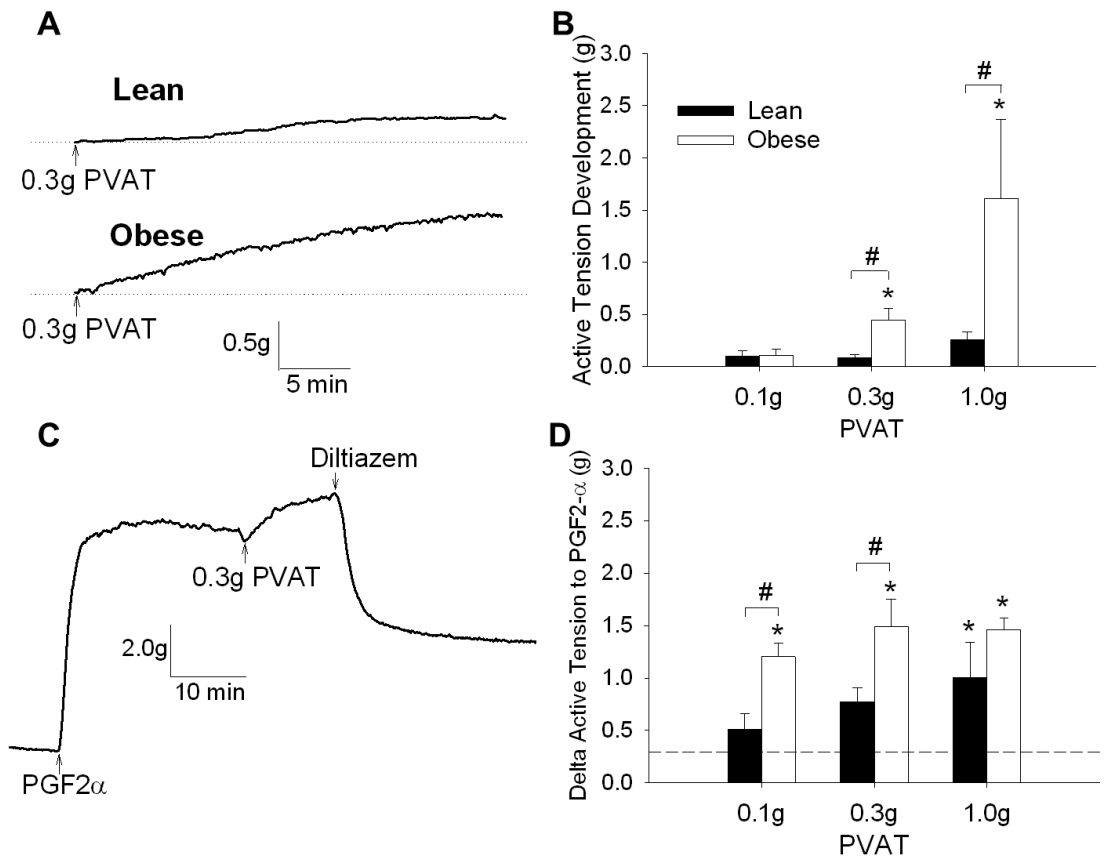

Figure 2.3 Effect of PVAT on baseline tension and response to PGF2α. A, Representative tracings of a lean and obese artery after addition of 0.3 g PVAT for 30 min. B, Addition of coronary PVAT (0.1-1.0g) to the organ bath increased tension in both lean and obese arteries and was dependent on the amount of coronary PVAT added to the bath. C, Representative tracing of a lean artery contracted with PGF2α to plateau, incubation with PVAT and treatment with diltiazem (10 μM). D, Delta active tension of arteries stimulated with PGF2α before and after the addition of coronary PVAT (0.1-1.0 g). **P* < 0.05 vs. average of paired time controls (represented by dashed line; 0.29 ± 0.08 g). #*P* < 0.05 lean vs. obese, same amount of PVAT.

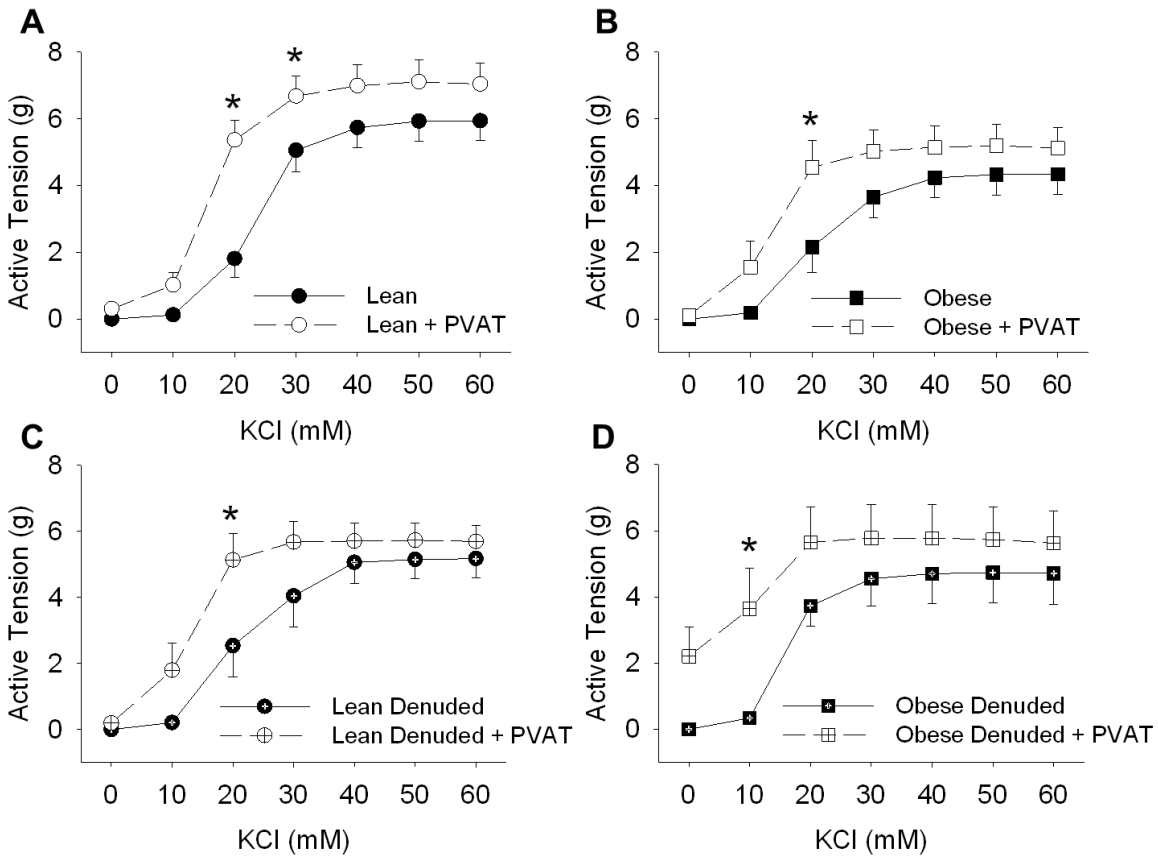

Figure 2.4 KCl dose-response curves in intact and denuded coronary arteries in the presence and absence of PVAT. Cumulative dose-response data of lean (A) and obese (B) arteries to KCl (10-60 mM) before and after coronary PVAT incubation (30 min). Arteries were incubated with coronary PVAT from the same animal on the same day. Cumulative dose-response data from denuded lean (C) and obese (D) vessels before and after PVAT incubation. *$P < 0.05$ vs. no PVAT-control at same KCl concentration.

