DISSERTATION

LIPIDS AND OXIDATIVE STRESS AS MEDIATORS OF ENDOTHELIAL PATHOPHYSIOLOGY IN OBESITY

Submitted by

Elise Laura Donovan

Department of Health and Exercise Science

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2011

Doctoral Committee:

Advisor: Benjamin F Miller
Co-Advisor: Karyn L Hamilton

Matthew S Hickey
Melinda A Frye

ABSTRACT

LIPIDS AND OXIDATIVE STRESS AS MEDIATORS OF ENDOTHELIAL PATHOPHYSIOLOGY IN OBESITY

Because obesity is a well established independent risk factor for diabetes and coronary artery disease (CAD), it is important to identify factors associated with obesity that are responsible for disease progression and interventions to decrease risk of developing obesity associated co-morbidities. Two of the many mediators of obesity associated risk for diabetes and CAD are oxidative stress and oxidized phospholipids, which have been implicated in vascular disease initiation and progression through endothelial cell activation, macrophage recruitment and advanced plaque rupture.

Plasma platelet activating factor acetylhydrolase (Lp-PLA_2) is an enzyme that circulates bound to LDL cholesterol and degrades platelet activating factor (PAF), a potent inducer of the platelet coagulation cascade and thrombosis. In addition, Lp-PLA_2 degrades oxidized phospholipids to lysophospholipid products and fatty acids that may also induce inflammatory changes in multiple cell types, including vascular endothelial cells.

Exogenous antioxidant supplementation has been examined as a means of decreasing vascular oxidative stress, however, exogenous antioxidants have little or no

effect on CVD outcomes, and in some cases it may increase mortality. A novel approach to protecting cells from oxidative stress is to increase cellular endogenous antioxidant defenses. NF-E2-related factor 2 (Nrf2) is a transcription factor that binds to the antioxidant response element (ARE) promoter region of many genes including phase II antioxidant enzymes. Protandim is a combination of phytochemicals that is thought to induce Nrf2 stabilization and translocation to the nucleus, with subsequent increases in phase II antioxidant enzymes and protection against oxidative stress.

The overall objectives of the three studies we performed were to 1) globally analyze obesity associated lipid and oxidative stress using lipidomics techniques 2) determine the effects of identified obesity associated oxidative and lipid stress on the vascular endothelium 3) determine whether Protandim treatment could protect vascular endothelial cells from an oxidative challenge, and 4) characterize Lp-PLA_2 in human adipose and skeletal muscle.

Experiment 1 tested the hypothesis that oxidized phospholipids would be greater in morbidly obese gastric bypass patients compared to lean surgical controls, and that global lipid profiles would differ between groups. To test this hypothesis we performed a combined targeted and global lipidomic analysis of plasma lipids from morbidly obese gastric bypass patients and lean controls. We identified a group of ether-linked lipids that were greater in obese subjects compared to lean, and further examined whether a representative lipid from this group induced pathophysiological phenotypic changes in vascular endothelial cells.

Experiment 2 tested the hypothesis that Protandim would protect human coronary artery endothelial cells (HCAEC) against an oxidative challenge by increasing phase II antioxidant enzymes in a Nrf2 dependent manner. To do this we performed a series of *in vitro* experiments treating HCAEC with Protandim and determined that Protandim induced Nrf2 nuclear localization, increased phase II antioxidant enzyme expression, and protected cells from undergoing apoptosis in response to an oxidative challenge. Silencing Nrf2 prior to the oxidative challenge inhibited the Protandim induced protection.

Experiment 3 tested the hypothesis that Lp-PLA_2 would be detectable in human adipose tissue and that Lp-PLA_2 would be greater in adipose from morbidly obese gastric bypass patients compared to lean. In addition, we examined whether adipose Lp-PLA_2 may be related to circulating Lp-PLA_2 activity, inflammation, and glucose intolerance.

We have identified ether-linked lipids that are elevated in obese subjects compared to lean. We found that Lp-PLA_2 is expressed in human adipose for the first time, adipose Lp-PLA_2 is co-localized with macrophages, and report relationships between Lp-PLA_2 and indices of glucose homeostasis and inflammation. Lastly, we found that Protandim protects endothelial cells from an oxidative challenge in a Nrf2 dependent manner. Collectively, these data provide insight into the oxidative and lipid stress milieu that occurs in obese subjects.

TABLE OF CONTENTS

CHAPTER I – INTRODUCTION AND EXPERIMENTAL HYPOTHESES

Introduction and Experimental Hypotheses

With rates of obesity increasing, and with an associated increased incidence of diabetes and cardiovascular disease (CVD), research in the area of obesity associated CVD pathology is a public health necessity. CVD is the leading cause of death in the United States, and coronary artery disease (CAD) is responsible for 68% of all CVD deaths [2]. Currently 65% of Americans are overweight, with over 30% of those individuals qualifying as obese [3]. The Framingham Heart Study has identified risk factors for CAD, including age, obesity, hypercholesterolemia, diabetes and hypertension. Obesity related hypertension, elevated low density lipoprotein (LDL) cholesterol, reduced high density lipoprotein (HDL) cholesterol, dyslipidemia (elevated triglycerides and ectopic fat distribution), and oxidative stress all contribute to CVD pathology, and specifically to atherosclerosis and cardiovascular remodeling. Increased carotid artery intima thickness (CIT), impaired ventricular function, and increased ventricular wall thickness have also all been observed in obese subjects compared to lean [4]. Insulin resistance, hyperglycemia, advanced glycation end product formation, and diabetes associated oxidative stress all also contribute to vascular disease [5-7]. While obesity associated pathophysiology and co-morbidities are complicated and

multi-faceted, two significant contributors to obesity-related CAD risk are reactive oxygen species (ROS) and dyslipidemia [4, 8-17]. The purpose of the projects described in this dissertation was to characterize the pathophysiology of obesity associated lipid and oxidative stress, and identify potential interventions to protect vascular endothelial cells against disease initiation and progression.

Lipid and Oxidative Stress

Oxidized phospholipids

Lipid oxidation can be ROS mediated or can occur enzymatically [18-20], resulting in a variety of products [19, 20]. Oxidized phospholipid components of LDL, which are increased in human obesity, can induce endothelial cell activation and pro-atherosclerotic changes in vascular endothelial cells [15]. Endothelial cell activation is a phenotypic shift characterized by expression of inflammatory mediators and cell adhesion molecules. Phospholipids containing arachidonate and linoleate, as well as unesterified arachidonate and linoleate, can be enzymatically oxidized by lipoxygenases (LOX), which may also cause endothelial cell activation [21-24] [19].

Phosphatidylcholine (PC) is the most abundant phospholipid product in mammalian cells, and PC oxidation products have been widely examined [25]. PCs that have polyunsaturated fatty acids, particularly arachidonate, at the SN-2 position are especially susceptible to oxidation, and this results in a variety of potential oxidized products. One such lipid is 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC), which is a major cell membrane constituent susceptible to oxidative damage.

PAPC and several products of PAPC oxidation including 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC), 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-sn-glycero-3-phosphorylcholine (PEIPC),1-palmitoyl-2-oxovaleroyl-sn-glycero-3-phosphorylcholine (POVPC), and lysophosphatidylcholine (lyso-PC) have been shown to activate endothelial cells and are implicated in atherosclerotic progression [15, 17, 26, 27]. These same oxidized phospholipids are also found deposited in atherosclerotic plaques [15, 17]. PAPC, POVPC, PGPC, and PEIPC are increased 10-25 fold in circulating modified LDL, and 3-6 fold in vascular lesions in rabbits fed an atherogenic diet. This increase in oxidized phospholipids in the vascular lesions is 10-20 times the amount needed to activate endothelial cells [15].

Oxidized phospholipids induce endothelial cell activation, in particular increasing expression of cell adhesion molecules and chemoattractant proteins involved in monocyte and neutrophil recruitment and binding. [9, 10, 12, 18, 28, 29]. For example, POVPC increases monocyte chemoattractant protein 1 (MCP-1) expression and monocyte binding to endothelial cells [27]. PGPC induces monocyte and neutrophil binding and vascular cell adhesion molecule-1 (VCAM-1) [15, 27]. PEIPC can stimulate an increase in MCP-1 in a dose dependent manner in human aortic endothelial cells (HAEC) [16]. Lyso-PC also stimulates MCP-1, as well as VCAM-1 and intracellular cell adhesion molecule (ICAM-1) [26]. PAPC alone does not induce ICAM-1, or VCAM-1, but does stimulate MCP-1 and monocyte binding [30].

Enzymatically oxidized lipids

Both non-esterified polyunsaturated fatty acids (PUFA), particularly arachidonate and linoleate and PUFA as components of phospholipids, are susceptible to oxidation by LOX [19]. The resulting products include hydroperoxyeicosatetraenoates (H(p)ETEs), hydroperoxyoctadecadienoates (H(p)ODEs), PL-HETEs and PL-HODEs which are formed in multiple cell types including macrophages, adipocytes, and endothelial cells. LOX can also directly oxidize LDL although the mechanisms by which this occurs are not fully understood [31]. LOX products may play a role in the beginning stages of atherosclerosis. A mouse model over-expressing 12/15 LOX, which resulted in elevated production of 12SHETE, also had increased MCP-1 and ICAM expression and increased monocyte binding to the endothelium [32]. A subsequent study found that 12/15LOX knockout mice crossbred to apoE-deficient mice, compared to atherosclerotic apoE deficient mice, showed a decrease in monocyte adhesion to the aorta, an effect that was mimicked by a LOX inhibitor [33]. HETE Isomer type may be important, as evidenced by 12SHETE, but not its stereoisomer 12RHETE (which is not a product of LOX peroxidation), increasing MCP-1 RNA and protein in macrophages [23]. In addition, LOX protein and products have been found localized in atherosclerotic lesions from humans [34]. Thus, whether formed via direct oxidation or enzymatically, it is clear that oxidized lipids in the endothelium contribute to the pathophysiology of atherosclerosis.

Platelet activating factor and Lp-PLA$_2$

Platelet activating factor (PAF) and the acetylhydrolases that degrade PAF have been implicated in diabetes and CAD. PAF, a pro-inflammatory ether-linked lipid, and

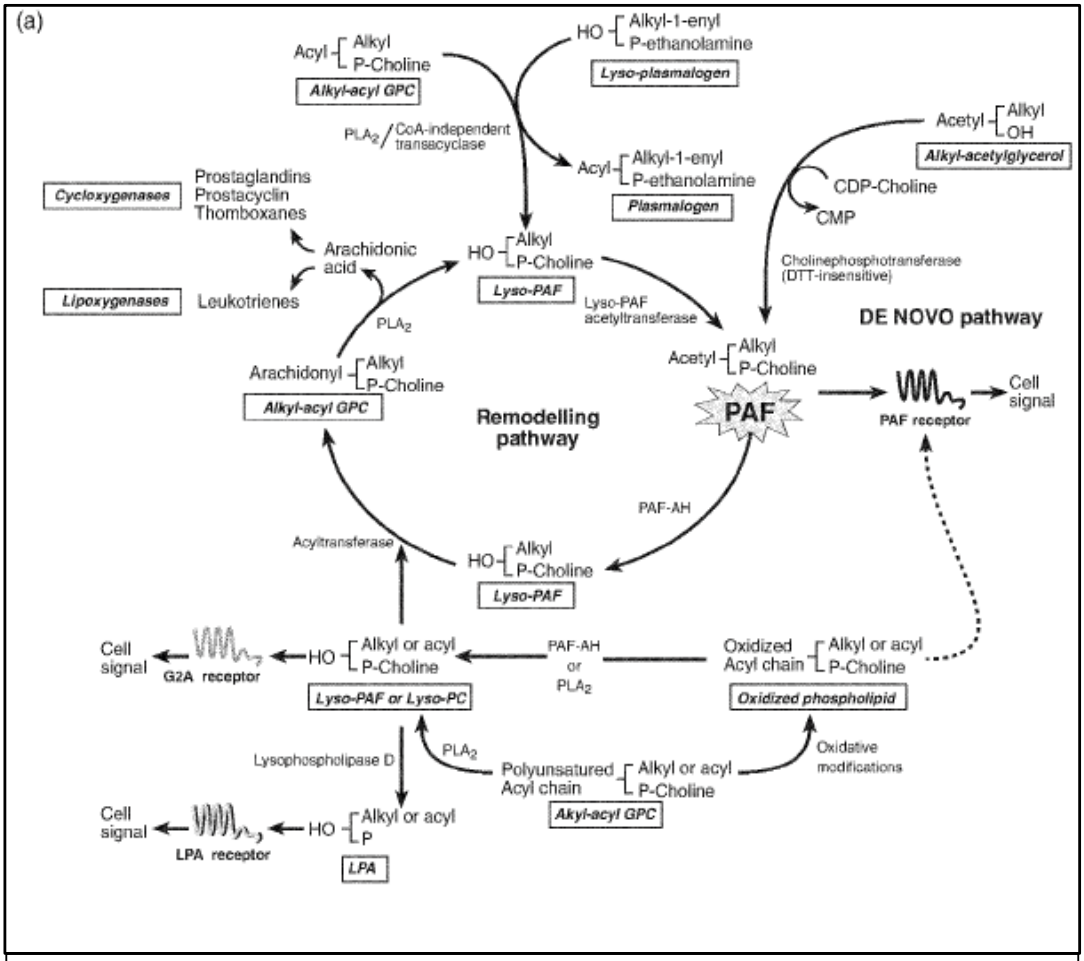


Figure 1. PAF metabolism including *de novo* and remodeling pathways.

plasma platelet activating factor acetylhydrolase/ lipoprotein associated phospholipase A$_2$ (Lp-PLA$_2$) may be involved in vascular cell pathology. Currently there is a lack of consensus as to whether Lp-PLA$_2$ is pro- or anti-inflammatory. PAF acts on multiple cell types, is a potent stimulator of the platelet coagulation cascade, inflammation, and thrombosis, and is constitutively synthesized through a *de novo* mechanism as well as

through a remodeling pathway (Figure 1 [1]). However, it is through the remodeling pathway that most inducible PAF synthesis occurs [35, 36]. Pro-inflammatory and pro-thrombotic mediators including TNF-α stimulate PAF synthesis through the remodeling pathway [37]. In the remodeling pathway, an sn-1 ether-linked phosphocholine, usually with an arachidonate or other polyunsaturated fatty acid at the sn-2 position, is acted on by a phospolipase resulting in the removal of the sn-2 chain. If the sn-2 chain is arachidonate, the arachidonate is then available for eicosanoid synthesis or other biological processes. The lyso-PAF that is left is acetylated by a lyso-PAF acetylhydrolase that transfers an acetyl group from acetyl CoA to the sn-2 position of the lyso-PAF [1]. Thus ether-linked phosphotidylcholine species function as substrates in the formation of PAF. Increased PAF also induces the expression of PAF-acetylhydrolases, however to what degree has yet to be fully determined [37].

Lp-PLA_2 is one member of a family of PAF acetylhydrolases (PAF-AH) that degrade PAF. Specifically Lp-PLA_2 specifically refers to the circulating form of PAF-AH [38]. While Lp-PLA_2 has been detected in several cell types including macrophages, rat hepatocytes and rat adipocytes in culture, circulating Lp-PLA_2 is generally thought to come from cells of hematopoetic origin, primarily macrophages [38]. Lp-PLA_2 is present in the circulation bound to either LDL or HDL. While it can transfer between LDL and HDL, Lp-PLA_2 activity is significantly decreased when bound to HDL [39]. Only approximately 1 in 1,000 LDL particles carries Lp-PLA_2 and apolipoprotein B-100 is essential for interaction between LDL and Lp-PLA_2. Lp-PLA_2 associates most strongly with small dense LDL, but activity is 2-4 fold lower when associated with small dense LDL

compared to other LDL fractions [40]. Lp-PLA_2 can also be glycosylated [41], though the function of this modification has not been thoroughly examined. The single study to examine the role of glycosylation found that it likely is involved in association of the enzyme with HDL; removal of Lp-PLA_2 glycosylation led to increased association of Lp-PLA_2 with HDL [41].