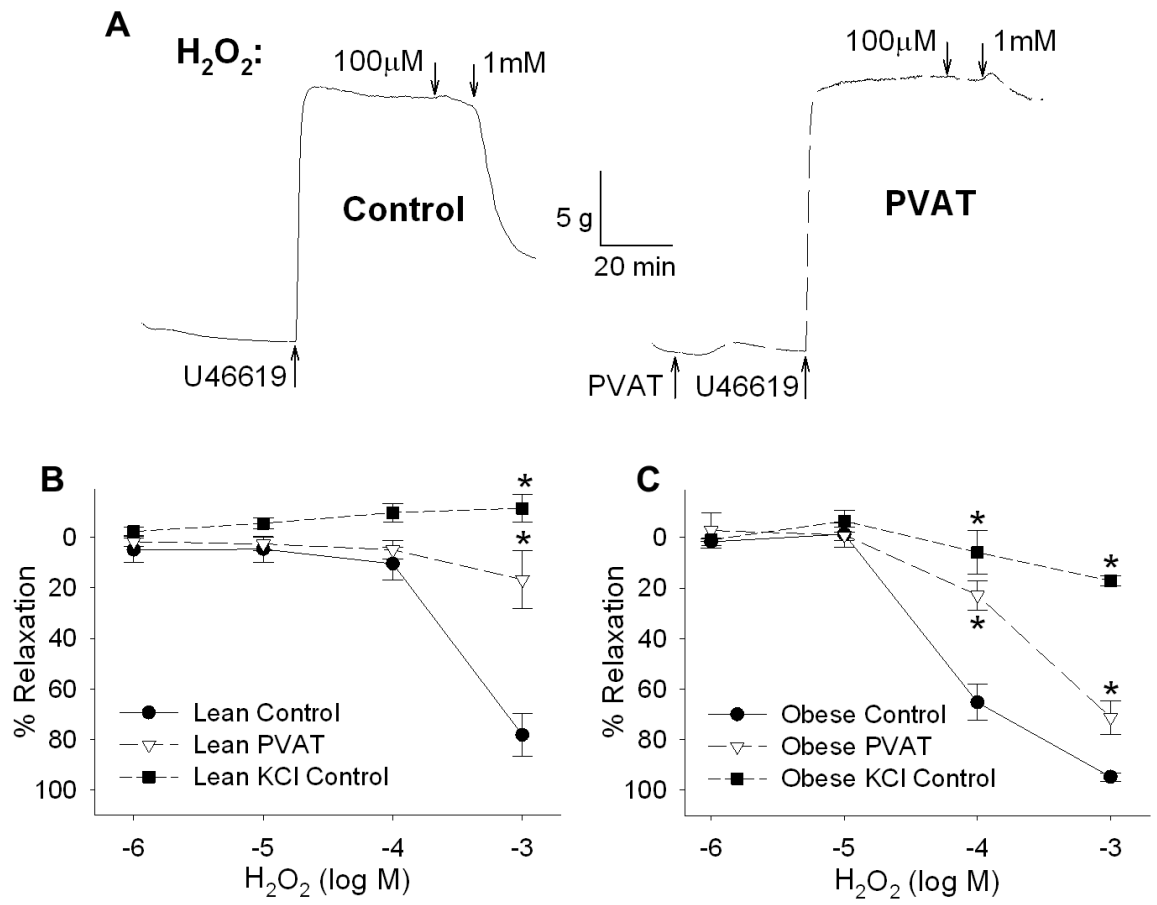

Figure 2.5 Effect of PVAT on coronary vasodilation to H_2O_2. A, Representative tracings of H_2O_2-induced relaxations of lean control arteries pre-constricted with 1 μM U46619 in the absence and presence of PVAT. Average percent relaxation of lean (B) and obese (C) control and PVAT-treated arteries to H_2O_2 after pre-constriction with either U46619 (1 μM) or KCl (60 mM). *P < 0.05 vs. control at same H_2O_2 concentration.

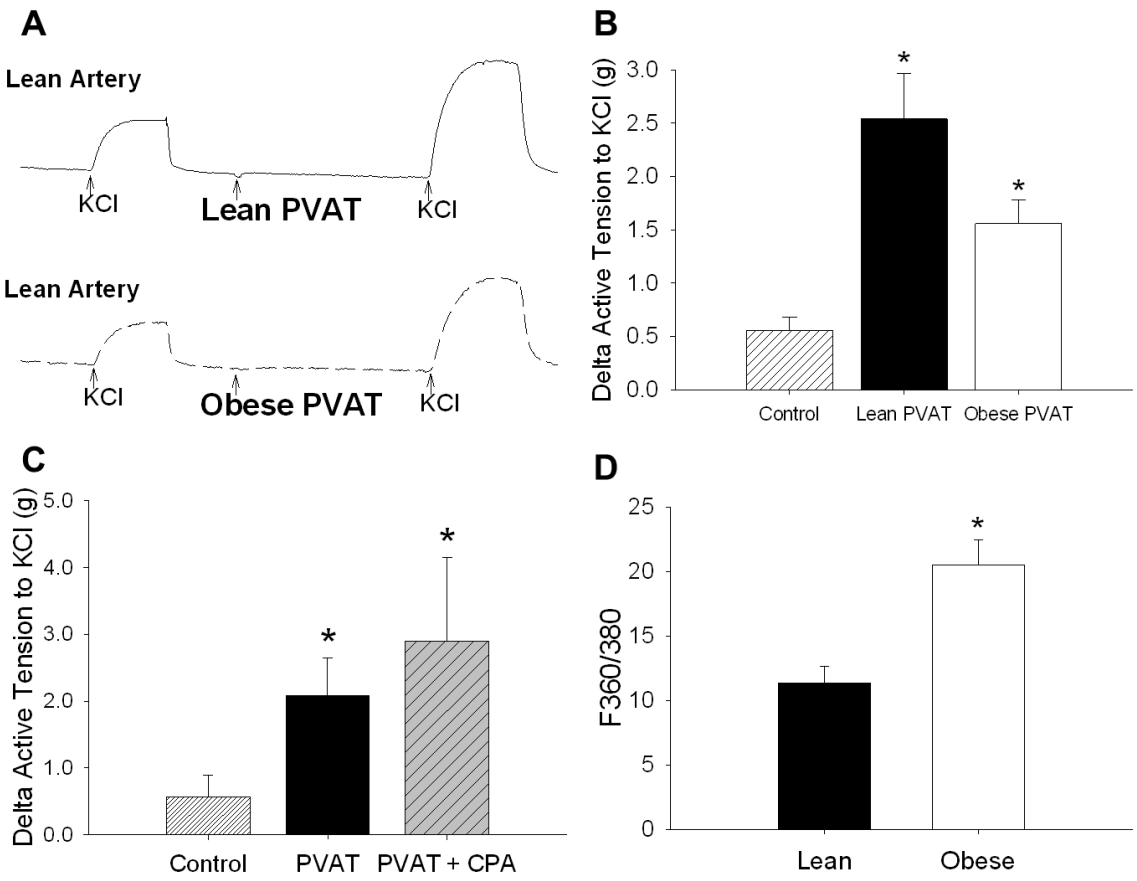

Figure 2.6 Vascular effects of lean vs. obese coronary PVAT. A, Representative tracings of lean arteries treated with 20 mM KCl, exposed to either lean or obese PVAT. B, Delta active tension (g) to 20 mM KCl of lean arteries exposed to time control, lean or obese PVAT. **P* < 0.05 vs. control. C, Delta active tension (g) to 20 mM KCl after exposure to SERCA inhibition with CPA (10 μM) *P* < 0.05 vs. control. D, F360/F380 ratio of fura-2 experiments after stimulation of isolated lean (n = 4) and obese (n = 5) coronary vascular smooth muscle with 80 mM KCl. **P* < 0.05 obese vs. lean.

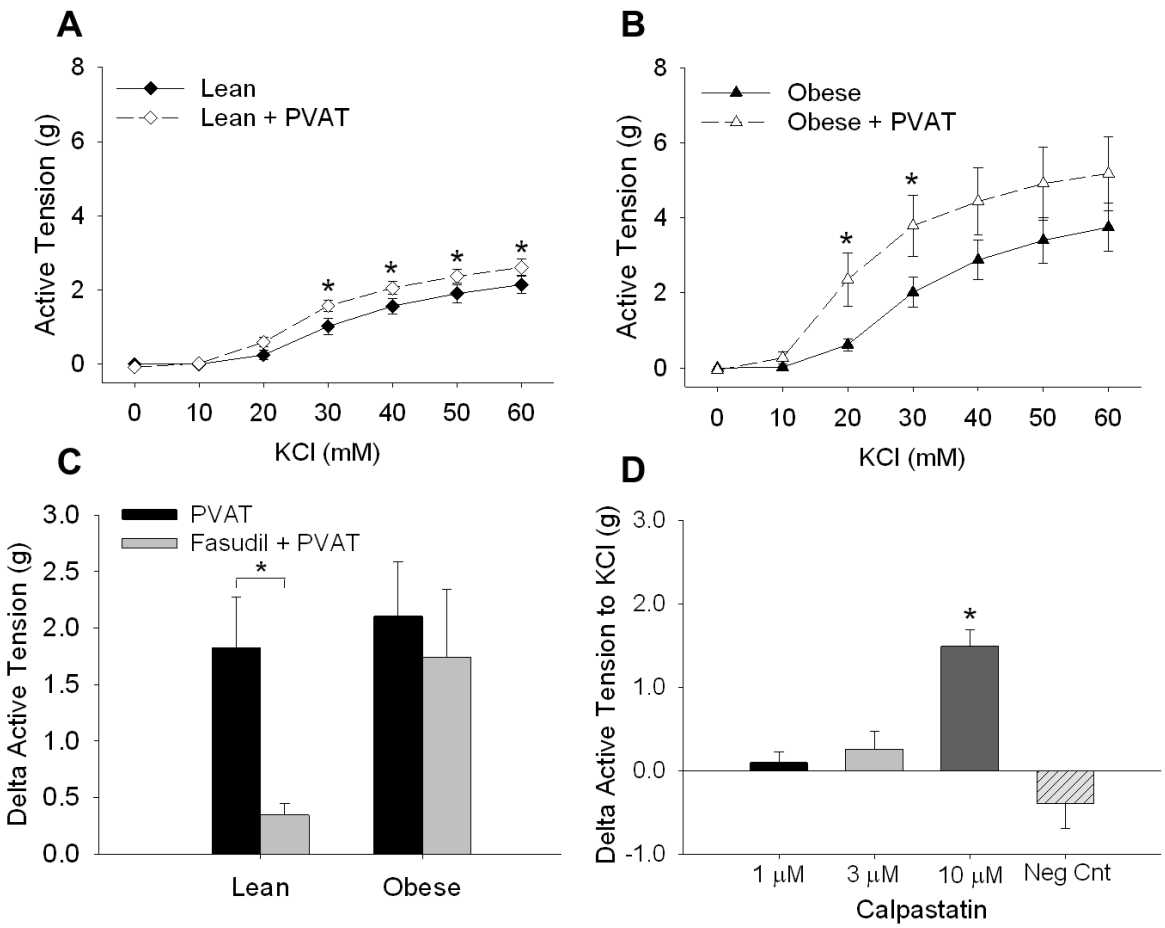

Figure 2.7 Effects of Rho kinase signaling and calpastatin on coronary artery contractions to KCl. Lean (A) and obese (B) arteries were incubated with 1 μM fasudil for 10 min prior to dose-responses to KCl (10-60 mM) in the absence and presence of coronary PVAT. **P* < 0.05 vs. no PVAT-control at same KCl concentration. C, Delta active tension (g) in response to 20 mM KCl in lean and obese PVAT control and PVAT + fasudil-treated arteries **P* < 0.05 vs. respective PVAT control. D, Delta active tension (g) to 20 mM KCl after incubation with increasing concentrations of calpastatin (1-10 μM) or scrambled calpastatin peptide (10 μM Neg Cnt) for 30 min. **P* < 0.05 relative to time control.

Chapter 3: Conclusion

Summary of the Findings

Unless dramatic changes are made to educating the general population about proper nutrition and improving physical and cardiovascular health, the pandemic of obesity will continue to spread worldwide. As obese children become adults, the dramatic prevalence of MetS will challenge healthcare systems to manage the rise in associated cardiovascular diseases. Although we are beginning to uncover some of the vast capabilities of adipose tissue as an endocrine and paracrine organ, the exact mechanism(s) by which increasing adiposity in obesity leads to cardiovascular disease is not yet understood.

Mounting evidence suggest phenotypic changes to PVAT could contribute to obesity associated cardiovascular disease. In particular, echocardiography[130], magnetic resonance imaging[129] and computed tomography[131, 132] reveal coronary PVAT is one of the best predictors of CAD, even more predictive of atherosclerotic burden that visceral adipose[92]. This is important because it suggests a novel, paracrine pathway to the development of vascular disease that may function independent of changes to visceral adipose and systemic adipokine levels. Furthermore, in addition to the increase in PVAT volume, studies reveal local inflammation and aberrant regulation of adipokines released from coronary PVAT may also exacerbate underlying vascular dysfunction in MetS[82, 95, 118, 137, 145]. The effects of adipokines on hypertension, endothelial function, cardiac pathology, atherosclerosis and inflammation implicate this local adipose depot in the

pathogenesis of vascular dysfunction, however, no one has established a causal link between PVAT and coronary artery disease.