In addition to degrading PAF, Lp-PLA_2 also hydrolyzes glycerophospholipids resulting in lysophospholipid and free fatty acid products. Lp-PLA_2 affinity is specific to species containing sn-2 fatty acid chains of 6 carbons or fewer, and glycerophospholipids with longer sn-2 fatty acid side chains that have undergone oxidative modification [40]. The products of this breakdown are lysophospholipids and in some cases oxidized fatty acid chains including arachidonic acid and its derivatives [40, 42-44]. Thus, whether Lp-PLA_2 degrading oxidized phospholipids is pro- or anti-inflammatory is debated as the lysophospholipid species that result from this breakdown also have detrimental effects on the endothelium. Lysophosphatidylcholine (LPC) is a component of oxidized LDL and its presence in oxidized LDL particles is 5-fold higher than in native LDL [44]. LPCs have been shown to increase plasminogen activator inhibitor-1, a potent prothrombotic and proatherogenic protein, in adipocytes [45]. In addition, LPCs can induce endothelial cell expression of cell adhesion molecules, an important step in endothelial cell activation and atherosclerotic progression [26] [46].

Several studies have shown an association between increased plasma Lp-PLA_2 activity and CAD, and drugs to inhibit Lp-PLA_2 activity in CAD patients are in phase III

clinical trials [47, 48]. Kinney *et al*. showed that Lp-PLA_2 activity is independently associated with coronary artery calcification [49], and Herrmann *et al.* found that elevated Lp-PLA_2 in carotid artery plaques results in higher risk for cardiac events [50]. Inhibition of Lp-PLA_2 in a swine model showed that inhibiting Lp-PLA_2 in diabetic and hypercholesterolemic animals decreased atherosclerotic lesion content of LPC and plaque area, and reduced the incidence of necrotic and unstable plaque phenotypes [51]. Decreasing necrotic core size and stabilizing lesions is of particular interest as necrotic core rupture induces thrombosis, and Lp-PLA_2 is elevated in advanced, necrotic plaques, co-localized with macrophages [52]. In addition, inhibition of Lp-PLA_2 reduced expression of inflammatory genes in whole coronary artery homogenates [51].

Other data suggest Lp-PLA_2 may be protective against CAD [53, 54]. Delivery of liver directed Lp-PLA_2 into apoE null mice increased circulating Lp-PLA_2 activity, and reduced macrophage recruitment and adhesion through decreasing PAF like lipids in VLDL. This effect is attributed to increased association of Lp-PLA_2 with HDL [54]. An additional study in apoE null mice using the same method for increasing Lp-PLA_2 showed an increase in plasma activity accompanied by a decrease in modified-LDL and LPC/PC ratio [53]. Following carotid artery denudation, animals that had received exogenous Lp-PLA_2 had less oxidized LDL, macrophages, and smooth muscle cells in the intimal space, in addition to a decreased lesion area and volume [53]. The decrease in lesion size was only significant in males suggesting a sex specific effect. Studies in humans have also shown sex differences in Lp-PLA_2 activity, with men having higher plasma activity than women. Post-menopausal women generally have increased activity

compared to pre-menopausal women, which can be decreased with some types of hormone therapy [55, 56]. Whether Lp-PLA_2 activity attenuates or exacerbates CAD may depend on other factors including total cholesterol, and whether inhibiting Lp-PLA_2 is beneficial or not may depend on the stage and severity of the plaque. It may be that higher Lp-PLA_2 activity is beneficial in preventing CAD initiation, but in advanced plaques it promotes rupture and thrombosis in mature plaques.

Data now show that Lp-PLA_2 activity may be predictive of diabetes in humans (in review), though mechanisms to explain this have yet to be examined. Both Lp-PLA_2 activity in insulin sensitive tissues and circulating Lp-PLA_2 may be involved. If increased Lp-PLA_2 activity results in increased degradation of phospholipids to lysophospholipids that induce inflammation in insulin sensitive tissues and the vascular endothelium, then inhibiting Lp-PLA_2 may be a beneficial intervention in diabetes and CVD. Two major insulin sensitive tissues involved in development of diabetes are skeletal muscle and adipose. It has been demonstrated that LPC can induce insulin resistance in skeletal muscle, an effect that was ameliorated by inhibiting LPC formation [57]. While Lp-PLA_2 has been found in rodent adipocytes [58], its role in adipose tissue and skeletal muscle has not been examined. As noted earlier, most circulating Lp-PLA_2 is thought to be macrophage derived, and macrophage infiltration of adipose tissue is a hallmark of obesity. Thus it is likely that Lp-PLA_2 is present and active in human adipose tissue though this has yet to be examined, and it is unknown whether tissue Lp-PLA_2 could be implicated in obesity associated diabetes.

Reactive oxygen species and antioxidant defense

Oxidation of lipids is one of the ways by which increased ROS production may contribute to chronic disease states. Broadly, the term ROS refers to compounds containing oxygen molecules that are chemically reactive [59]. ROS function normally as cell signaling molecules and are generated through multiple mechanisms including the mitochondrial electron transport chain, uncoupled nitric oxide synthase, xanthine oxidase, and NADPH oxidase. When production of ROS exceeds antioxidant defense oxidative stress occurs. The most commonly described ROS include the hydroxyl radical (OH^-), superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Production of ROS is normally tightly countered by extensive intracellular antioxidant systems that include iron containing species, thiols, and antioxidant enzymes. Well-characterized antioxidant enzymes include but are not limited to superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase, peroxiredoxins and heme oxygenase. Oxidized LDL is a common systemic marker of oxidative stress, while several other markers are commonly used as indicators of oxidative damage to lipids, proteins, and nucleotides. Malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) are commonly used as indicators of lipid peroxidation. Protein carbonyls are a common marker of oxidative damage to proteins, and 8-hydroxy-2'-deoxyguanosine (8-OHdG) is used as a marker of DNA oxidation. There is still a lack of consensus on the utility of circulating markers of oxidative stress such as oxidized-LDL and MDA as clinical predictors of future coronary events [60].

Oxidative stress in obesity

There is a significant body of literature demonstrating increased oxidative stress with obesity. As visceral adipose mass increases, secretion of inflammatory adipokines by adipocytes and resident macrophages increases. Proinflammatory cytokines such as TNF-α, IL-6, and IL-8 have been shown to increase ROS production in multiple tissues and cell types including adipose, monocytes/macrophages, and endothelial cells [61]. Furukawa *et al*. showed that increased obesity was strongly associated with markers of systemic oxidative stress [62]. In addition, adipose tissue ROS production increased with obesity and elevated circulating free fatty acids. The increase in adipose tissue ROS occured through increased NADPH oxidase expression, and this increase in oxidative stress led to increased secretion of inflammatory adipokines including IL-6, plasminogen activator inhibitor-1 (PAI-1), and MCP-1 [62]. Importantly, in obese mice, inhibition of NADPH oxidase led to decreased adipose ROS production, attenuated ROS-induced increases in inflammatory cytokines and improved blood glucose and insulin concentrations [62]. Increased vascular NADPH oxidase expression has been observed in obese subjects compared to lean with concomitant elevation in markers of protein oxidative damage and inflammation [14]. Collectively these data support a scenario where increased adipose tissue secretes proinflammatory cytokines, which increase adipose and systemic oxidative stress. This oxidative stress leads to additional inflammatory cytokine production and a continued vicious cycle occurs leading to progression of systemic metabolic disease. Weight loss can improve the systemic and tissue pro-oxidative profile. In bariatric surgery patients, oxidative stress and

inflammation markers improve with significant weight loss [63]. In addition, one year post-surgery after significant weight loss, plasma MDA was significantly decreased while total serum antioxidants were increased, and indicators of systemic inflammation and glucose status were improved [63].

Obesity associated oxidative stress, lipid stress, and the endothelium

Increased oxidative stress leads to oxidized LDL cholesterol, which has a well-established role in the initiation and progression of atherosclerosis and endothelial cell activation [64]. LDL cholesterol is oxidized and taken up by macrophages in the vessel wall leading to increased inflammation, foam cell production, and macrophage and endothelial cell apoptosis. Oxidized LDL adversely affects adipose tissue as well. Oxidized LDL, and specifically the LPC component of oxidized LDL, induces adipokine production in adipose. Specifically, LPC induces PAI-1, which is a potent prothrombotic cytokine, in a ROS dependent manner [45].

In the endothelium, the role of NOX superoxide production is currently a topic of research because NOX expression and activity might be causally related to vascular disease. NOX enzymes are comprised of multiple subunits, which assemble in plasma membranes and generate O_2^- radicals [65]. To date, seven NOX isoforms have been identified [65]. In vascular endothelial cells NOX2 and NOX4 are the primary isoforms [65]. Superoxide generated by these isoforms plays a role in regulating cellular function, including proliferation and angiogenesis, as well as entering the circulation and

permeating the sub-endothelial space where it can induce oxidative damage [65]. In obese rats, superoxide production by NOX precedes development of myocardial dysfunction [66]. In primates with diet-induced atherosclerosis, lesion severity was related to increased superoxide production [67]. When these primates were returned to a normal diet, superoxide production returned to normal with concomitant decreases in NOX activity and NOX subunit expression [67].

The increase in oxidized phospholipids with obesity may contribute to CVD pathology through increasing NOX activity and superoxide production. Oxidized phospholipids can increase superoxide generated by NOX, and can increase NOX expression and localization to membranes in endothelial cells [13]. Treatment of endothelial cells with ox-PAPC resulted in increased NOX activity, which was abrogated by RNA silencing of NOX, and by pharmacological inhibition of NOX [11]. Ox-PAPC has also been shown to increase recruitment of cytosolic NOX subunits to the membrane [68], superoxide production by NOX4, and NOX4 subunit expression in bovine aortic endothelial cells [13]. Ox-PAPC induces increases in MCP-1 and expression of other pro-inflammatory genes through NOX4 activation and subsequent up-regulation of O_2^- signaling pathways *in vitro* [11]. *In vivo*, a knockout mouse model for the p47 subunit of NOX and ApoE had smaller atherosclerotic lesion areas than ApoE(-/-) only mice, regardless of whether animals were fed an atherosclerotic diet. [69]. In addition, atherosclerotic progression in apoE null mice is attenuated by inhibiting both macrophage and endothelial cell NOX dependent superoxide production [70].

There is also evidence indicating a relationship between HETE products and NOX in endothelial cells [71, 72]. 20-HETE activates NOX by stimulating subunit translocation to the membrane, and increases NOX superoxide production in bovine pulmonary artery endothelial cells [72]. In addition, 20-HETE has been shown to increase superoxide in pulmonary artery endothelial cells via NADPH oxidase activation [72], and increases in monocyte MCP-1 expression and secretion stimulated by 12-HETE were attenuated by NOX inhibition [23].

Studies in humans confirm the relationship between oxidized phospholipids, NOX, obesity and CVD [6, 14, 73, 74]. Compared to non-obese controls, overweight and obese subjects demonstrate increased NOX subunit expression in the vascular endothelium, as well as augmented oxidative stress [14]. NOX subunit expression and NOX activity are greater in vessels of diabetics undergoing coronary artery bypass [6]. Increased NOX activity is associated with clinical risk factors for atherosclerosis in humans including hypercholesterolemia and diabetes [73]. NOX subunit expression is elevated in lesions of coronary arteries in bypass graft patients, particularly in the vicinity of macrophages, and NOX expression levels correlate with severity of atherosclerosis [74].

Impaired antioxidant defense systems may contribute to a pro-oxidant environment in endothelial cells. SOD activity is decreased in coronary arteries of humans with clinical CAD compared to controls [75]. Heme oxygenase and glutathione peroxidase are also decreased in the aortas of high fat diet-fed animals [76]. Total

plasma antioxidant capacity is decreased in morbidly obese subjects compared to lean, but improves after bariatric surgery [77]. Antioxidant defenses increase in response to increased ROS production, however with chronic overproduction of ROS the capacity of antioxidant systems to respond is decreased, diminishing overall antioxidant capacity.

In summary, there is a role for oxidized lipids and ROS in endothelial cell activation and pathogenesis of vascular disease. However, much research is still needed to fully understand how obesity-related oxidative and lipid stress leads to diabetes and CVD, and thus understand how to intervene to decrease obesity and CAD-related mortality.

Obesity and Oxidative Stress Interventions

Bariatric surgery

With the increasing prevalence of obesity and the subsequent risk of co-morbidities, bariatric surgeries, including Roux-en-Y gastric bypass and laparoscopic banding, have emerged over the past decade as a safe and relatively effective obesity treatment. Current data indicate that successful surgeries result in an average loss of 60% of excess weight [78]. Epidemiological reports show that CVD risk, as classified by Framingham criteria, decreases significantly as early as six months following surgery [79]. To qualify for Roux-en-Y gastric bypass, a patient must have a body mass index greater than 40 kg/m^2 or a BMI greater than 35 kg/m^2 with co-morbidities. The bariatric population is a useful model for studying the pathophysiology of morbid obesity

because of the unique physical characteristics of the subjects, rapid and predictable weight loss, and the ability to obtain valuable tissue during surgery that is not accessible in a standard clinical laboratory.

Antioxidant interventions

Interventions to combat oxidative stress have included supplementation with exogenous antioxidants that have been largely ineffective. Clarke *et al.* performed a meta-analysis on eight trials examining beta-carotene or vitamin E supplementation in healthy individuals and those at risk for CVD, and reported no protective effects of supplementation on future development of CVD [80]. Other studies report similar findings as well as data indicating that vitamin A, vitamin E and beta carotene may increase mortality in some cases [81-83], and that vitamin C can be pro-oxidant [84].

An alternative approach to combat oxidative stress is to activate the antioxidant response element (ARE) binding transcription factor NF-E2-related factor 2 (Nrf2). Nrf2 activation leads to induction of endogenous phase II antioxidant enzymes and protection from oxidative stress. Nrf2 binds to the ARE in the promoter regions of several hundred genes including many phase II antioxidant enzymes [85, 86]. Nrf2 is constitutively expressed but is marked for ubiquitination by association with Kelch-like ECH-associated protein 1 (Keap1). Activation of Nrf2 occurs when it is released from Keap1, translocates to the nucleus, heterodimerizes with small Maf or Jun proteins, binds the ARE, and initiates transcription of the associated genes. Multiple mechanisms

for Nrf2 activation have been proposed (reviewed in [85]). Electrophilic or oxidative modification to Keap1 cysteine thiol groups leads to dissociation of Nrf2 binding by Keap1 [87]. Direct phosphorylation of Nrf2 by a number of kinases, including several from the MAPK family, can also cause dissociation of Nrf2 from Keap1 [88-90]. The exact role of direct Nrf2 phosphorylation is not completely understood, as others have reported that phosphorylation of Nrf2 by multiple MAPKs may have a limited contribution to Nrf2 activation [91].

Protandim is a commercially available dietary supplement consisting of phytochemicals derived from five widely studied medicinal plants including silymarin from milk thistle, curcumin from turmeric, bacopa extract, ashwaganda, and green tea extract. It is thought that the five components of Protandim act synergistically to induce Nrf2 [92]. Phytochemical compounds, including the combination in Protandim, likely activate Nrf2 and induce phase II antioxidant enzyme expression through activation of various kinases with subsequent Nrf2 phosphorylation [93]. In humans, supplementation with Protandim significantly decreased circulating TBARS, and significantly increased erythrocyte SOD and catalase activity [94]. Data from *in situ* experiments in human saphenous veins show that Protandim increased SOD and catalase activity in addition to HO-1 activity, while decreasing superoxide and markers of lipid peroxidation [95]. Significant Nrf-2 dependent HO-1 induction by Protandim has also been shown in multiple cell lines *in vitro*, with increases in HO-1 being as high as 8.5 fold over baseline [92]. Thus, phytochemical induced Nrf2 activation is a potential

therapeutic intervention against endothelial cell oxidative stress and associated vascular disease initiation and progression.

Hypotheses and Experiments

The overall objectives of these projects were to investigate obesity associated lipid and oxidative stress, and examine strategies to protect coronary artery endothelial cells against these stressors.

Experiment 1- We hypothesized that lipid profiles would be significantly different in plasma from morbidly obese humans compared to lean, and that lipids that are elevated in obese subjects would induce inflammatory changes in cultured endothelial cells. In addition, we hypothesized that oxidized phospholipids would be greater in plasma from obese subjects compared to lean.

The first experiment was designed to use a lipidomic analysis to compare lipid distribution and oxidation in samples from lean control subjects and morbidly obese gastric bypass patients. The analysis was a combined targeted and shotgun approach. The targeted portion examined oxidized phospholipids that are known to induce endothelial cell pathogenic phenotypes. The shotgun portion involved a sample wide examination of lipid species to determine what is different between lean and obese, to identify biomarkers or pathological lipids that have not been characterized yet in this capacity.

Experiment 2- We hypothesized that Protandim would activate Nrf2 and induce phase II antioxidant enzyme expression in human coronary artery endothelial cells (HCAEC), and that Protandim treatment prior to an oxidative challenge would protect HCAEC in a Nrf2 dependent manner.

The second experiment was designed to examine whether Protandim can protect human coronary endothelial cells (HCAEC) from an oxidative challenge, and if that protection is Nrf2 mediated. We first examined Nrf2 nuclear localization and antioxidant enzyme protein expression in response to Protandim. To determine whether Protandim effects on Nrf2 and antioxidant enzymes protected cells from an oxidative challenge, we examined cell death in response to an oxidative challenge with and without Protandim treatment. Last, to determine if Nrf2 mediated protection, we knocked down Nrf2 prior to Protandim treatment, then examined cell death in response to an oxidative challenge.

Experiment 3- We hypothesized that Lp-PLA_2 would be expressed in human adipose tissue, and that expression would be greater in tissue from obese subjects compared to lean. We also hypothesized that Lp-PLA_2 expression would correlate with plasma Lp-PLA_2 activity, plasma inflammatory markers and indices of glucose tolerance.

The third experiment was designed to examine the presence of Lp-PLA_2 in different adipose depots from lean and obese subjects, and determine cell type distribution of Lp-PLA_2 within those adipose depots. In addition, plasma Lp-PLA_2 activity,

plasma TNF-α and HbA1C were measured to determine if there is a relationship between adipose Lp-PLA_2 and markers of inflammation and glucose intolerance.

Main conclusions

These projects describe novel investigations into lipid and oxidative stress in obesity, how these factors affect endothelial cells, and how phytochemicals in Protandim can protect endothelial cells from oxidative stressors. Experiment 1 showed that while ox-PC is not elevated in obese subjects compared to lean, a group of ether-linked phospholipids are greater in the serum of obese subjects compared to lean. In addition, these lipids induce cell adhesion molecule protein expression in endothelial cells. Experiment 2 showed that Protandim induces Nrf2 nuclear localization and phase II antioxidant enzyme protein expression, and protects endothelial cells from an oxidative challenge in a Nrf2 dependent manner. Experiment 3 showed that Lp-PLA_2 is expressed in adipose tissue, that adipose tissue Lp-PLA_2 is preferentially localized to adipose resident macrophages, and that glycosylation of adipose Lp-PLA_2 correlates with indices of glucose homeostasis.

Collectively, these data provide insight into the oxidative and lipid stress milieu that exists in obese subjects, novel information regarding tissue Lp-PLA_2 expression and relation to indices of glucose homeostasis and inflammation, and evidence supporting Protandim as an intervention to protect against endothelial oxidative stress.

CHAPTER II- MANUSCRIPT I

LIPIDOMIC ANALYSIS OF HUMAN PLASMA REVEALS NOVEL LIPIDS OF INTEREST THAT ARE ELEVATED IN MORBIDLY OBESE HUMANS COMPARED TO LEAN.

Title: Lipidomic analysis of human plasma reveals novel lipids that are greater in morbidly obese humans compared to lean.

Authors: [1]Elise L. Donovan, [2]Matthew R Lewis, [3]Stefan M Pettine, [1]Matthew S Hickey, [1]Karyn L. Hamilton*, [1]Benjamin F. Miller*

Affiliations: [1]Department of Health and Exercise Science, Colorado State University, [2]Proteomics and Metabolomics Facility, Department of the Vice President for Research, Colorado State University, [3]Bariatric Center of the Rockies, Fort Collins, Colorado

*Co-Principal Investigators

Corresponding Author:

Benjamin F. Miller

220 Moby B Complex 1582

Colorado State University

Fort Collins, CO 80523

970-491-3291 (phone)

benjamin.f.miller@colostate.edu

Dyslipidemia and oxidative stress are characteristic of obesity and diabetes, and lipid peroxidation appears to play a major role in cardiovascular disease. We performed a lipidomic analysis to explore differences in lipid profiles between plasma from lean and obese subjects, and used *in vitro* methods to examine a role for the identified lipids in endothelial cell pathophysiology. Plasma was collected from 15 morbidly obese and 13 lean control subjects. Lipids were extracted from plasma and analyzed using LC/MS, and MS/MS to characterize lipid profiles and identify lipids that are elevated in obese subjects compared to lean. Orthogonal partial least squares- discriminant analysis (OPLS-DA) modeling showed that lipid profiles were significantly different in obese subjects compared to lean. Analysis of lipids that were driving group separation in the OPLS-DA model and that were significantly elevated in the obese group led to identification of a group of ether-linked phosphatidylcholine and phosphatidylethanolamine lipids of interest. Treatment of human coronary artery endothelial cells with the ether-linked phosphatidylethanolamine induced expression of cell adhesion molecules, a hallmark of endothelial cell activation. We also scanned our data for oxidized phospholipids that have previously been shown to induce endothelial cell activation *in vitro*, and did not find any significant differences between groups *in vivo*. These data suggest a role for ether-linked lipids in obesity associated dyslipidemia and vascular disease.

Keywords: Lipidomics, obesity, dyslipidemia, endothelial cells, oxidized phospholipids.

Introduction

Dyslipidemia and oxidative stress are characteristic of obesity and diabetes, and lipid peroxidation appears to play a major role in diabetes, cardiovascular disease, and cancer [1, 2]. Lipid oxidation can be mediated by reactive oxygen species (ROS) or occur enzymatically, resulting in a variety of potential products. The mechanisms of oxidized phospholipid formation have been extensively reviewed [3, 4]. Obesity is associated with increased oxidized LDL that leads to inflammation and oxidative stress in vascular endothelial cells and contributes to the pathophysiology of the metabolic syndrome and atherosclerosis [5, 6]. The chronic inflammatory state associated with morbid obesity and the metabolic syndrome further increases the oxidative modification of phospholipids [7]. Collectively, this creates a "vicious cycle" of inflammation, oxidative stress, and lipid stress, which contributes to obesity-associated pathology. Ectopic fat deposition in morbidly obese individuals also contributes to the pathogenesis of vascular and metabolic disturbance by inhibiting insulin action and disrupting lipid metabolism in the liver, pancreas, kidney and skeletal, smooth and cardiac muscle[8]. This "lipotoxicity", also including the sequelae associated with abnormal oxidation of lipids, contributes to inflammation and oxidative stress in multiple tissues [9] [10, 11].

Phosphatidylcholine (PC) species that contain polyunsaturated fatty acids, particularly arachidonate, at the SN-2 position are especially susceptible to free radical oxidation resulting in a variety of potential oxidized products. One such lipid

is 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC), which is a common cell membrane constituent and circulates within cholesterol particles. PAPC and several products of PAPC oxidation including 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC), 1-palmitoyl-2-(5,6-epoxyisoprostane E_2)-sn-glycero-3-phosphorylcholine (PEIPC),1-palmitoyl-2-oxovaleroyl-sn-glycero-3-phosphorylcholine (POVPC), and lysophosphatidylcholine (lyso-PC) have been shown to induce cell adhesion molecule expression and inflammatory mediator secretion in endothelial cells and have been implicated in atherosclerotic progression [12-17] . In addition, oxidized phospholipids are found deposited in atherosclerotic plaques [18, 19]. PAPC, POVPC, PGPC, and PEIPC are increased 10-25 fold in modified LDL and 3-6 fold in lesions in rabbits fed an atherogenic diet [19]. This increase in oxidized phospholipids is 10-20 times the amount needed to induce pro-atherogenic phenotypic shifts in endothelial cells [19]. Additionally, endothelial cell apoptosis may be an independent risk factor for thrombosis [20], and PAPC, PGPC, and lyso-PC induce endothelial cell apoptosis [21-23] thus contributing to plaque progression and rupture. In addition to up-regulating expression of inflammatory mediators and cell adhesion molecules, oxidized phospholipids can induce expression of NADPH oxidase and production of reactive oxygen species (ROS), directly increasing cellular oxidative stress [24] [25]. Because PC is the most abundant cellular phospholipid, most oxidation products detected contain the choline head group [26]. However, other lipid classes and additional oxidized phospholipids are likely involved in vascular pathology, and it is

likely that interactive effects between oxidized phospholipids contribute to CVD/metabolic disease pathophysiology.

Lipidomics is broadly defined as the large-scale study of pathways and networks of cellular lipids in biological systems. Lipidomics can be used to examine the presence and structure of the range of lipid species within different tissues making it an attractive method for examining distribution of phospholipids in tissue, as well as for globally examining differences in lipids between subject groups. A broad examination of oxidized lipid species that are elevated and potentially contribute to metabolic and cardiovascular disease pathology in morbidly obese humans has not been performed. A lipidomic study of this nature could provide insight into the link between obesity and pathogenesis of atherosclerosis and facilitate discovery of lipid biomarkers that increase disease risk.

The purpose of this study was to: 1) use a shotgun lipidomics approach to examine global lipid distribution in plasma from lean and morbidly obese humans to identify lipids that are greater in the morbidly obese population that may contribute to metabolic and cardiovascular pathology, 2) examine the effects of lipids identified in our shotgun analysis as elevated in obese subjects in an *in vitro* endothelial cell model to determine whether they induce disease related phenotypic changes, and 3) use a targeted lipidomics approach to determine if there is a difference in distribution of oxidized phospholipids in plasma from lean and morbidly obese humans. We hypothesized that lipid profiles would be significantly different in plasma from morbidly obese humans compared to lean, and that lipids identified in

our shotgun approach that are elevated in obese subjects compared to lean would induce inflammatory changes in endothelial cells. In addition, we hypothesized that oxidized phospholipids shown previously *in vitro* to cause endothelial cell production of inflammatory mediators and cell adhesion molecules would be greater in plasma from obese humans compared to lean.

Materials and Methods

Ethics approval

The Institutional Review Boards of Colorado State University and Poudre Valley Hospital approved this protocol (CSU protocol #- 05-116H, PVH protocol #-07-874). Each volunteer was informed of the potential risks and written consent was obtained prior to enrollment. The study followed the guidelines set forth by the Declaration of Helsinki.

Study overview

A total of 15 morbidly obese gastric bypass patients, and 13 lean controls age 18-60 were recruited to participate through the Northern Colorado Surgical Associates (NCSA) of the Bariatric Center of the Rockies. Bypass patients were required to have a body mass index (BMI) > 40 kg/m^2, while controls were required to have a BMI < 30 kg/m^2. There were no other exclusion criteria allowing the subject population to be heterogeneous, and thus differences in lipid profile between groups would more closely represent the general clinical population. Subjects completed medical and exercise questionnaires prior to undergoing a venipuncture blood draw following a 12-hour fast. Blood was collected in vacutainer tubes containing EDTA, 0.5ml was aliquoted for HbA1C analysis, the remainder was centrifuged (1200g, 15 min, 4°C) and stored at -80°C until lipid extractions were performed.

Subject characteristics

Participant characteristics are shown in Table 1. We were unable to obtain body composition data because subject size exceeded the capacity of the available dual energy x-ray absorptiometry (DEXA) equipment. Height and weight were measured at the surgical center, while other demographic and health information was obtained through a health history questionnaire. Whole blood samples were sent to the University of Colorado Denver Clinical Translational Research Center for analysis of hemoglobin A1C (HbA1C) using a DCA Vantage analyzer (Siemens, Deerfield, IL).

Lipid extraction

Lipid extractions were performed using the methyl-tert butyl ether (MTBE) method described by Matyash *et al.* [27], with all steps performed in the Captair Pyramid glove box (Erlab, Rowley, MA) under argon gas to prevent exposure to oxygen and subsequent oxidation of lipids. The MTBE method is analogous to the traditional Folch and Bligh Dyer methods, except the organic lipid containing phase is present above the aqueous phase eliminating the need to collect the organic layer from beneath the aqueous layer. We ran a Bligh Dyer extraction and an MTBE extraction side by side to confirm that lipid yields and content were indeed equivalent prior to proceeding with the MTBE method. Briefly, 200µl plasma was added to 80µl of mass spec grade H_2O in a glass tube with a Teflon lined cap, followed by addition of 1.5ml molecular grade methanol. The tubes were then vortexed, followed by the addition of 5ml molecular grade MTBE, and then rocked for 1hr at room temperature. Following the incubation, 1.25ml mass spec grade H_2O

was added and the tubes were vortexed and centrifuged at 1,000 x g for 10min. The upper organic phase was collected, placed in another glass tube with a Teflon lined cap, and dried under nitrogen gas. Samples were re-suspended in 1ml LC-MS grade methanol, and topped with high purity argon gas. Samples were stored for no longer than one week at -20°C prior to chromatographic analysis.

Liquid chromatography/mass spectroscopy (LC/MS)

Both the targeted and shotgun analysis were performed on lipid extracts were separated by reversed phase ultra performance liquid chromatography (UPLC) on an Acquity instrument (Waters, Milford MA). Solvent A consisted of an 89:5:5:1 mixture of water, isopropyl alcohol, acetonitrile and 500mM ammonium acetate respectively. Solvent B consisted of a 50:49:1 mixture of isopropyl alcohol, acetonitrile, and 500mM ammonium acetate, respectively. All solvents were LC-MS grade (Fisher, Optima LCMS). One microliter injections were loaded to a 1.0x100mm Acquity BEH C8 column held at 50°C with a 140 µl/min flow of 100% solvent A. Solvent A was held at 100% for 0.1 min, followed by a linear gradient to 40% Solvent A over 0.9 min. A second linear gradient to 100% Solvent B was achieved over 10 minutes, followed by a 3 min hold at 100% Solvent B. The chromatographic system was returned to initial conditions via a 0.1 min linear gradient to 100% Solvent A, followed by a 5.9 min equilibration prior to the subsequent injection. Total run time was 20 min. Eluate was introduced to a Q-ToF Micro mass spectrometer (Waters/Micromass, Manchester, UK) via electrospray in either the positive or negative ionization mode. Capillary voltages were held at 3000 V and 2200 V in positive and negative ionization modes, respectively. In both

modes the sample cone voltage was held at 30 V, the collision energy was held at 7 V, the source temperature was held at 130°C, and the desolvation temperature was held at 300°C. Data were acquired over the 100-1200 m/z range in all analyses, at a rate of 2 scans/sec. The sample set was analysed in independently randomized technical duplicate for each ionization mode. Both the UPLC and mass spectrometer were operated using MassLynx software version 4.1 (Waters, Milford, MA).