With these earlier studies in mind, the central focus of the present investigation was to investigate the potential role of coronary PVAT in the development of smooth muscle dysfunction. Specifically, our goal was to elucidate the mechanisms by which lean and MetS PVAT-derived factors influence vascular smooth muscle ion channel function and the coronary PVAT proteome. The major findings of our investigation are summarized:

Aim 1: Test the hypothesis that coronary PVAT augments tension development of coronary arteries and is potentiated in MetS. Results from these studies indicate that PVAT-derived factors are capable of influencing coronary vasomotor tone in lean “healthy” arteries. Independent of any changes associated with MetS, addition of lean PVAT to a cleaned isolated coronary artery increased baseline tension, and augmented constriction of coronary arteries to other pharmacological stimuli (**Figure 2.3**). These effects were dependent upon the amount of adipose tissue present and were observed in denuded arteries, suggesting the effect of PVAT in the coronary circulation is volume-dependent, endothelial-independent and mediated by direct action on coronary vascular smooth muscle cells.

Our data support that the vascular effects of PVAT vary according to the anatomical location as coronary and mesenteric adipose tissue potentiated contraction of coronary arteries, whereas subcutaneous adipose and rat periaortic

adipose tissue attenuated constriction relative to the time control rings in coronary and rat aorta, respectively (**Figure 2.2**). To specifically examine the role of potential "ADRFs" in coronary PVAT, adipose tissue was added to coronary arteries pre-constricted with PGF2α. We found that the addition of PVAT to these pre-constricted arteries augmented coronary artery tension, suggesting the predominance of adipose derived constricting factors in coronary PVAT of Ossabaw swine.

Experiments to examine the vasoregulatory effects of PVAT in the setting of MetS demonstrated that MetS PVAT also increased baseline tension and PGF2α mediated contractions of isolated coronary arteries, but that the increases relative to the amount of PVAT added were significantly elevated compared to the lean-controls (**Figure 2.3**). Taken together, our findings related to Aim 1 provide novel evidence that factors released from coronary PVAT are capable of augmenting coronary vasomotor tone, independent of effects on endothelium. In addition, the effect of coronary PVAT derived constricting factors is augmented in the setting of obesity/MetS, suggesting these factors are altered in disease.

Aim 2: Identify and examine the potential coronary PVAT-derived factors that mediate the vascular effects in lean vs. MetS coronary arteries. To accomplish this aim we performed global proteomic assessment of coronary PVAT from lean and MetS swine. This examination represents the first systematic examination of the coronary PVAT proteome and revealed substantial alterations in the proteome in the setting of obesity. Overall, we detected alterations in 186

proteins ($P \leq 0.05$) in obese vs. lean PVAT (complete listing of 1,472 quantified non-redundant proteins provided in Supplemental Table 1). Ingenuity Pathway Analysis software revealed several proteins involved in cellular growth and proliferation (51 molecules) and cellular movement (39 molecules). However, increases in RhoA (2.9-fold) and calpastatin (1.6-fold) were of particular interest as these pathways are directly linked to smooth muscle contraction, Ca^{2+} sensitization, and both are implicated in the progression of hypertension[154, 155]. Further studies to examine the effects of calpastatin, a known endogenous calpain inhibitor[155, 158] revealed that this protein augments contractions of isolated coronary arteries to an extent similar to coronary PVAT (**Figure 2.7**). Although calpastatin has been implicated as an agonist of L-type calcium channels in ventricular cardiomyocytes[158], this study is the first investigate its effects on vascular reactivity. A potential role for RhoA is discussed in Aim 3. Overall, the marked alterations in the expression profile of the coronary PVAT proteome in MetS swine uncovers new potential therapeutic target proteins (e.g. calpastatin) and signaling pathways that should be further explored.

Aim 3: Identify the specific vascular smooth muscle signaling pathways and end effector ion channels that mediate the vascular consequences of PVAT in MetS. To examine potential mechanisms by which coronary PVAT augments smooth muscle contraction, we first examined the effect of PVAT on lean vs. MetS coronary vasodilation in response to H_2O_2; which has been previously shown to induce dilation via activation of K^+ channels[162]. Our

findings revealed that H_2O_2-mediated vasodilation was markedly attenuated by the presence of coronary PVAT (**Figure 2.5A**) and that this inhibitory effect was much more prominent in tissues obtained from lean (**Figure 2.5B**) vs. obese (**Figure 2.5C**) swine. More recent experiments documented similar inhibitory effects of PVAT on coronary vasodilation in response to adenosine (**Figure 3.1**). Interestingly, vasodilation to adenosine in the absence of PVAT was diminished in lean vs. MetS coronary arteries ($P \leq 0.001$). In contrast, previous *in vivo* studies revealed that the increase in coronary blood flow to adenosine was diminished in MetS v. Lean swine[163], while the responses of pressurized arterioles to the adenosine analog 2-chloroadenosine (2-CAD) were not different between early stage MetS v. lean swine[164]. These differences in the effect of PVAT indicate that the MetS significantly alters the adipocytokine factors released from PVAT (Aim 2), the vascular effects of these factors, and/or the underlying mechanisms of smooth muscle contraction and dilation.

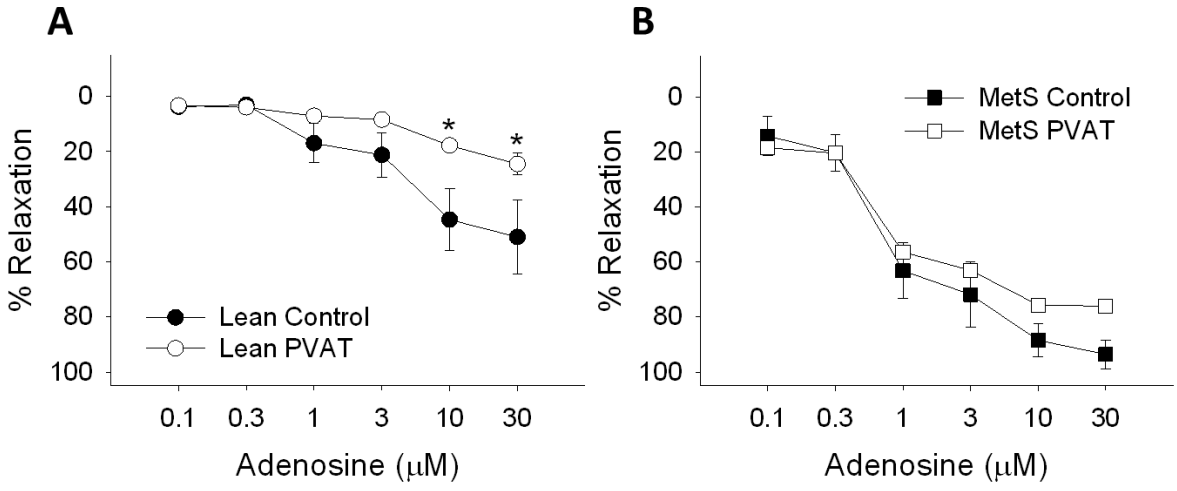

Figure 3.1 Effect of PVAT on coronary vasodilation to Adenosine. Average percent relaxation of lean (A) and MetS (B) control and PVAT-treated arteries to Adenosine after pre-constriction with U46619 (1 μM). *$P < 0.05$ vs. control at same Adenosine concentration.