Peak detection and integration were performed using MarkerLynx software (Waters, Milford MA). Chromatographic peaks eluting between 0 and 14 min with m/z values between 100 and 1200 were detected using a mass window of 0.07 Daltons and a retention time window of 0.1 min. Apex Track peak parameter settings were used, with peak width and baseline noise set for automatic calculation. A threshold of 10 counts per scan and a noise elimination level of 6 were implemented to minimize the detection of spectral noise, and the deisotope option was enabled to exclude isotopic peaks from the final data matrix of detected features and their intensities across all samples.

OPLS modeling and lipid identification

For the shotgun analysis, multivariate statistical analysis was performed using SIMCA-P^{+} software (Umetrics Kinnelton, NJ). We used principal components analysis (PCA), and combined PLS and orthogonal single correction discriminant analysis (OPLS-DA) to analyze the LC/MS data. In short, PCA is an unbiased analysis of the total metabolite content (in our analysis the total plasma lipid profile) within a set of samples. PCA detects variance among samples and provides sample clusters

based on similarity of molecular profile. OPLS-DA is an extended form of partial least squares-discriminant analysis (PLS-DA) that, in addition to explaining overall differences between classes, separates predictive and non-predictive variation [28]. Data were modeled to visualize discrimination between the obese and lean control groups using the OPLS-DA model and scores plot of the first and second components. Goodness of fit was quantified by R^2X and R^2Y, and predictability by Q^2Y.

There were factors in addition to obesity that could influence and potentially weaken our model because of the heterogeneity of the subject population. However lipids that emerge as significantly different between lean and obese despite the heterogeneity best represent the general clinical population. To account for potential model weakness, a strict analysis and reduction to specific lipid ions of interest was performed using the models and scores plots from the OPLS-DA analysis, along with the raw LC/MS data. This analysis allowed us to identify lipids that were significantly different between lean and morbidly obese subjects as well as those lipids driving the difference between the groups. First, a list was compiled of all the ions falling between 0.5 and 1 on the scores plots from both positive and negative mode because these ions were driving the difference between groups in the OPLS-DA model. An additional list was compiled containing all ions that were significantly different between groups based on t-tests comparing feature abundance. All ions on the t-test list with a difference of less than 10 fold between obese and lean control groups were excluded, as were the features with abundances too low to be distinguished as actual lipid ions, allowing us to narrow the list to

approximately the most significantly different 10% of the ions. The remaining ion features on the t-test list were cross-referenced with the ion features on the scores plot list. A final list was compiled of ion features that were present on both the scores plot list and final t-test list. These were the lipid ions that were both driving the difference between groups observed in the OPLS modeling, and were statistically significantly different between groups. Ions on this list were further examined using tandem mass spectroscopy (MS/MS).

Tandem mass spectroscopy and lipid identification

Samples were analyzed by MS/MS as described above for the initial LC/MS analysis with the exception of selective mass filtering with the quadrupole for our ions of interest, and a collision energy of 40 V. Fragmentation patterns were analyzed and cross referenced to Lipid Maps mass spectrometry peak prediction resources [29], and matched with published spectra where available for identification of lipids of interest. Standards were commercially available (Avanti Polar Lipids, Alabaster, AL) for three of the tentatively identified lipids of interest, 1-0-1'-(Z)-Octadecenyl-2-Arachidonoyl-sn-Glycero-3-Phosphoethanolamine, 1-Palmitoyl-2-Linoleoyl-sn-Glycero-3-Phosphocholine, and 1-octadecanoyl-2-(5Z, 8Z, 11Z, 14Z-eicosatetraenoyl)-sn-Glycero-3-Phospho-(1'-myo-inositol))and we performed MS/MS on these to confirm our tentative identifications. Following confirmation of our tentative identifications, we used the ether-linked phosphoethanolamine standard in cell culture treatments to determine if it induced pathological phenotypic changes in endothelial cells. Treatment was done with this standard because it was the ether-linked standard that was available.

Cell culture and lipid treatments

Primary human coronary artery endothelial cells (HCAEC) (Lonza, Walkersville, MD) were grown in endothelial cell growth medium (EBM-2) containing 5% FBS and manufacturer recommended supplemental growth factors, antibiotics and antimycotics. All assays were performed on cells at 80-100% confluence, between passages 3 and 9. Lipid treatments included normal medium vehicle controls, and the 1-0-1'-(Z)-Octadecenyl-2-Arachidonoyl-sn-Glycero-3-Phosphoethanolamine (PE) standard. This PE standard was used for endothelial cell treatments as a representative ether-linked lipid. Treatment concentration for the PE standard was 25μg/ml, with all treatments performed for 4 hrs and repeated a minimum of 3 times in duplicate or triplicate.

Western blot analyses

HCAEC were seeded in 65mm polystyrene cell culture dishes and grown to at least 80% confluence prior to lipid treatment. Following treatment, cells were scraped in RIPA buffer (50mM Tris, 0.15M NaCl, 1% Na deoxycholic acid, 1mM EGTA, 1% NP40) containing protease and phosphatase inhibitors, and sonicated 3 x 10 sec. Protein concentrations were determined using a BCA assay, and samples were diluted to equal concentrations with Laemmli sample buffer . Samples were separated on 7.5 or 10% polyacrylamide gels (depending on the molecular weight of the protein of interest) at 125 V, and transferred to nitrocellulose membranes (BioRad, Hercules, CA) for 1 hr at 50 V. Membranes were blocked for 1 hr in Superblock (Thermo Scientific, Rockford, IL), then incubated with primary antibodies against VCAM(1:200) and ICAM(1:200) followed by the appropriate

HRP-conjugated secondary antibodies. Membranes were developed by chemiluminescence using SuperSignal West Dura substrate (Thermo Scientific, Rockford, IL), with digital images obtained using the Biospectrum UVP system (Upland, CA). All signals were normalized to β-actin obtained from the same blot, and expressed as the percent of the normal medium control condition.

ELISA analysis

Monocyte chemoattranctant protein-1(MCP-1) in the cell culture medium was determined using a sandwich ELISA (R & D, Minneapolis, MN). The analysis was performed according to manufacturer instructions. The lower limit of detection was 31.2 pg/ml and average CV was 2.2%.

Statistical analysis

Modeling and analysis of LC/MS data are described above. Comparison of ion abundance between lean and control groups was performed using unpaired t-tests. Lipid treatment effects on HCAEC were compared using one-way ANOVA with Tukey's *post-hoc* where applicable. Significance was set *a priori* at $p \leq 0.05$.

Results

Subject characteristics

There was no difference in average age between groups, but average BMI was significantly higher ($p<0.01$) in the morbidly obese group (49.87 ± 11.27 kg/m^2) compared to lean (25.76 ± 4.39 kg/m^2) and HbA1C was significantly higher ($p<0.01$) in the morbidly obese group (6.41 ± 0.35 % of total) compared to lean (5.15 ± 0.10 % of total) (Table 1).

Global lipidomic analysis

For both the shotgun and targeted analysis we analyzed lipid extracts from lean and obese subjects using LC/MS. Representative chromatography and spectroscopy are presented in Supplementary Figure 1. We then began the analysis for the shotgun approach with the principal component analysis (PCA). PCA models (Figure 1) for positive and negative mode poorly classified the lean and obese groups, which was not unexpected given the heterogeneity of the groups. The PCA model eliminated outliers prior to the biased OPLS-DA analysis. OPLS-DA modeling was performed on the remaining lean and obese lipid profile data obtained in both positive and negative mode. The positive mode model and scores plot are shown in Figure 2A, the negative mode model and scores plot are shown in Figure 2B. R^2X, R^2Y, and Q^2Y of the positive mode model were 0.298, 0.839, and 0.259 respectively, while R^2X, R^2Y, and Q^2Y of the negative mode model were 0.49, 0.658, and 0.216 respectively. The R values indicate the goodness of fit of the x and y axis

components, the Q value indicates the predictability of the model. While the greater the R and Q values the better, our values are reasonable based on other analyses in heterogeneous diseased human populations [30]. Based on the OPLS-DA models and statistical analysis as described above, a list of 43 lipid ions that were significantly different between groups and driving the group separation in the OPLS-DA model was compiled for further examination, 26 of those were higher in obese subjects (Supplementary Table 1).

Lipid ion of interest identification

MS/MS analysis was performed on all lipid ions of interest. Following MS/MS analysis, screening of spectra, comparison of fragments in Lipid Maps, comparison to published spectra, and elimination of ions that were unidentifiable or became obsolete, 22 ions of interest were tentatively identified. Numerous tentatively identified ions were present as multiple adducts. Table 2 shows the final list of ions of interest that were greater in obese subjects and of interest for further study. We discovered that multiple ether-linked phospholipids were significantly higher in the obese group compared to lean and were driving group separation in the OPLS-DA model. Figure 3 shows a representative MS/MS spectrum of PE(P-18:0/20:4(5Z,8Z,11Z,14Z)) an ether-linked phosphatidylethanolamine (PE) that was identified in negative mode, and a previously published spectrum of the same ion.

To confirm our tentative ion identifications, we purchased the three standards that were available from our list to analyze via MS/MS and compare the

spectra of our unknown ions to the spectra of the standards. Spectra from all three standards matched the spectra of our unknowns confirming the identity of those three ions. Figure 4A shows the negative mode MS/MS spectrum of PI(18:0/20:4(5Z,8Z,11Z,14Z)) from our sample and Figure 4B the negative mode MS/MS spectrum of the PI(18:0/20:4(5Z,8Z,11Z,14Z)) standard. Major identifying peaks include 885 (parent ion), 303 (20-4 arachidonic acid), 283 (18-0 steric acid), and 241(inositol head group). Because the exceptional finding from the shotgun analysis was the emergence of the group of ether-linked lipids, we next sought to examine what potential effects ether-linked lipids may have on endothelial cells, to obtain insight into the relationship between obesity associated dyslipidemia and vascular disease. From the list of ether-linked lipids, we were able to perform *in vitro* cell culture treatments using the p-PE standard.

Endothelial cell response to ether-linked phosphatidylethanolamine

We used PE-(P-18:0/20:4(5Z,8Z,11Z,14Z)) (p-PE) to treat HCAEC. p-PE induced increases in the cell adhesion molecules ICAM (158% of control, $p<0.05$) and VCAM (144% of control, $p<0.05$) (Figure 5). Cell culture medium was collected for analysis of the secreted inflammatory mediator MCP-1. MCP-1 did not differ in medium collected from cells incubated in normal medium, with PAPC or oxidized PAPC, and those that were exposed to p-PE (Figure 6).

Targeted analysis of oxidized phosphatidylcholine

Because previous *in vitro* data indicate that oxidized phosphatidylcholine (ox-pc) products cause pathological phenotypic changes in endothelial cells, we

targeted pc and ox-pc in our LC/MS data from morbidly obese and lean plasma using a targeted approach. We scanned all ions in the positive mode analysis for any that could be PC, PGPC, POVPC, or PEIPC based on mass. While none of the best potential matches for these lipids were between 0.5 and 1 in our OPLS-DA models, PC appeared to be significantly higher in obese subjects compared to lean ($p= 0.040$). Of the best matches for the ox-pc species, only those for PGPC were close to significantly elevated in the obese compared to lean ($p=0.052$, and 0.055), while the best potential matches for PEIPC were not significant ($p=0.707$, and 0.661) and no ions that could represent POVPC were detected (Table 3).

Discussion

Because obesity is a major independent risk factor for coronary artery disease, and a major component of that risk is dyslipidemia, we sought to characterize the lipid profiles from morbidly obese human subjects compared to lean. We were able to combine *in vivo* and *in vitro* methods and further examine the role of lipids that were identified and elevated in obese subjects in pathophysiology of endothelial cells. The major findings of this study were that a group of ether-linked lipids was elevated in plasma from morbidly obese subjects compared to lean, and an ether-linked phosphatidylethanolamine increased cell adhesion molecule protein but not the inflammatory mediator MCP-1 in cultured endothelial cells. In addition, targeted oxidized PAPC products previously shown to alter endothelial cell phenotype were not elevated in our obese group compared to lean.

Ether-linked lipids

Importantly, there was a significant difference in the lipid profiles between our lean and obese subjects. Following our conservative and strict approach to narrowing down the total lipid profile to a small list of lipids of interest, a group of ether-linked phospholipids that were elevated in obese subject plasma compared to lean that were driving the separation of groups in our model emerged. Ether-linked lipids are characterized by an ether linkage between the glycerol backbone and one or both fatty acid side chains (usually the sn-1 position) as opposed to an ester linkage. A subclass of ether-linked lipids are plasmalogens which are characterized

by a vinyl ether linkage at the sn-1 position with an ester linkage at the sn-2 position.

Ether-linked lipids have been recently characterized in macrophages, lens cells, adipose, liver, and serum [31-33]. Kotronen *et al.* performed a lipidomic analysis on serum, liver, subcutaneous adipose, and intraabdominal adipose from a group of obese subjects [32]. Results showed that ether-linked PEs were higher in liver compared to both adipose depots, but ether-linked PCs were higher in adipose depots. In this analysis ester PCs were more abundant in all tissues compared to ether-linked, but ether-linked PEs were more abundant than esters in all tissues. Ether-linked PCs and PEs have also been characterized in macrophages, along with ether-linked lipids with other head groups, though this analysis did not attempt to examine the role of such lipids [33]. Other recent work profiling lipid composition of LDL found that plasmalogen PEs are decreased in metabolic syndrome patients compared to lean, as well as in diabetics compared to lean, and the proportion of plasmalogen PE negatively correlates with waist circumference. [34] In the present study we did not have enough subjects to stratify results based on diabetes in our obese group. Collectively, these data and ours support further examination of why ether-linked phospholipids are altered between lean and obese subjects and what the role of ether-linked lipids in metabolic disease may be.

Physiological roles of ether-linked lipids

Lipidomics often functions as a hypothesis generating technique where biomarkers of interest emerge from large data sets indicating further analysis of

physiological roles of identified features. Our findings and those described above support further investigation of the discrepancies in ether-linked lipids between lean and obese subjects, as well as diabetics and non-diabetics, as there is a lack of consensus about whether ether-linked lipids are pro-or anti-oxidant and inflammatory. Ether-linked phospholipids are present in most cell membranes and studies of their function have ranged from signaling in brain cells to antioxidant properties [35-38]. Ether-linked lipids are more susceptible to oxidation compared to ester-linked lipids ([37, 38]). Khaselev and Murphy found that ether-linked plasmalogens are preferentially oxidized over ester-linked phospholipids, possibly due to structural differences leaving the polyunsaturated sn2 side chains more exposed [39], and lower bond dissociation energies of ether linkages [36]. Decreased plasmalogens in LDL from patients with the metabolic syndrome and diabetes have been observed and suggested as an indicator of oxidative stress [34]. Plasmalogen deficiency has also been linked to Alzheimers disease [35]. Whether ether-linked lipids function as ROS scavengers or are simply more susceptible to oxidation remains to be seen. However if ether-linked lipids are scavengers and are preferentially synthesized in response to a pro-oxidant environment or altered membrane fluidity, this may be of interest in reference to obese and diabetic populations where increased oxidative stress is often a problem.

Ether-linked lipids may be pro-inflammatory. Ether-linked lipids can serve as arachidonic acid reservoirs. Oxidation products of ether-linked lipids include proinflammatory lysophospholipids and ether-linked lipids are platelet activating factor (PAF) precursors. Ether-linked lipids, particularly plasmalogens have a high

proportion of arachidonic acid at the sn-2 position, and are involved in membrane remodeling as well as intracellular arachidonic acid metabolism [40, 41]. Arachidonic acid released from the sn-2 position of ether and ester lipids can be enzymatically oxidized to form prostaglandins, prostacyclins, thromboxanes, and leukotrienes, all of which are pro-inflammatory [42, 43]. In addition, the remaining lysophospholipids serve as precursors to PAF formation and can be pro-inflammatory and pathological. Lysophosphocholines (LPCs) have been shown to increase plasminogen activator inhibitor-1, a potent prothrombotic and proatherogenic protein [11]. In addition, LPCs can induce endothelial cell expression of cell adhesion molecules, an important step in endothelial cell activation and atherosclerotic progression [9] [44]. Ether-linked lipids with arachidonic acid at the sn-2 position serve as PAF synthesis precursors, as removal of the sn-2 fatty acid chain is the first step in the PAF formation remodeling pathway. PAF is an ether-linked species with a choline headgroup, an ether-linked fatty acid at the sn-1 position and an acetyl group at the sn-2 position. PAF is a potent stimulator of the platelet coagulation cascade, thrombosis, and inflammation. PAF is synthesized in response to external pro-inflammatory and pro-thrombotic mediators including TNF-α [45]. PAF has been implicated in atherogenesis because it activates and recruits inflammatory cells, and in advanced plaque rupture because it initiates the coagulation cascade in platelets (reviewed in [46]). PAF synthesis occurs through the remodeling of sn-1 ether-linked phospholipids where through a series of enzymatic reactions the sn-2 side chain is removed and an acetyl group is attached in its place. What is unknown is whether PAF formation through the

remodeling pathway only occurs in response to external stimuli, or if remodeling formation of PAF can be driven by excess substrate availability. The ether-linked phospholipids, particularly those with arachidonic acid at the sn-2 position and choline head groups may serve as substrates for PAF formation and thus be characterized as pro-inflammatory.

pPE and HCAEC

To address whether ether-linked lipids can induce phenotypic changes in endothelial cells, we performed *in vitro* experiments using a commercially available ether-linked PE that we identified as elevated in obese subjects. The pPE that we tested stimulated cell adhesion molecule expression in human coronary artery endothelial cells, a key step in atherogenesis. We cannot conclude based on these data that the effects of this plasmalogen phosphatidylethanolamine are representative of other ether-linked lipids, including those ether-linked phosphatidylcholines that were also identified during our analysis. However, our data suggest that this lipid may induce changes in endothelial cells indicative of vascular disease, and support further analysis of the role of ether linked lipids in the vascular endothelium.

Oxidized phospholipids

The finding that phosphatidylcholine oxidation products that have been shown *in vitro* to induce disease related phenotypic changes in endothelial cells were not elevated in our obese subjects was unexpected. Because oxidized phosphatidylcholine products have been found in modified LDL and atherosclerotic

lesions from high fat fed rabbits [19] and oxidized LDL has been found in lesions isolated from human vessels [47], we hypothesized they would be elevated in morbidly obese subjects compared to lean. Our findings do not discredit the data indicating detrimental effects of oxidized PAPC on endothelial cells, however oxidized PAPC products did not drive the separation between lean and obese groups in our model. It could be that a modest difference is enough to be biologically relevant. Our data support the importance of examining other classes of lipids and presence of oxidized phospholipids in human plasma and LDL.

Lipidomic and metabolomic analyses

Several recent studies have used lipidomic and metabolomic techniques to examine differences between diseased subject groups and healthy controls. As noted above Kotronen et al. performed lipidomic analysis on multiple tissues in subjects with metabolic syndrome and diabetes [32]. Wang et al. also examined plasma from diabetics using lipidomic techniques [48]. Kim et al performed metabolic profiling of overweight and obese subjects compared to lean and identified several lysophospholipids in overweight and obese subjects in addition to differences in fatty acid profiles between groups that generate questions related to altered metabolism in obesity [49]. Additional metabolic profiling in gastric bypass subjects before and after surgery has provided insight into the metabolic changes that occur with surgically induced weight loss [50].

While lipidomics and metabolomics studies don't necessarily address mechanistic questions, information obtained using these techniques provides

information on global differences between groups and often drives hypothesis development for mechanistic questions. Each lipidomic and metabolomic analysis is performed using unique methods and parameters, and generates unique results contributing to global understanding of group differences and metabolism. Limitations in our analysis include a small sample size, and a model with weaker than ideal predictability. In addition, while all preparation and analysis of samples following collection was performed under inert gas, we did not use an internal antioxidant. Regardless, the emergence of the ether-linked lipids of interest is strongly supported. Data in our lipidomic analysis show elevated ether-linked lipids in morbidly obese subjects compared to lean and support further investigation of the role this class may play in obesity associated vascular and metabolic disease.

Acknowledgements

The authors would like to thank Shanyn Willis, Leora Garcia, Dr. Melinda Frye, Dr. Chris Bell, and Dr. Corey Broeckling from CSU, as well as Drs. Robert Quaid, Michael Roller, James Dickinson, and all nurses and staff at Northern Colorado Surgical Associates and the Bariatric Center of the Rockies for their contributions and assistance.

Funding Source

CSU Proteomics and Metabolomics Facility Academic Enrichment Program

Figure Legends

Figure 1. Principal components analysis models for all ions for all subjects detected in positive and negative mode.

Figure 2. OPLS-DA models and representative scores plots from positive mode analysis (A) and negative mode analysis (B). Black circles in the OPLS model represent lean controls, red squares represent obese pre-operative subjects. Each triangle on the scores plot represents an ion that was detected. The red boxes show the ions that were screened for further analysis.

Figure 3. Representative spectrogram of the ion m/z 750.5399 from MS/MS analysis that was identified as the plasmalogen phosphatidylethanolamine PE(P-18:0/20:4(5Z,8Z,11Z,14Z)), along with a previously published MS/MS spectrogram of the same species. Peak patterns match with the exception of a fragment of 331 in the previously published spectra resulting from presence of a minor additional isomer.

Figure 4. MS/MS spectrogram of ion m/z 885.5499 (PI(18:0/20:4(5Z,8Z,11Z,14Z)) from our samples (A), and the spectrogram of the standard purchased based on the initial tentative identification of the ion (B). Fragmentation patterns match at major peaks 885-parent ion, 581- parent ion with loss of sn-2 arachidonic acid side chain m/z 303, 303- liberated arachidonic acid, 283- liberated stearic acid, and 241-inositol head group.

Figure 5. Effects of lipid treatment on HCAEC cell adhesion molecule protein expression. pPE increased ICAM protein (158% of control, $p < 0.05$) and VCAM

protein (144% of control, $p<0.05$) in HCAEC. Data are presented as Mean $\pm$ SE. * = $p<0.05$ compared to control.

Figure 6. Effects of lipid treatment on HCAEC MCP-1 secretion. No differences were detected between treatments. Data are presented as Mean $\pm$ SE.

Supplementary Figure 1. Representative LC/MS chromatogram and spectrogram. The chromatogram x-axis shows minutes 6 to 14 of total run time, while the spectrogram x-axis shows the m/z of ions detected between 8 and 8.5 minutes.

Tables

Table 1. Subject Characteristics

	Control	**Obese**
	n=13	n=15
	(F=11, M=2)	(F=10, M=5)
Age (yr)	42	44
Height (in)	65 ± 3	67±3
Weight (kg)	71±14	142 ± 33 *
BMI (kg/m^2)	25.76± 4.39	49.87± 11.27 *
BMI min	20	41
BMI max	31	87
HbA1C (% of total)	5.15±0.10	6.41±0.35 *
Current smokers	1	2
Former smokers	11	4
Statins	1	2
Anti-Hypertensives	6	2
Hypoglycemics	4	0
Anti-anxiety and anti-depressants	3	4

* = $p<0.01$ compared to control. Data are presented as Mean ± SE

Table 2. Identifications of ions from MS/MS analysis

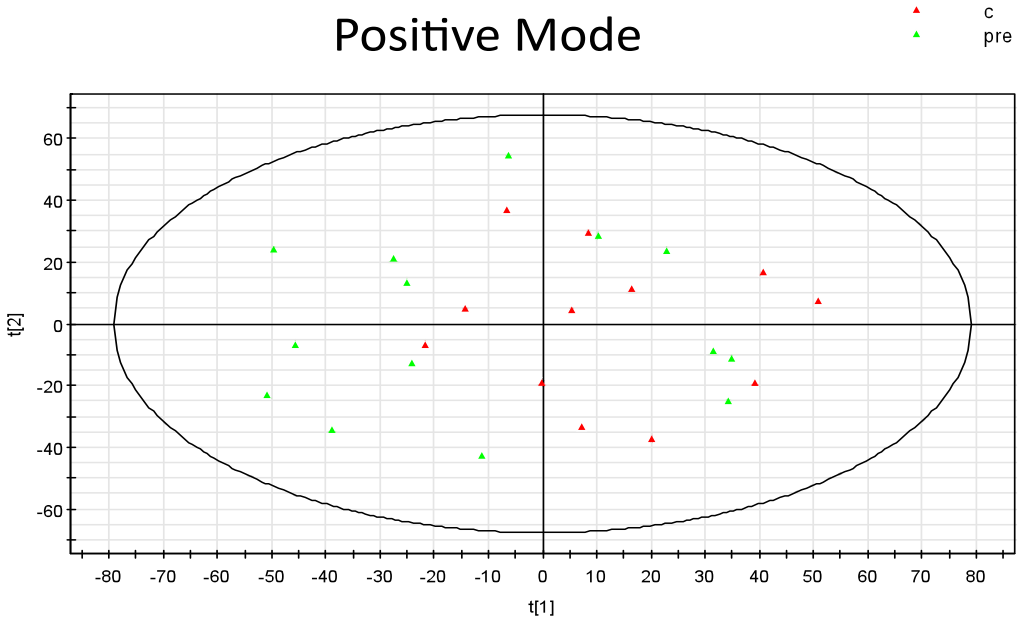
Ion of Interest from Original LC/MS analysis	Tentative ID based on MS/MS Analysis
750.5399	PE(P-18:0/20:4(5Z,8Z,11Z,14Z))
838.5599	PE(18:2(9Z,12Z)/20:2(11Z,14Z))
829.57	PC(16:0/18:2(9Z,12Z))
802.5599	PC(O-16:0/18:2(9Z,12Z))
816.57	PC(O-16:0/18:2(9Z,12Z))
830.59	PC(O-18:0/18:2(9Z,12Z))
844.5999	PC(O-18:0/18:2(9Z,12Z))
832.5999	PC(O-18:0/18:1(9Z))
868.5999	PC(O-18:0/20:4(5Z,8Z,11Z,14Z))
870.6199	PC(O-18:0/20:3(8Z,11Z,14Z))
885.5499	PI(18:0/20:4(5Z,8Z,11Z,14Z))

Table 3. Oxidized PAPC products from positive mode analysis

p-values were calculated using an unpaired t-test comparison of group means for each ion. Ave [c] (lean control) and [pre] (obese pre-surgical) values are average relative abundance of each ion for each group expressed in arbitrary units. C= control, O= obese pre-surgical. Scores plot locations represent the coordinates of the location of each ion on the positive mode scores plot shown in Figure 2.

Phospholipid	**Expected m/z**	**Best Match**	**P-value**	**Scores Plot Location**	
PAPC	782.7	11.10_782.6904	0.04	x = 0.005	y = 0.4
PGPC	610.2	9.53_610.1888	0.052	x = 0.01	y = 0.4
		9.40_610.1828	0.055	x = 0.1	y = 0.525
POVPC	594.3	No ions detected at this mass			
PEIPC	828.6	8.31_828.5536	0.707	x = 0	y = 0
		8.32_828.6107	0.661	x = 0	y = 0

Figure 1.


Positive Mode
c
pre
t[2]
t[1]
60
40
20
0
-20
-40
-60
-80
-70
-60
-50
-40
-30
-20
-10
0
10
20
30
40
50
60
70
80

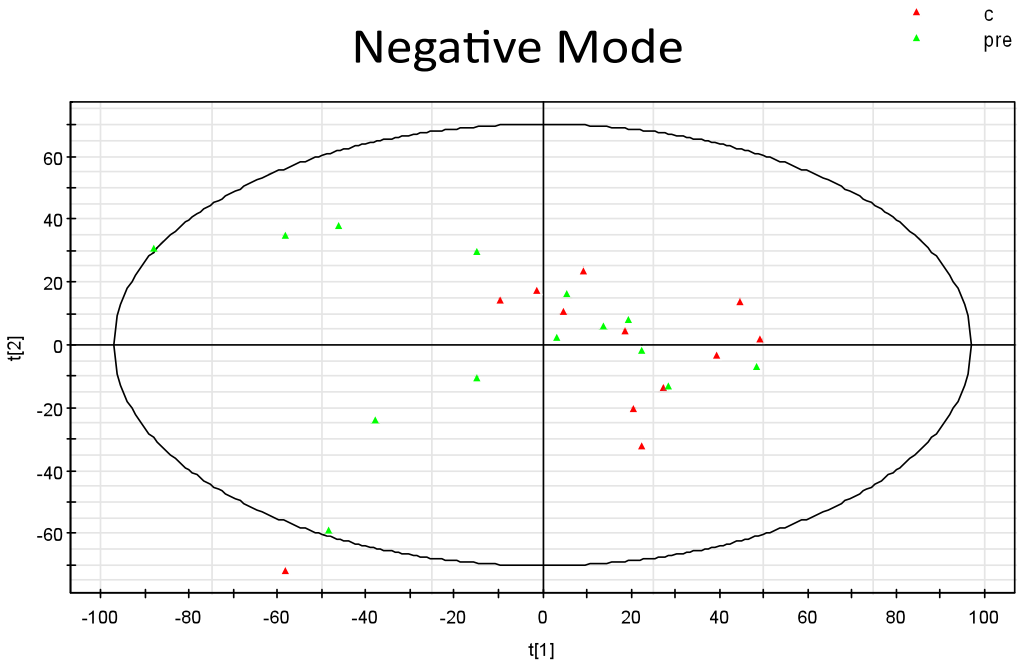

Negative Mode
c
pre
t[2]
t[1]
60
40
20
0
-20
-40
-60
-100
-80
-60
-40
-20
0
20
40
60
80
100

Figure 2.