In studies to examine these potential mechanisms we found that Rho kinase expression was increased 2.9-fold in MetS PVAT supernatant, but interestingly that Rho kinase inhibition in MetS tissues did not reduce tension development nor influence the effect of PVAT on MetS contractions to KCl as observed in lean coronary arteries (**Figure 2.7A and B**). These data, which indicate that the MetS alters the regulatory pathways responsible for vascular smooth muscle contraction, are further supported our Ca^{2+} imaging experiments which demonstrated that the rise in intracellular Ca^{2+} concentration in response to KCl-induced depolarization was significantly elevated in isolated coronary vascular smooth muscle cells from MetS vs. lean swine (**Figure 2.6D**). In addition, our "crossover" studies also support that the vascular effect of PVAT is dependent on the underlying phenotypic state of the coronary vascular smooth muscle (**Figure 2.6A and B**).

Taken together, these studies indicate that the expression and effect of PVAT-derived factors differs substantially between vascular beds and is influenced by underlying disease states (see schematic diagram in **Figure 3.2**). Additional studies to delineate which PVAT-derived factors (e.g. calpastatin) are influencing coronary vascular reactivity could provide novel targets for intervention before progression into overt atherosclerosis. In addition, future studies on coronary vascular smooth muscle ion channels and signaling pathways will need to consider the influence of this proximal adipose depot.

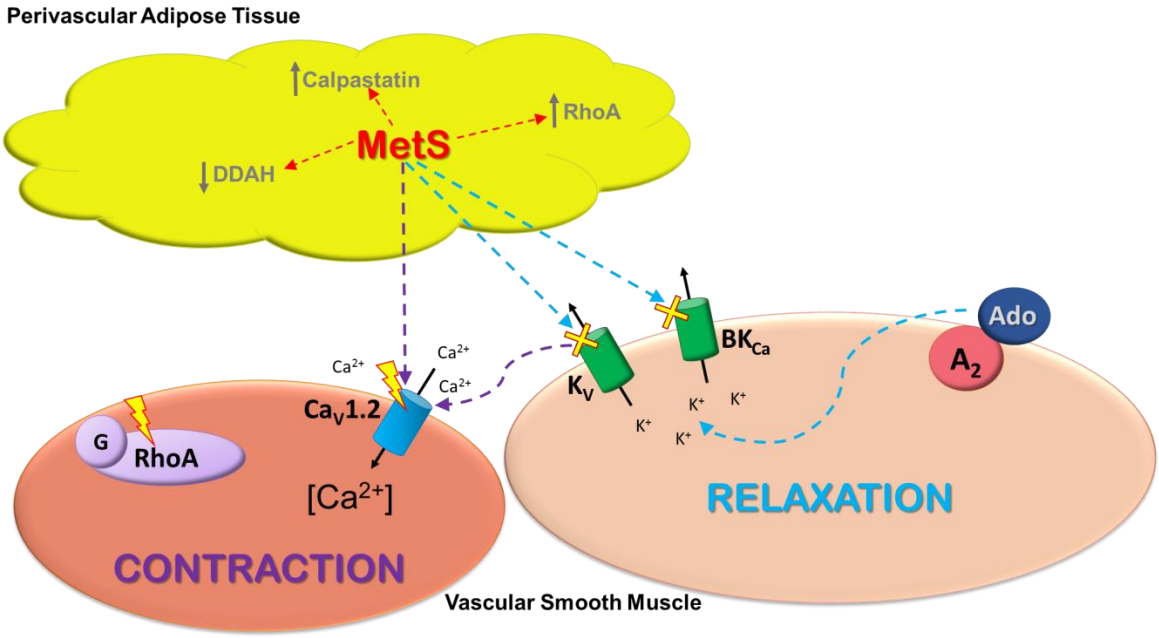

Figure 3.2 Schematic representing proposed mechanisms of coronary PVAT action on vascular smooth muscle reactivity. Proteomics revealed increased Calpastatin, RhoA and decreased DDAH protein expression in MetS PVAT supernatants vs. lean. Our data propose PVAT increases contraction by releasing factors that converge on $Ca_V1.2$ channels increasing its activity. PVAT also attenuates relaxation to H_2O_2 and adenosine, which is proposed to occur via inhibition of BK_{Ca} and K_V channel activity. Additionally, inhibition of K^+ channel activity in vascular smooth muscle couples to increased $Ca_V1.2$ channel activity, potentiating constriction even further.

Future Directions and Proposed Studies

This investigation uncovered many novel observations regarding the role of PVAT on coronary smooth muscle vascular reactivity; however, several key issues still require elucidation. In particular, we have yet to identify the specific PVAT-derived factors that facilitate the effects observed in lean vs. MetS coronary arteries. In addition we have yet to narrow down the specific signaling cascades involved in converging on smooth muscle $Ca_V1.2$ and K^+ channels. However, identification of these channels have provided a starting point with which subsequent investigations involving inhibition of smooth muscle signaling pathways may elucidate key mediators involved. In addition, our translational pig model will help to facilitate studies that knock down or up-regulate specific PVAT-derived targets that may be mediating vascular dysfunction in MetS. Together, these proposed issues will help to advance our understanding of how perivascular adipose-derived adipokines contribute to coronary artery disease.

Coronary Smooth Muscle Signaling

Our studies identified several ion channels that function to mediate changes in vascular tone in the presence of PVAT. Subsequent experiments to identify the specific smooth muscle signaling pathways involved in activation of these ion channels may uncover mechanisms by which PVAT can influence coronary reactivity. In addition, understanding how PVATs effect on signaling pathways differs in MetS may help to unravel the specific vascular smooth muscle alterations that occur as a result of phenotypic changes to PVAT.

Several signaling pathways converge on the contractile apparatus in vascular smooth muscle. Our data show normal rho kinase signaling may be less active in MetS vascular smooth muscle cells, however, overall tension development between lean and MetS was unchanged. This suggests there may be compensatory signaling to maintain contraction in the absence of rho kinase activity. This could explain why expression of RhoA was augmented in supernatant from MetS vs. lean swine (i.e. compensatory increases in expression with decreased activity/contribution). Future studies to address the relative activity and functional expression of signaling intermediates in these vasoregulatory pathways may elucidate changes required for progression into atherosclerosis in the setting of MetS.

Examination of Specific Targets

There is a need to further characterize the link between coronary PVAT-derived factors and the initiation and progression of coronary atherogenesis in obesity/MetS. Based on recent data from our laboratory implicating a potential role for both leptin and calpastatin in endothelial and smooth muscle dysfunction in MetS, we are currently planning to conduct future studies to locally knockdown and overexpress these proteins in coronary PVAT using lentiviral vectors delivered with a microinjection catheter.