A. B.

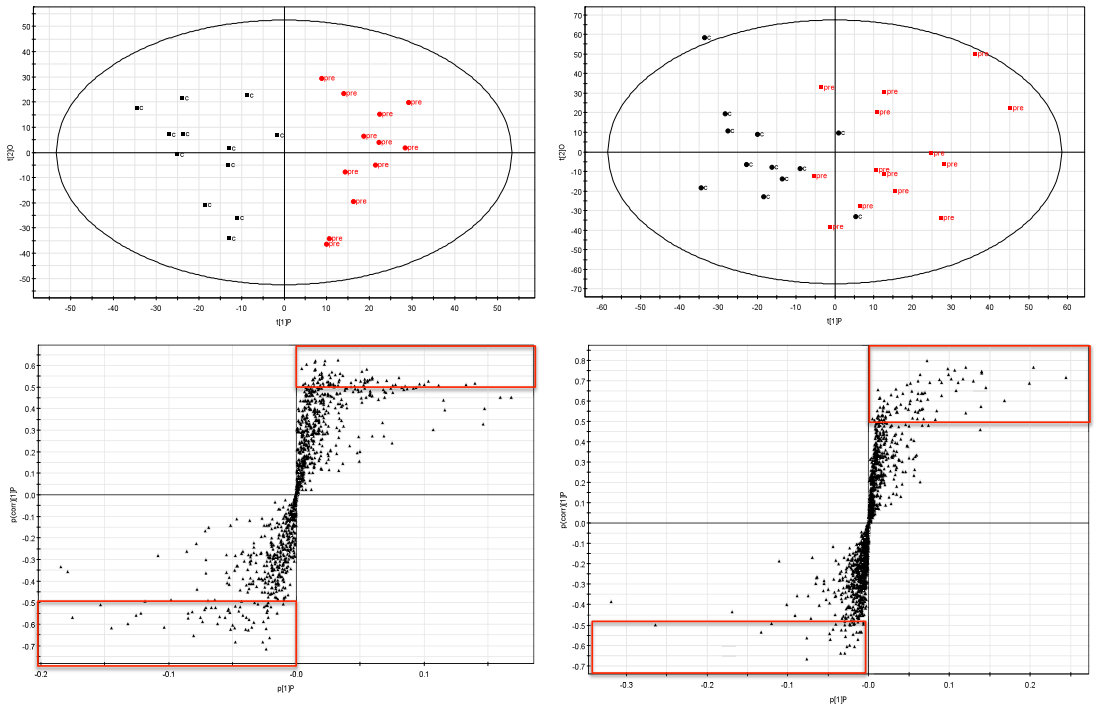


Figure 3.

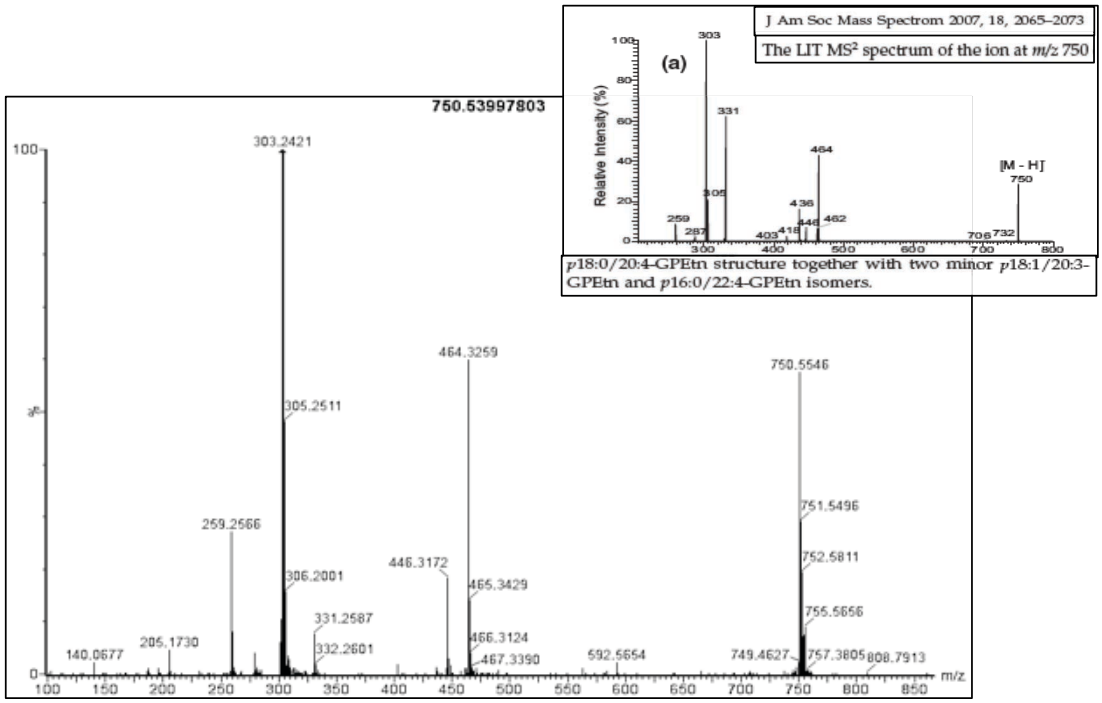
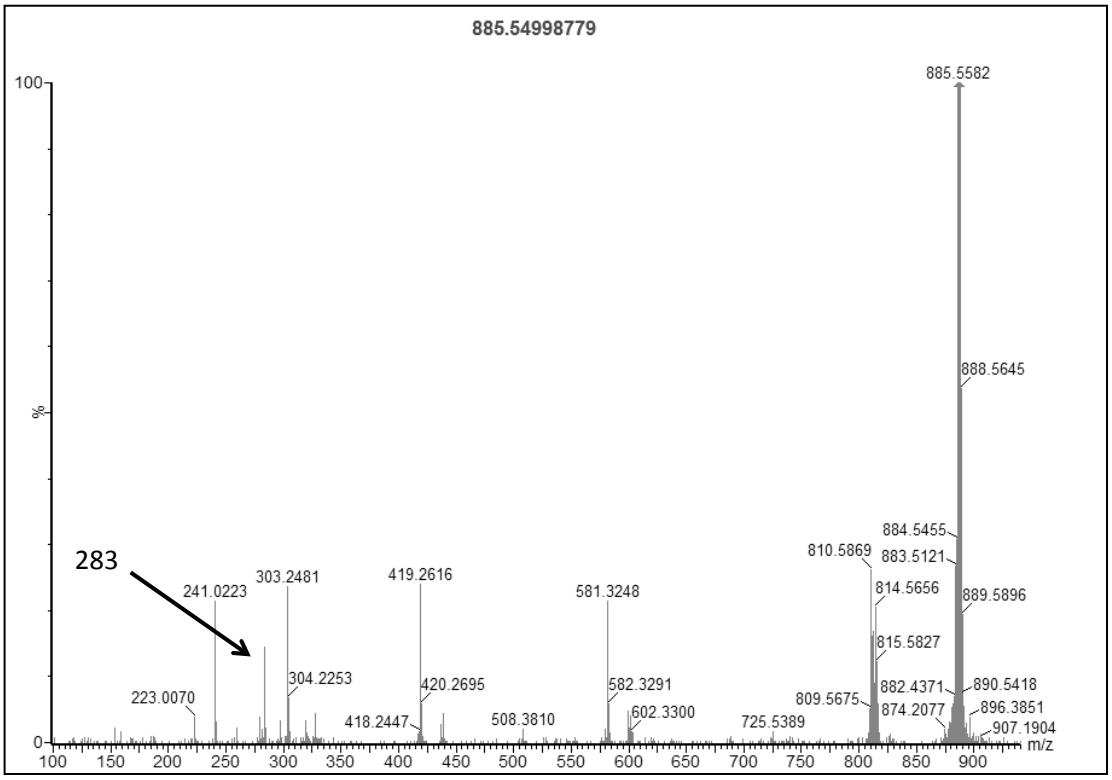

J Am Soc Mass Spectrom 2007, 18, 2065–2073
The LIT MS² spectrum of the ion at m/z 750
(a)
Relative Intensity (%)
[M - H]
750
p18:0/20:4-GPEtn structure together with two minor p18:1/20:3-GPEtn and p16:0/22:4-GPEtn isomers.
750.53997803
303.2421
464.3259
750.5546
305.2511
751.5496
259.2566
752.5811
446.3172
306.2001
465.3429
331.2587
755.5656
466.3124
140.0677
205.1730
332.2601
467.3390
592.5654
749.4627
757.3805
808.7913
m/z

Figure 4.

A.


885.54998779
100
%
0
283
885.5582
888.5645
884.5455
810.5869
883.5121
814.5656
889.5896
815.5827
241.0223
303.2481
419.2616
581.3248
304.2253
420.2695
582.3291
223.0070
809.5675
882.4371
890.5418
874.2077
896.3851
418.2447
508.3810
602.3300
725.5389
907.1904
m/z
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900

B.

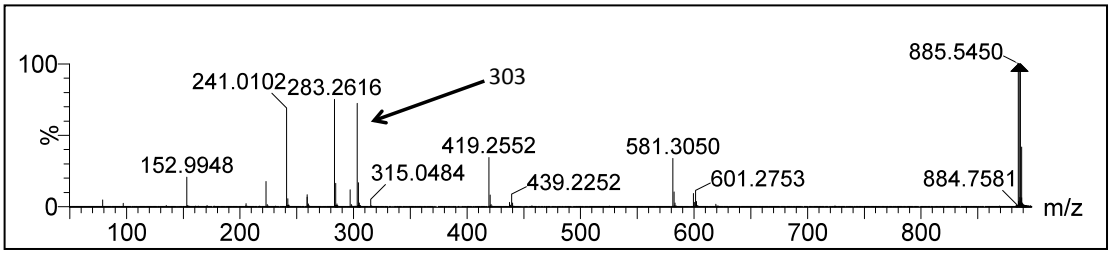

100
%
0
885.5450
241.0102
283.2616
303
419.2552
581.3050
152.9948
315.0484
439.2252
601.2753
884.7581
m/z
100 200 300 400 500 600 700 800

Figure 5.

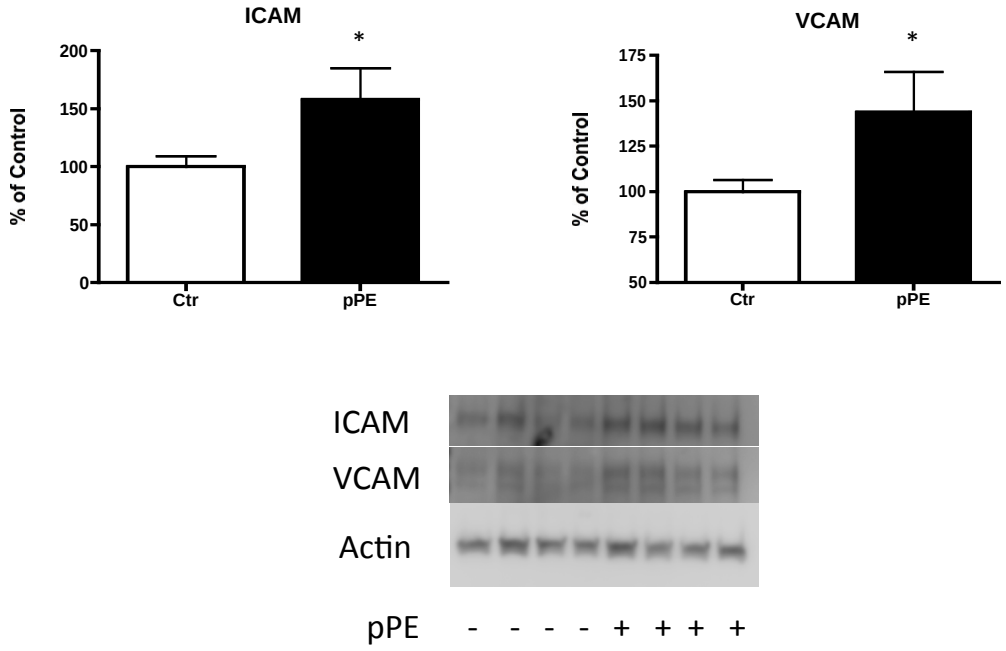
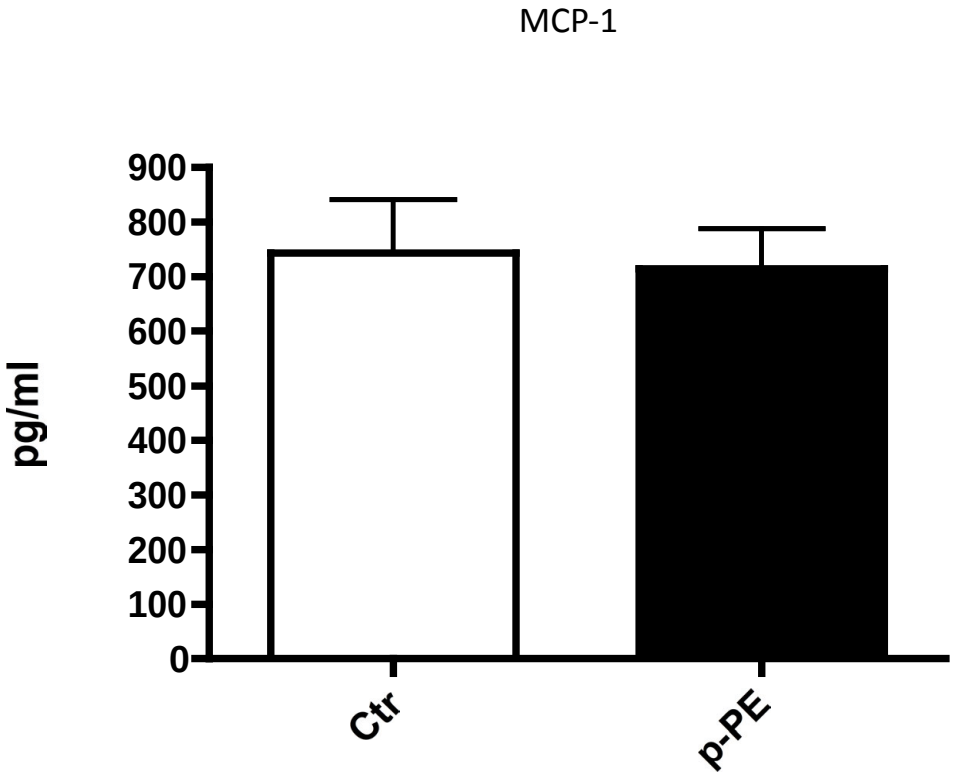
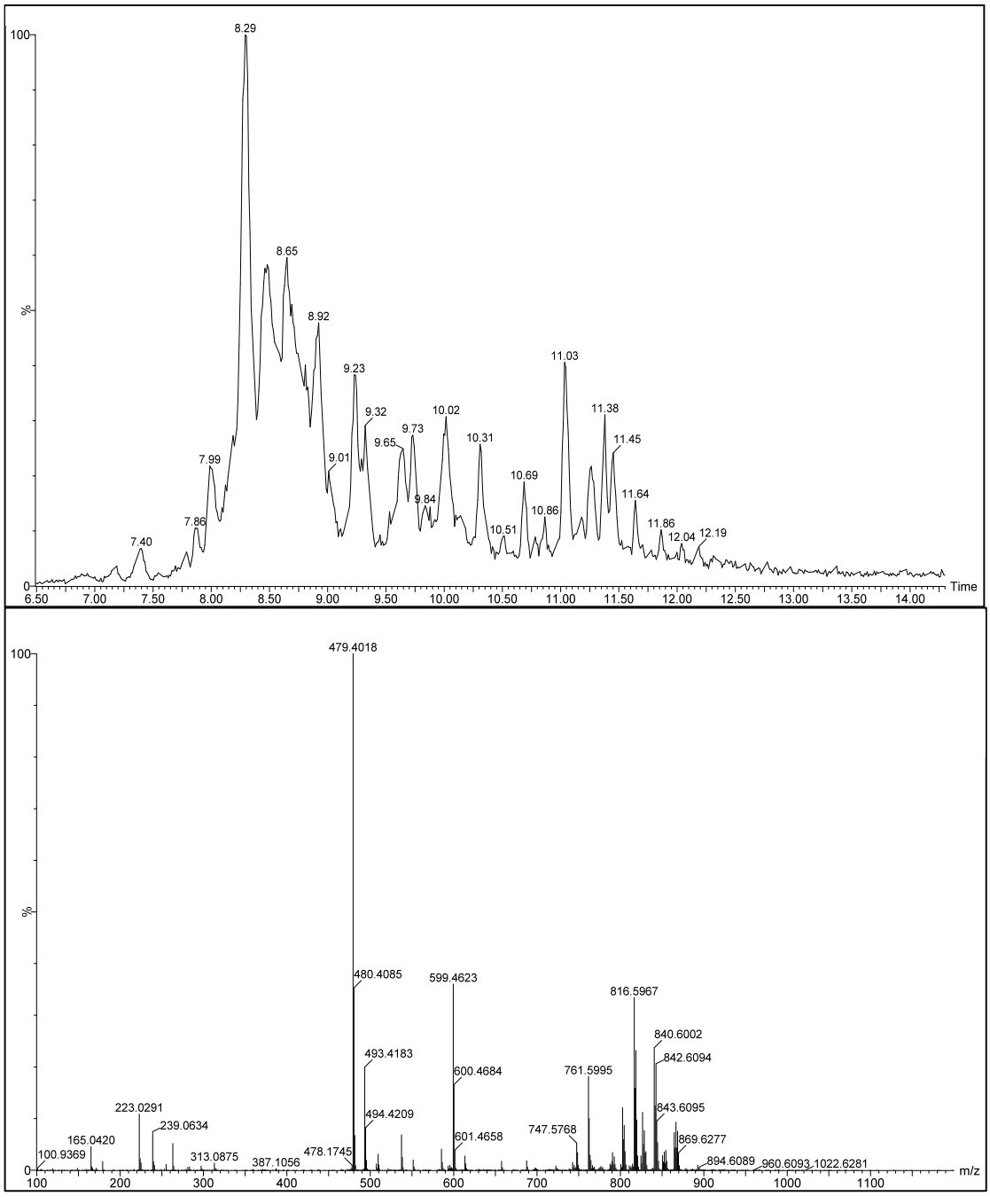

ICAM
*
200
150
100
50
0
% of Control
Ctr
pPE
VCAM
*
175
150
125
100
75
50
% of Control
Ctr
pPE
ICAM
VCAM
Actin
pPE - - - - + + + +

Figure 6.



Supplementary Data

Figure 1.



Supplementary Table 1. Ions of interest identified in LC/MS analysis that were selected for MS/MS. p-value was calculated using an unpaired t-test comparison of group means for each ion. Ave [c] (lean control) and [pre] (obese pre-surgical) values are average relative abundance of each ion for each group expressed in arbitrary units. C= control, O= obese pre-surgical

Positive Mode					**Negative Mode**				
Ret. Time and Ion	**P-value**	**Ave [c]**	**Ave [pre]**	**Higher in**	Ret. Time and Ion	**P-value**	**Ave [c]**	**Ave [pre]**	**Higher in**
7.99_612.1680	0.046	0.004	0.054	O	9.26_829.5643	0.022	0.0139	0.1783	O
9.52_237.0804	0.033	0.032	0.849	O	9.99_666.6038	0.000	6.9303	11.9490	O
11.51_864.8034	0.012	7.398	11.860	O	9.21_750.5424	0.016	8.2493	13.7307	O
3.35_205.0854	0.025	15.968	12.118	C	11.44_917.7758	0.042	7.4802	13.9198	O
7.19_366.3747	0.018	19.978	12.180	C	9.22_832.6023	0.005	8.8548	14.4645	O
4.52_522.3565	0.041	17.113	12.594	C	9.27_817.6412	0.013	9.6848	15.3987	O
5.20_304.2618	0.044	15.000	12.668	C	9.03_856.6022	0.009	10.4531	16.2154	O
3.35_301.1414	0.044	16.211	13.217	C	11.44_903.7659	0.026	9.9010	16.7782	O
10.09_719.5164	0.013	18.992	13.423	C	7.78_733.5486	0.004	11.9465	17.6002	O
12.12_1128.3085	0.039	6.868	13.549	O	10.31_711.6237	0.030	12.3959	18.1693	O
4.60_930.5785	0.027	21.794	15.768	C	11.25_901.7457	0.038	12.6943	20.4485	O
6.42_338.3426	0.026	25.424	17.522	C	9.34_527.4475	0.036	15.3125	22.4955	O
3.35_149.0242	0.023	25.520	19.548	C	10.30_708.6421	0.021	17.4746	26.3310	O
7.23_344.2969	0.041	28.497	21.618	C	8.11_838.5567	0.039	17.6692	27.8192	O
8.70_734.5697	0.015	27.614	23.764	C	10.30_694.6343	0.013	24.1369	36.2948	O
9.30_811.6669	0.016	43.954	33.723	C	9.02_870.6202	0.028	30.8649	44.7140	O
3.80_702.4395	0.026	63.843	47.216	C	8.91_830.5908	0.003	32.4771	45.3975	O
9.94_705.4997	0.028	65.940	47.692	C	8.01_885.5468	0.011	45.6587	62.1103	O
9.60_813.6826	0.012	81.572	63.719	C	8.89_868.6058	0.010	41.5375	63.8662	O
					8.47_802.5587	0.019	49.2173	64.2907	O
					6.90_493.3676	0.036	121.6714	97.4658	C
					8.91_844.6054	0.005	94.6827	130.2282	O
					8.46_816.5744	0.031	161.8050	208.0984	O
					8.70_599.4461	0.029	729.2035	615.2231	C

References

1. Dandona, P., et al., Metabolic Syndrome. Circulation, 2005. 111(11): p. 1448-1454.

2. Negre-Salvayre, A., et al., Pathological aspects of lipid peroxidation. Free Radical Research. 44(10): p. 1125-1171.

3. Bochkov, V.N., et al., Generation and biological activities of oxidized phospholipids. Antioxid Redox Signal. 12(8): p. 1009-59.

4. Niki, E., Lipid peroxidation: physiological levels and dual biological effects. Free Radic Biol Med, 2009. 47(5): p. 469-84.

5. Holvoet, P., et al., Association Between Circulating Oxidized Low-Density Lipoprotein and Incidence of the Metabolic Syndrome. JAMA: The Journal of the American Medical Association, 2008. 299(19): p. 2287-2293.

6. Njajou, O.T., et al., Association between oxidized LDL, obesity and type 2 diabetes in a population-based cohort, the Health, Aging and Body Composition Study. Diabetes/Metabolism Research and Reviews, 2009. 25(8): p. 733-739.

7. Scheffer, P.G., et al., LDL oxidative modifications in well- or moderately controlled type 2 diabetes. Diabetes Metab Res Rev, 2004. 20(4): p. 298-304.

8. Unger, R.H., et al., Lipid homeostasis, lipotoxicity and the metabolic syndrome. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 1801(3): p. 209-214.

9. Kume, N., M.I. Cybulsky, and M.A. Gimbrone, Jr., Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. J Clin Invest, 1992. 90(3): p. 1138-44.

10. Leitinger, N., et al., Role of group II secretory phospholipase A2 in atherosclerosis: 2. Potential involvement of biologically active oxidized phospholipids. Arterioscler Thromb Vasc Biol, 1999. 19(5): p. 1291-8.

11. Kuniyasu, A., et al., Oxidized LDL and lysophosphatidylcholine stimulate plasminogen activator inhibitor-1 expression through reactive oxygen species generation and ERK1/2 activation in 3T3-L1 adipocytes. Biochim Biophys Acta. 1811(3): p. 153-62.

12. Berliner, J.A. and N.M. Gharavi, Endothelial cell regulation by phospholipid oxidation products. Free Radic Biol Med, 2008. 45(2): p. 119-23.

13. Berliner, J.A., N. Leitinger, and S. Tsimikas, The role of oxidized phospholipids in atherosclerosis. J Lipid Res, 2009. 50 Suppl: p. S207-12.

14. Berliner, J.A. and A.D. Watson, A role for oxidized phospholipids in atherosclerosis. N Engl J Med, 2005. 353(1): p. 9-11.

15. Bochkov, V.N., Inflammatory profile of oxidized phospholipids. Thromb Haemost, 2007. 97(3): p. 348-54.

16. Fu, P. and K.G. Birukov, Oxidized phospholipids in control of inflammation and endothelial barrier. Transl Res, 2009. 153(4): p. 166-76.

17. Leitinger, N., Oxidized phospholipids as triggers of inflammation in atherosclerosis. Mol Nutr Food Res, 2005. 49(11): p. 1063-71.

18. Watson, A.D., et al., Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. J Biol Chem, 1997. 272(21): p. 13597-607.

19. Subbanagounder, G., et al., Determinants of bioactivity of oxidized phospholipids. Specific oxidized fatty acyl groups at the sn-2 position. Arterioscler Thromb Vasc Biol, 2000. 20(10): p. 2248-54.

20. Xu, F., et al., Endothelial cell apoptosis is responsible for the formation of coronary thrombotic atherosclerotic plaques. Tohoku J Exp Med, 2009. 218(1): p. 25-33.

21. Fruhwirth, G.O. and A. Hermetter, Mediation of apoptosis by oxidized phospholipids. Subcell Biochem, 2008. 49: p. 351-67.

22. Sordillo, L.M., et al., Enhanced 15-HPETE production during oxidant stress induces apoptosis of endothelial cells. Prostaglandins Other Lipid Mediat, 2005. 76(1-4): p. 19-34.

23. Lu, J., et al., Mediation of electronegative low-density lipoprotein signaling by LOX-1: a possible mechanism of endothelial apoptosis. Circ Res, 2009. 104(5): p. 619-27.

24. Lee, S., et al., A role for NADPH oxidase 4 in the activation of vascular endothelial cells by oxidized phospholipids. Free Radic Biol Med, 2009. 47(2): p. 145-51.

25. Rouhanizadeh, M., et al., Oxidized-1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine induces vascular endothelial superoxide production: implication of NADPH oxidase. Free Radic Biol Med, 2005. 39(11): p. 1512-22.

26. Catala, A., Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. Chem Phys Lipids, 2009. 157(1): p. 1-11.

27. Matyash, V., et al., Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lipid Res, 2008. 49(5): p. 1137-46.

28. Bylesjˆ, M., et al., OPLS discriminant analysis: combining the strengths of PLS-DA and SIMCA classification. Journal of Chemometrics, 2006. 20(8-10): p. 341-351.

29. Fahy E, S.M., Cotter D and Subramaniam S. , LIPID MAPS online tools for lipid research. LIPID MAPS Online Tools, 2007. 35: p. W606-12.

30. Castro-Perez, J.M., et al., Comprehensive LC-MS E lipidomic analysis using a shotgun approach and its application to biomarker detection and identification in osteoarthritis patients. J Proteome Res. 9(5): p. 2377-89.

31. Deeley, J.M., et al., Identification of abundant alkyl ether glycerophospholipids in the human lens by tandem mass spectrometry techniques. Anal Chem, 2009. 81(5): p. 1920-30.

32. Kotronen, A., et al., Comparison of lipid and fatty acid composition of the liver, subcutaneous and intra-abdominal adipose tissue, and serum. Obesity (Silver Spring). 18(5): p. 937-44.

33. Ivanova, P.T., S.B. Milne, and H.A. Brown, Identification of atypical ether-linked glycerophospholipid species in macrophages by mass spectrometry. J Lipid Res. 51(6): p. 1581-90.

34. Colas, R., et al., LDL from obese patients with the metabolic syndrome show increased lipid peroxidation and activate platelets. Diabetologia. 54(11): p. 2931-40.

35. Han, X., D.M. Holtzman, and D.W. McKeel, Jr., Plasmalogen deficiency in early Alzheimer's disease subjects and in animal models: molecular characterization using electrospray ionization mass spectrometry. J Neurochem, 2001. 77(4): p. 1168-80.

36. Lessig, J. and B. Fuchs, HOCl-mediated glycerophosphocholine and glycerophosphoethanolamine generation from plasmalogens in phospholipid mixtures. Lipids. 45(1): p. 37-51.

37. Engelmann, B., C. Brautigam, and J. Thiery, Plasmalogen phospholipids as potential protectors against lipid peroxidation of low density lipoproteins. Biochem Biophys Res Commun, 1994. 204(3): p. 1235-42.

38. Vance, J.E., Lipoproteins secreted by cultured rat hepatocytes contain the antioxidant 1-alk-1-enyl-2-acylglycerophosphoethanolamine. Biochim Biophys Acta, 1990. 1045(2): p. 128-34.

39. Khaselev, N. and R.C. Murphy, Susceptibility of plasmenyl glycerophosphoethanolamine lipids containing arachidonate to oxidative degradation. Free Radic Biol Med, 1999. 26(3-4): p. 275-84.

40. Chilton, F.H. and R.C. Murphy, Remodeling of arachidonate-containing phosphoglycerides within the human neutrophil. Journal of Biological Chemistry, 1986. 261(17): p. 7771-7777.

41. Tamby, J.P., P. Reinaud, and G. Charpigny, Preferential esterification of arachidonic acid into ethanolamine phospholipids in epithelial cells from ovine endometrium. J Reprod Fertil, 1996. 107(1): p. 23-30.

42. Nanda, B.L., et al., PLA2 mediated arachidonate free radicals: PLA2 inhibition and neutralization of free radicals by anti-oxidants--a new role as anti-inflammatory molecule. Curr Top Med Chem, 2007. 7(8): p. 765-77.

43. Ricciotti, E. and G.A. FitzGerald, Prostaglandins and inflammation. Arterioscler Thromb Vasc Biol. 31(5): p. 986-1000.

44. Lee, H., et al., Lysophospholipids increase ICAM-1 expression in HUVEC through a Gi- and NF-kappaB-dependent mechanism. Am J Physiol Cell Physiol, 2004. 287(6): p. C1657-66.

45. Howard, K.M., et al., Lipopolysaccharide and platelet-activating factor stimulate expression of platelet-activating factor acetylhydrolase via distinct signaling pathways. Inflamm Res. 60(8): p. 735-44.

46. Ninio, E., Phospholipid mediators in the vessel wall: involvement in atherosclerosis. Curr Opin Clin Nutr Metab Care, 2005. 8(2): p. 123-31.

47. Vickers, K.C., et al., Relationship of lipoprotein-associated phospholipase A2 and oxidized low-density lipoprotein in carotid atherosclerosis. J Lipid Res, 2009.

48. Wang, C., et al., Plasma phospholipid metabolic profiling and biomarkers of type 2 diabetes mellitus based on high-performance liquid chromatography/electrospray mass spectrometry and multivariate statistical analysis. Anal Chem, 2005. 77(13): p. 4108-16.

49. Kim, J.Y., et al., Metabolic profiling of plasma in overweight/obese and lean men using ultra performance liquid chromatography and Q-TOF mass spectrometry (UPLC-Q-TOF MS). J Proteome Res. 9(9): p. 4368-75.

50. Mutch, D.M., et al., Metabolite profiling identifies candidate markers reflecting the clinical adaptations associated with Roux-en-Y gastric bypass surgery. PLoS One, 2009. 4(11): p. e7905.

CHAPTER III – MANUSCRIPT II

PROTANDIM INDUCED NRF2 ACTIVATION PROTECTS HUMAN CORONARY ARTERY ENDOTHELIAL CELLS AGAINST AN OXIDATIVE CHALLENGE

Title: Protandim induced Nrf2 activation protects human coronary artery endothelial cells against an oxidative challenge

Authors: [1]Elise L. Donovan, [2]Joe M. McCord, [1]Benjamin F. Miller, [1]Karyn L. Hamilton

Affiliations: [1]Department of Health and Exercise Science, Colorado State University, Fort Collins, Colorado; [2]Pulmonary Sciences and Critical Care Medicine, University of Colorado Denver, Aurora, CO

Corresponding Author:

Karyn L. Hamilton

220 Moby B Complex 1582

Colorado State University

Fort Collins, CO 80523

970-491-3961 (phone)

970-491-0445

Karyn.hamilton@colostate.edu

Protandim treatment is a potential therapeutic intervention against endothelial cell oxidative stress and associated vascular disease initiation and progression. We hypothesized that Protandim treatment would induce NF-E2-related factor 2 (Nrf2) nuclear localization and phase II antioxidant enzyme protein expression in human coronary artery endothelial cells (HCAEC), providing protection against an oxidant challenge in a Nrf2 dependent manner. Protandim treatment induced Nrf2 nuclear localization, and increased HO-1 (778 % of control, $p < 0.01$), SOD1 (125.9 % of control, $p = 0.015$), NQO1 (126 % of control, $p < 0.01$) and Grx (119.5 % of control $p = 0.07$) in HCAEC. Treatment of HCAEC with H_2O_2 induced apoptosis in 34% of cells while pre-treatment with Protandim resulted in protection against the H_2O_2 challenge with only 6% apoptotic cells ($p < 0.01$). Nrf2 silencing significantly decreased the Protandim induced increase in HO-1 protein expression ($p<0.01$). Nrf2 silencing also significantly decreased the protection afforded by Protandim against H_2O_2 induced apoptosis ($p<0.01$ compared to no RNA, and $p<0.05$ compared to control RNA). These results show that the phytochemicals in Protandim induce Nrf2 nuclear localization and antioxidant enzyme expression, and protect HCAEC from an oxidative challenge in a Nrf2 dependent manner.