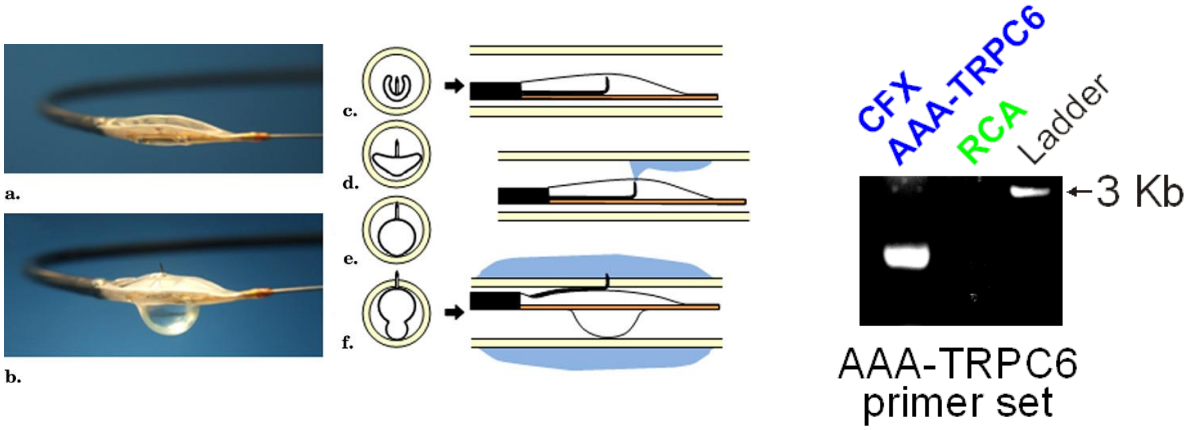

Figure 3.3. Coronary Perivascular Transfection. A) Picture and schematic of Mercator Micro-Injection Catheter. When the desired injection site is reached in the coronary artery, the balloon is inflated with saline to allow lentiviral vector injection through the blood vessel wall, directly into the surrounding perivascular adipose tissue. This keeps the concentration high near the target site only. B) Reporter assay confirming lentiviral vector expression in the circumflex artery (CFX) and no expression in the control, right coronary artery (RCA). TRPC6 figure provided by Dr. Alexander Obukhov.

Utlizing these catheters and angiography, siRNA directed at proteins such as leptin or calpastatin could be delivered to the adjacent PVAT surrounding a coronary artery (**Figure 3.3**). Additionally, a GFP-labeled artery delivered to an alternative conduit artery would allow a pig to serve as its own control. These target-specific studies would help to determine whether these proteins contribute to processes such as endothelial and smooth muscle dysfunction in the face of a high fat diet. During several months of local knock down or up regulation with or without the atherogenic diet, coronary artery stenosis could be measured *in vivo* using intravascular ultrasound to examine whether either of these proteins participates in plaque development. Additional functional isometric tension studies, western blots and immunohistochemistry could be performed *in vitro* to closely examine the influence of these targets over the course of disease progression.

Stromal Vascular Cell Types

In our proteomic assessment, we were careful not to assume all of the proteins were derived from adipocytes. Stromal vascular cells include pre-adipocytes, monocytes and macrophages, fibroblasts and vascular endothelial and smooth muscle cells that compose the small arteries in the PVAT itself. As these cell types produce their own chemical messengers, delineating which vascular mediators are coming from which of the cell types may help to elucidate a cell-specific messenger that could be targeted in MetS. Specifically, macrophage polarization has been shown to occur in adipose tissue with obesity[88]. Further characterization of the differential adipokine secretion in M1 versus M2 macrophages could help to elucidate the link between obesity, inflammation and vascular dysfunction.

Concluding Remarks

Despite the growing pandemic, the pathophysiologic mechanisms linking obesity, MetS and CVD remain poorly understood. We have found that obesity and MetS are associated with vascular endothelial dysfunction and smooth muscle ion channel dysregulation. The central goal of this investigation was to examine the extent to which local PVAT derived factors modulate was a coronary vascular smooth muscle function and potentially to the development of CAD in MetS. Results from these investigations illustrate that PVAT-derived factors impair coronary vascular smooth muscle function by converging on ion channels that promote vasoconstriction. These investigations are the first to globally characterize

alterations to the PVAT secretome in MetS. Importantly, perivascular adipose-derived calpastatin production may increase in MetS and drive the enhanced constriction observed in the presence of PVAT. Above all, the present findings suggest that local PVAT contributes to the normal control of coronary tone and the contribution of PVAT changes as disease progresses, and highlight the potential diagnostic benefit of monitoring coronary PVAT expansion and protein expression.

Appendix

Supplementary Methods

Isolation of coronary artery rings

Lean and obese swine hearts were excised upon sacrifice and the aorta cannulated to perfuse the coronary tree with 4°C, Ca^{2+}-free Krebs solution (131.5 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 10 mM glucose) in order to rinse the excised heart of blood and blood proteins. After perfusion, coronary arteries from lean and obese swine were grossly dissected from the heart, and further isolated from surrounding myocardium and adventitia using a dissecting microscope. Adjacent adipose was cleaned of myocardium and stored in ice-cold Ca^{2+}-free Krebs for later use in the protocols outlined. Following adventitial removal, arteries were cut into 3 mm rings and mounted in water-jacketed organ baths filled with a Ca^{2+}-containing Krebs solution (131.5 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 10 mM glucose, 4 mM $CaCl_2$) at 37°C (**Figure 2.1**). Optimal length (passive tension) was assessed by contractions of isolated arteries to 60 mM KCl. Passive tension was increased in gram increments until there was <10% change in active tension development to 60 mM KCl (typical optimal passive tension equaled ~4 g). Once the arteries had stabilized at their respective baseline tensions they were subjected to the following protocols outlined.

Vascular effects of adipose tissue from different anatomical beds

In order to obtain control responses, clean isolated coronary artery rings were contracted with 20 mM KCl absence of adipose tissue. After the artery contractions stabilized, the rings were washed and allowed to return to baseline passive tension. The arteries were then incubated with no adipose tissue (i.e. time-control, n = 10) or 0.3 g of adipose tissue obtained from coronary (n = 5), subcutaneous (n = 5), or mesenteric (n = 3) depots for 30 min. Following this incubation period, contractile responses to 20 mM KCl then repeated. Active tension development of the same artery to repeated administration of KCl is reported as “delta active tension”; i.e. the difference in active tension development to KCl in the presence of PVAT minus the active tension development in the absence of PVAT (**Figure 2.2A**). Thus, paired comparisons of a single artery with and without PVAT were performed to determine the effects of PVAT on coronary vasomotor responsiveness. Identical studies were also performed in rat aortic rings (n = 3) that were exposed to rat peri-aortic adipose tissue.

Bioassay experiments with coronary PVAT supernatant

Bioassay experiments were conducted on clean coronary artery rings that had not been previously exposed to PVAT. In these studies, coronary artery responses to 20 mM KCl were compared in the same artery in the absence of PVAT (control) and following the addition of a filtered (0.2 μm filter) supernatant containing Ca^{2+}-Krebs buffer that had been incubated with 0.3g of coronary PVAT for 30 min.

Effect of coronary PVAT on baseline tension and response to PGF2-α

To examine the vasoactive effects of coronary PVAT on baseline coronary artery tension, weighed quantities of lean ((Control, n = 21) (0.1 g, n = 9) (0.3 g, n = 20) (1.0 g, n = 7)) and obese ((Control, n = 17) (0.1 g, n = 4) (0.3 g, n = 15) (1.0 g, n = 4)) coronary PVAT were added to organ baths containing clean (PVAT free) coronary arteries at 37°C. In these studies, lean coronary arteries were exposed to PVAT from the same lean swine while obese coronary arteries were also exposed to PVAT from the same obese swine (i.e. paired artery-PVAT responses from the same lean or obese animals were compared). Baseline tension of the arteries exposed to these different quantities of PVAT was then followed over a 30 min time period. Coronary artery responses to PGF2α (10 μM) were also obtained in the absence and presence of lean ((Control, n = 7) (0.1 g, n = 8) (0.3 g, n = 6) (1.0 g, n = 6)) and obese ((Control, n = 5) (0.1 g, n = 3) (0.3 g, n = 4) (1.0 g, n = 6)) coronary PVAT.

Diltiazem (10 μM) or Nifedipine (0.1 μM) was added to selected baths at the plateau of the KCl or PGF2α + PVAT contraction to examine the conuMtribution of voltage-dependent Ca^{2+} ($Ca_V1.2$) channels to these contractions.

Effects of lean vs. obese PVAT on KCl Dose-Responses in intact and denuded arteries

Coronary contractions to increasing concentrations of KCl (10-60 mM) were examined before and after incubation with coronary PVAT (0.3 g for 30 min) in intact (lean, n = 8; obese, n = 6) and endothelium denuded arteries (lean, n = 7; obese, n = 8). The endothelium was removed by gently rubbing fine-tip forceps along the lumen of the artery and confirmed by <30% relaxation to 1 μM bradykinin. To determine whether adding extracellular K^+ increases active tension when osmolarity is maintained by simultaneously removing equimolar Na^+, a modified Krebs was used that substituted equimolar Na^+ for 20 or 60 mM K^+. This equimolar replacement did not significantly change tension development compared to paired responses without substitution (P = 0.154 at 20 mM; P = 0.122 at 60 mM), suggesting the effects of KCl are independent of osmolarity changes with application of extracellular KCl (**Figure A**). Additional control experiments also revealed no significant changes in the tension development of isolated coronary arteries exposed to 10-60 mM NaCl (instead of KCl), further supporting that the coronary effects of KCl are not related to changes in osmolarity. As outlined, paired artery-PVAT responses from the same lean or obese animals were compared in these studies.

Effects of lean vs. obese PVAT on H_2O_2-mediated coronary vasodilation

The effects of PVAT on coronary vasodilation to H_2O_2 (1 μ–1 mM) were examined in isolated arteries pre-constricted with either KCl (60 mM) or U46619 (1 μM). In these experiments, coronary arteries were incubated without or with 0.3 g of coronary PVAT from the same animal (lean, n = 5 or obese, n = 9) for 30 min prior to pre-contraction. PVAT was absent in arteries contracted with KCl (lean, n = 3; obese, n = 3). Once coronary contractions to KCl or U46619 stabilized, increasing concentrations of H_2O_2 were added to the baths in a cumulative manner.

Crossover study: Vascular effects of lean vs. obese PVAT

To further examine the vascular effects of lean vs. obese coronary PVAT on artery contractions to 20 mM KCl, additional "crossover" experiments were performed in lean coronary arteries that were incubated for 30 min with 0.3 g of coronary PVAT obtained from either a lean (n = 7) or obese (n = 11) animal sacrificed on the same day; i.e. lean coronary arteries were exposed to coronary PVAT obtained from a lean and obese swine (**Figure 2.6A**). Delta active tension was determined in response to repeated administration of 20 mM KCl before and after incubation of PVAT in the same artery. Similarly, to determine the effects of coronary PVAT-derived factors on sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), coronary contractions to 20 mM KCl before and after PVAT incubation were examined in the presence of the SERCA inhibitor cyclopiazonic acid (CPA, 10 μM, n = 6).

Effects of PVAT on Rho kinase and calpastatin on coronary artery contractions to KCl

Similar to studies outlined, concentration response studies to KCl (10-60 mM) were conducted in lean (n = 6) and obese (n = 8) arteries in the presence of the Rho kinase inhibitor, fasudil (1 μM). In these experiments, fasudil was added 10 min prior to the KCl responses in the absence and presence of 0.3 g of PVAT[147] (30 min incubation) in the same coronary artery.

Further studies were also performed to examine the effects of 30 min incubation of calpastatin ((1 μM, n = 3) (3 μM, n = 7) (10 μM, n = 4) Calbiochem)) or negative (scrambled) calpastatin peptide (10 μM, n = 3, Calbiochem) on coronary artery contractions to 20 mM KCl.

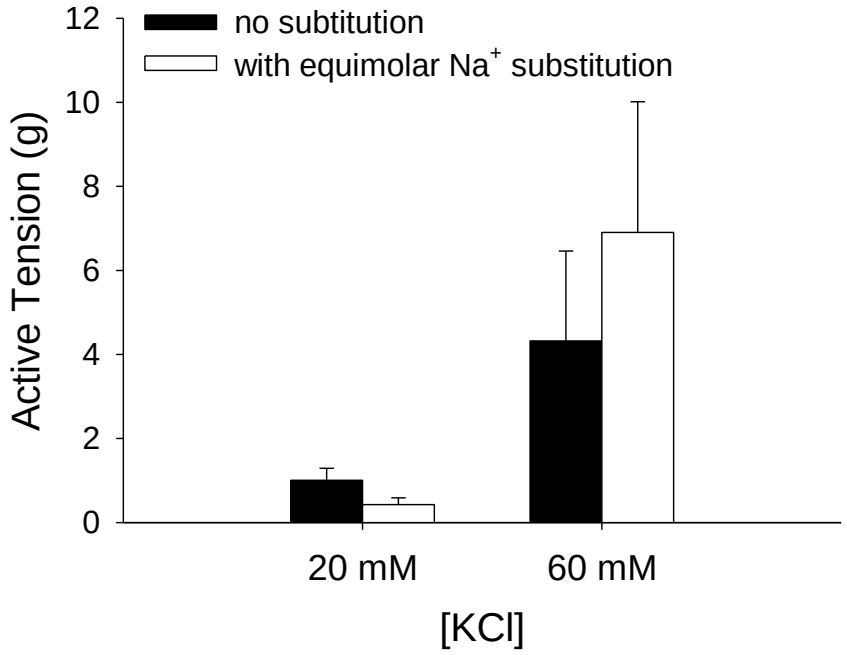

Figure A KCl contractions with equimolar Na^+ substitution. Equimolar replacement of K^+ for Na^+ did not significantly change tension development of isolated coronary arteries (n = 3) when compared to paired responses without equimolar substitution (*P* = 0.154 at 20 mM; *P* = 0.122 at 60 mM).

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Curriculum Vitae

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Education

DePauw University
2009, B.A., Biology

Indiana University
2013, Ph.D., Cellular and Integrative Physiology

Professional Organizations

Memberships

2010-present	American Physiological Society
2010-present	American Association for the Advancement of Science
2010-2012	Project SEED
2011-present	Society for Experimental Biology and Medicine
2011-2013	Preparing Future Faculty

Committee Memberships

2011-2013	Indiana Physiological Society, Elected Student Councilor
2012-2013	Indiana Physiological Society Education Committee

Honors and Awards

2011	Abstract Award, Indiana Physiological Society Meeting
2012	Abstract Award, Indiana Physiological Society Meeting
2013	SEBM Young Investigator Award, Experimental Biology Meeting
2013	APS Cardiovascular Section Research Recognition Award, Experimental Biology Meeting

Publications

Peer-Reviewed Journal Articles

1. Berwick ZC, Dick GM, Moberly SP, **Kohr MC**, Sturek M, Tune JD. Contribution of voltage-dependent K+ channels to metabolic control of coronary blood flow. *J. Mol. Cell Cardiol*. April; 52(4):912-919, 2012.

2. Berwick ZC, Moberly SP, **Kohr MC**, Kurian M, Morrical E, and Tune JD. Contribution of voltage-dependent K+ and Ca2+ channels to coronary pressure-flow autoregulation. *Basic Res Cardiol*., 107:1-11, 2012.

3. Payne GA, **Kohr MC**, Tune JD. Epicardial perivascular adipose tissue as a therapeutic target in obesity-related coronary artery disease. *Br. J. Pharmacol.* Feb; 165(3):659-669, 2012.

4. Moberly SP, Berwick ZC, **Kohr MC**, Mather K, Tune JD. Intracoronary glucagon-like peptide 1 preferentially augments glucose uptake in ischemic myocardium independent of changes in coronary flow. *Experimental Biology of Med.,* 237:334-342, 2012.

5. **Owen MK**, Witzmann FA, McKenney ML, Lai X, Berwick ZC, Moberly SP, Alloosh M, Sturek M, Tune JD. Perivascular adipose tissue potentiates contraction of coronary vascular smooth muscle: Influence of obesity. *Circulation*. DOI: 10.1161/CIRCULATIONAHA.112.001238. 2013.

6. Moberly SP, Mather KJ, Berwick ZC, **Owen MK**, Hutchins GD, Green MA, Ng Y, Considine RV, Perry KM, Chisholm RL, Tune JD. Impaired cardiometabolic responses to glucagon-like peptide 1 in obesity and type 2 diabetes mellitus. *Br. J. Pharmacol*. In Press.

7. Berwick ZC, Dick GM, O'Leary HA, Bender SB, Goodwill AG, Moberly SP, **Owen MK**, Miller SJ, Obukhov AG, Tune JD. Contribution of electromechanical coupling between KV and CaV1.2 channels to coronary dysfunction in metabolic syndrome. *Basic Res Cardiol.* In Press.

Manuscripts in Preparation

1. Casalini ED, **Owen MK**, Goodwill AG, Moberly SP, Berwick ZC, Tune JD. Role of hydrogen sulfide in the regulation of coronary blood flow. *In Preparation.*

2. Goodwill AG, Casalini ED, **Owen MK**, Conteh A, Sassoon D, Shatagopam K, Dick GM, Tune JD. Role of voltage-dependent Kv7 channels in the regulation of coronary blood flow. *In Preparation.*

3. McKenney ML, **Owen MK**, Alloosh M, Schultz KA, Tune JD, Sturek MS. Dysfunction of coronary smooth muscle Ca2+ regulation in the progression of metabolic syndrome and coronary artery disease in Ossabaw miniature swine. *In Preparation.*

Published Abstracts Presented at National Meetings

1. **Kohr MC**, Payne GA, Lai X, Witzmann FA, and Tune JD. Altered protein expression of coronary perivascular adipose tissue in metabolic syndrome. Experimental Biology, 2011.

2. Moberly SP, Berwick ZC, **Kohr MC**, Svendsen M, Mather KJ, Tune JD. Intracoronary Infusion of Glucagon-like peptide 1 acutely enhances myocardial glucose uptake during ischemia in canines. Experimental Biology, 2011.

3. **Kohr MC**, Lai X, Moberly SP, Berwick ZC, Witzmann FA, Tune JD. Augmented coronary vasoconstriction to epicardial perivascular adipose tissue in metabolic syndrome. Experimental Biology, 2012.

4. McKenney ML, **Kohr MC**, Alloosh MA, Schultz KA, Bell LN, Tune JD, Sturek MS. Dysfunction of coronary smooth muscle Ca2+ regulation in the progression of metabolic syndrome and coronary artery disease in Ossabaw miniature swine. Experimental Biology 2012.

5. Berwick ZC, Dick GM, Bender SB, Moberly SP, **Kohr MC**, Goodwill AG, Tune JD. Contribution of Cav1.2 channels to coronary microvascular dysfunction in metabolic syndrome. Experimental Biology, 2012.

6. Berwick ZC, Moberly SP, **Kohr MC**, Morrical EB, Kurian MM, Goodwill AG, Tune JD. Contribution of voltage-dependent potassium and calcium channels to coronary pressure-flow autoregulation. Experimental Biology, 2012.

7. Moberly SP, Berwick ZC, **Kohr MC**, Mather K, Tune JD. Cardiac responses to intravenous glucagon-like peptide 1 are impaired in metabolic syndrome. Experimental Biology, 2012.

8. **Owen MK**, Krenzke R, Dick GM, Tune JD. Perivascular Adipose Tissue impairs H2O2-mediated vasodilation in the coronary circulation. Experimental Biology 2013.

9. Casalini ED, **Owen MK**, Goodwill AG, Moberly SP, Berwick ZC, Tune JD. Role of hydrogen sulfide in the regulation of coronary blood flow. Experimental Biology 2013.

10. Goodwill AG, Casalini ED, **Owen MK**, Conteh A, Sassoon D, Shatagopam K, Dick GM, Tune JD. Role of voltage-dependent Kv7 channels in the regulation of coronary blood flow. Experimental Biology 2013.

Grants and Fellowships

T32 5T32DK064466-09, National Institutes of Health, Research Training Program in Diabetes and Obesity (Pre-doctoral, 2011-2013); **Effects of epicardial perivascular adipose tissue on coronary vascular smooth muscle function in metabolic syndrome.**

Teaching Assignments

IUPUI

F557 Physiology II — 2011 (Fall)
Physiology of the Circulation: Control Mechanisms

IU School of Medicine

F503 Basic Human Physiology — 2012 (Fall)
Hemodynamics
Vascular Tone
Regulation of Blood Pressure
Cardiovascular Disease

Service

K-12 Outreach

St. Malachy School

6th Grade (Digestive System) — 2012
"How to make poop" (45 students)

6th - 8th Grades (Body Systems) — 2012 – 2013
Heart Dissection Lab (135 students)

St. Simon School

7th Grade (Digestive System) — 2013
"How to make poop" (52 students)

Community

Mentor, Project SEED Summer Research Internship Program for Economically Disadvantaged High School Students:

Jacob Burton-Edwards — 2010
Micah Brown — 2011
Carmen Hu — 2012

Other Professional Activities

Invited Seminar Presentations

2012 – *Augmented coronary vasoconstriction to epicardial perivascular adipose tissue in metabolic syndrome*
INPhys Meeting, Ball State University, Muncie, IN

2012 – *Fat and the Heart: Linking Obesity to Cardiovascular Disease*
DePauw University, Greencastle, IN

2013 – *Study of Digestive and Regulatory Processes through Exploration of Fasted and Postprandial blood Glucose*
Experimental Biology Meeting, Boston, MA