Keywords: Protandim, NF-E2-related factor 2 (Nrf2), oxidative stress, endothelial cells

Introduction

Oxidative stress has been implicated in many chronic diseases including Alzheimer's, diabetes, and coronary artery disease (CAD) [1-4]. In CAD, increased production of ROS and oxidative damage in the vascular endothelium contribute to disease initiation and progression. Specifically, increased vascular superoxide causes oxidation of lipids, decreased nitric oxide availability, increased expression of adhesion molecules and inflammatory mediators, and recruitment of monocytes to the endothelium[5-8]. Endothelium bound superoxide dismutase is also decreased in CAD patients compared to healthy controls, impairing the cellular response to excessive ROS production [9]. Atherosclerotic coronary arteries isolated from humans display increased superoxide production compared to non-atherosclerotic human coronary arteries, and in a mouse model of atherosclerosis, attenuation of superoxide production by decreased expression of NADPH oxidase results in a decrease in atherosclerotic lesion size [10, 11].

Initial studies examining the effects of decreasing oxidative stress in several different diseases, including cardiovascular disease, have used exogenous antioxidant supplements such as vitamins C and E. However, data showed no protective effects of exogenous antioxidants, and in some cases supplementation increased mortality [12-14].

A novel approach to decreasing disease associated oxidative stress involves bolstering endogenous antioxidant defense systems rather than relying on exogenous antioxidant supplementation. Protandim is a commercially available

dietary supplement consisting of phytochemicals derived from five widely studied medicinal plants including silymarin from milk thistle, curcumin from turmeric, bacopa extract, ashwaganda, and green tea extract. It is thought that the five phytochemical components of Protandim have a synergistic effect to induce phase II antioxidant enzymes and protect cells from oxidative stress through activation of the transcription factor NF-E2-related factor 2 (Nrf2). Nrf2 binds to the antioxidant response element (ARE) in the promoter region of several hundred genes including many phase II antioxidant enzymes [15, 16].

Nrf2 is constitutively expressed but is marked for ubiquitination by association with Kelch-like ECH-associated protein 1 (Keap1). Activation of Nrf2 occurs when it is released from Keap1, translocates to the nucleus, heterodimerizes with small Maf or Jun proteins, binds the ARE, and initiates transcription of the associated genes. Multiple mechanisms for Nrf2 activation have been proposed including oxidation of Keap1 and phosphorylation of Nrf2 itself (reviewed in [15]). Electrophilic or oxidative modification to Keap1 cysteine thiol groups leads to dissociation of Nrf2 from Keap1 [17]. Direct phosphorylation of Nrf2 by a number of kinases, including several from the MAPK family can also cause dissociation of Nrf2 from Keap1[18-20]. Phytochemical compounds including those in Protandim likely activate Nrf2 and induce phase II antioxidant enzyme expression through activation of various kinases with subsequent Nrf2 phosphorylation [21].

Although acute activation of Nrf2 occurs *in vivo* in response to oxidized phospholipid signaling, increased ROS production, hyperglycemia, and shear stress

[22-24], in chronic disease states the antioxidant response is often insufficient to maintain redox balance and prevent disease progression [24-26]. For example, Landmesser *et al.* report increased SOD activity in young hypercholesterolemic subjects compared to age matched controls[9]. In contrast, decreased SOD activity was observed in coronary arteries from CAD patients compared to age matched controls. It was recently reported that Protandim protected a human saphenous vein ex vivo culture from oxidative stress induced hyperplasia and vessel wall thickening [27]. Thus, phytochemical induced Nrf2 activation is a potential therapeutic intervention against endothelial cell oxidative stress and associated vascular disease initiation and progression. However the effects of Protandim on Nrf2 in human coronary artery endothelial cells are not known.

The purpose of this study was to determine 1) if treatment with Protandim induces Nrf2 nuclear translocation and phase II antioxidant enzyme protein expression in human coronary artery endothelial cells (HCAEC), 2) if treatment with Protandim protects HCAEC from apoptosis induced by an oxidant challenge, and 3) if Nrf2 mediates Protandim induced protection from an oxidative challenge. We hypothesized that Protandim treatment would induce Nrf2 nuclear localization and phase II antioxidant enzyme protein expression, and Protandim treatment prior to an oxidant challenge would afford cells protection in a Nrf2 dependent manner.

Materials and methods

Materials

HCAEC and cell culture reagents were purchased from Lonza (Walkersville, MD). Heme oxygenase-1 (HO-1) antibody was from Affinity Bioreagents (Golden, CO), nitroquinone oxidoreductase (NQO1) and glutathione reductase (Grx) antibodies and appropriate HRP conjugated secondary antibodies were purchased from AbCam, (Cambridge, MA). Nrf2, Cu-Zn superoxide dismutase (SOD1), and actin antibodies, HRP and FITC conjugated appropriate secondary antibodies, and Nrf2 siRNA and control RNA were purchased from Santa Cruz Biotech (Santa Cruz, CA). PrimeFect transfection reagent and PrimeFect diluent were from Lonza (Walkersville, MD). The Pierce BCA assay kit for determining protein concentrations, and protease and phosphatase inhibitors were from Thermo Scientific (Rockford, IL). TUNEL assay kits were from Roche (Indianapolis, IN).

Culture of HCAEC

Primary HCAEC were grown in endothelial cell growth medium (EBM-2) containing 5% FBS and manufacturer recommended supplemental growth factors, antibiotics and antimycotics. All assays were performed on cells at 80-100% confluence, between passages 3 and 9.

Protandim and H_2O_2 preparation and treatment

Protandim is a phytochemical composition containing *W. somnifera*, *B. monniera* (45% bacosides), *S. marianum, Ca. sinesis* (98% polyphenols and 45% (-)-

epigallocatechin-3-gallate), and curcumin (95%) from turmeric *(Cu. longa)* (LifeVantage Corp., Littleton, CO). Protandim extract was prepared by mixing 500mg Protandim in 5ml 95% ethanol. The mixture was rocked overnight at room temperature, and then centrifuged for 15 min at 3,000 x g. The resulting supernatant contains ethanol extracted Protandim at a concentration of 100mg/ml. The Protandim extract was diluted to 20µg/ml with complete cell culture medium for all treatments following initial concentration response experiments that showed 20µg/ml was the lowest concentration that significantly increased phase II antioxidant enzyme protein expression. Cells not treated with Protandim were treated with 95% ethanol as a vehicle control, with a maximum ethanol concentration in the growth medium of 0.02% (2µl in 10ml). H_2O_2 (30% W/W) was diluted in complete cell growth medium to a final concentration of 1.25 µM for all treatments. Protandim treatments ranged from 1 hr to 12 hrs as indicated, and H_2O_2 treatments were 4 hrs unless otherwise indicated. In Protandim removal experiments, Protandim was removed, cells were washed with PBS, and then incubated in normal cell culture medium for the indicated duration.

Western blot analyses

HCAEC were seeded in 65mm polystyrene cell culture dishes and grown to at least 80% confluence prior to Protandim treatment. Following treatment, cells were scraped in RIPA buffer (50mM Tris, 0.15M NaCl, 1% Na deoxycholic acid, 1mM EGTA, 1% NP40) containing protease and phosphatase inhibitors, and sonicated 3 x 10 seconds. Protein concentrations were determined using a BCA assay, and

samples were diluted to equal concentrations with Laemmli sample buffer. Samples were separated on 10% polyacrylamide gels at 125 volts, and transferred to nitrocellulose membranes (BioRad, Hercules, CA) for 1 hr at 50 volts. Membranes were blocked for 1 hr in Superblock (Thermo Scientific, Rockford, IL), then incubated with primary antibodies against HO-1 (1:500), SOD1(1:500), NQO1(1:1000), and Grx(1:5000) followed by the appropriate HRP-conjugated secondary antibodies. Membranes were developed by chemiluminescence using SuperSignal West Dura substrate (Thermo Scientific, Rockford, IL), with digital images obtained using the Biospectrum UVP system. All signals were normalized to β-actin obtained from the same blot, and expressed as the percent of the vehicle control (no Protandim) condition.

Immunocytochemistry for Nrf2 nuclear localization

HCAEC were grown to confluence on coverslips in 35mm polystyrene cell culture plates coated with 2μg/cm^2 fibronectin prior to Protandim treatment. Cells were initially treated with Protandim for 1 hr, 2 hrs, 4 hrs, 8 hrs, and 12 hrs to determine the optimal duration of treatment for visualizing Nrf2 nuclear localization. Following time course experiments, all Protandim treatments were for 1hr. Cells were washed with PBS, fixed for 30 min in 4% paraformaldehyde, washed with PBS, and then permeabilized in cold acetone for 30 min. Cells were blocked for 1 hr in 5% bovine serum albumin with 0.5% goat serum, and then incubated with Nrf2 primary antibody (1:100) for 1 hr at room temperature. Cells were washed with PBS then incubated for 45 min in FITC conjugated secondary antibody at room

temperature in the dark. The coverslips were mounted on slides using DAPI containing mounting medium for identification of cell nuclei, and visualized by fluorescence microscopy (Nikon TE2000).

TUNEL assay to assess apoptosis

A TdT-mediated dUTP nick end labeling (TUNEL) assay was used to assess HCAEC apoptosis in response to an oxidative challenge. Cells were grown to confluence on fibronectin coated coverslips prior to Protandim and H_2O_2 treatment. Cells were washed with PBS, fixed for 30 min in 4% paraformaldehyde, washed again with PBS, then permeabilized for two min in 0.1% Triton X-100 with 0.1% sodium citrate. Cells were washed again with PBS and incubated in TUNEL reagent for 1.5 hrs. The coverslips were then mounted on slides using DAPI containing mounting medium to identify cell nuclei. Signals were visualized using fluorescence microscopy (Nikon TE2000).

Nrf2 silencing

Prior to transfection, 250µL PrimeFect Diluent was mixed with 5µL PrimeFect transfection reagent and incubated at room temperature for 15 min. Nrf2 siRNA or control RNA was added to the transfection solution for a final concentration of 50nM, and incubated on the bench for 15 min. The transfection solution was applied to cells grown in antibiotic free media to 70-80% confluence, along with 1.25mL antibiotic free growth medium. The volume of transfection reagent used caused minimal distress to the cells as assessed by minimal changes to cell

morphology. After 24 hrs, the transfection solution was removed and the cells were rinsed with PBS, treated with Protandim, and assayed as indicated.

Statistical analysis

Unpaired t-tests were used to compare control vs. Protandim treatments. A two by three treament (Protandim and no Protandim) by condition (normal medium, control RNA, and NRf2 siRNA) ANOVA with *a priori* linear contrasts of means was used to analyze Nrf2 silencing experiments. Percent protection was analyzed using one-way ANOVA. Statistical significance was set at $p<0.05$

Results

Antioxidant enzyme protein expression is increased in response to Protandim treatment.

HCAEC were treated with Protandim concentrations of 0 to 50μg/ml in 5μg/ml increments to determine a profile of Nrf2 activation as measured by an increase in HO-1 protein content. HO-1 protein increased from non-detectable in control conditions to 8-10 fold at 20-30μg/ml, while concentrations higher than 30μg/ml induced morphological changes (Figure 1A). We used the 20μg/ml concentration in all subsequent treatments. HO-1 protein was visible after 1 hr, and became significant and sustained from 4 hrs through the longest treatment period of 12 hrs (Data not shown). To confirm treatment concentration and duration on multiple antioxidant enzymes we determined that treatment with 20μg/ml Protandim for 12 hrs increased HO-1 (778 % of control $\pm$ 82.25 $p < 0.01$), SOD1 (125.9 % of control $\pm$ 6.05 $p < 0.01$), NQO1 (126 % of control $\pm$ 6.5 $p < 0.01$), and Grx trended towards significance (119.5 % of control $\pm$ 7.00 $p < 0.05$) (Figure 1B). All subsequent treatments used Protandim at a concentration of 20μg/ml for 12 hrs unless otherwise noted.

Protandim stimulates Nrf2 nuclear localization

HCAEC were treated with Protandim for 12 hrs and subsequently visualized with immunocytochemistry to determine Nrf2 nuclear localization. Nrf2 content and nuclear localization increased as soon as 1 hr of Protandim treatment. These

increases remained at 2 hrs, 4 hrs, 8 hrs, and 12 hrs (12 hrs was the longest treatment duration examined) (Figure 2). After removal of Protandim Nrf2 content and nuclear localization were decreased within 15 min (Figure 2).

Protandim protects HCAEC from oxidative challenge induced apoptosis

HCAECs were treated for 12 hrs with Protandim or vehicle control followed by a 4 hr exposure to 1.25 μM hydrogen peroxide and induction of apoptosis as measured by TUNEL assay. Importantly, Protandim was removed prior to hydrogen peroxide exposure. In vehicle controls apoptosis was induced in 34% of cells (Figure 3A and 3B) while pre-treatment with Protandim resulted in only 6% apoptotic cells ($p < 0.01$ compared to vehicle control) (Figure 3A and 3B).

Nrf2 silencing diminishes Protandim induced increases in HO-1 and protection from an oxidative challenge.

We used siRNA to silence Nrf2 prior to Protandim treatment to determine if Protandim induced increases in antioxidant enzyme expression and protection occur through Nrf2 activation. Nrf2 silencing prior to Protandim treatment significantly decreased ($p<0.01$) Protandim induced increases in HO-1 expression compared to both the no RNA conditions, and control RNA treatment. (Figure 4A and 4B). There were no differences between no RNA and control RNA conditions with or without Protandim.

We then silenced Nrf2 prior to Protandim treatment and an oxidative challenge. In cells receiving no RNA and cells that received control RNA, 30 - 40% of

cells underwent apoptosis (Figure 5 and 6A). Nrf2 siRNA treatment prior to H_2O_2 resulted in apoptosis in 80% of cells ($p<0.0001$) compared to no RNA conditions and control RNA (Figure 5 and 6A). In both no RNA and control RNA conditions, Protandim treatment prior to H_2O_2 resulted in significantly fewer apoptotic cells ($p<0.0001$ compared to normal conditions and control) (Figure 5 and 6A). The number of apoptotic cells in the no RNA condition compared to the control RNA condition was not significantly different with or without Protandim ($p=0.413$ no Protandim, $p=0.093$ with Protandim). Protandim prior to Nrf2 siRNA also significantly protected cells from apoptosis ($p=0.023$), however the amount of protection afforded by the Protandim in this condition was significantly less than in no RNA and control RNA conditions ($p<0.01$ and $p<0.05$) (Figure 5, 6A and 6B).

Discussion

The major findings of this study were that Protandim treatment of HCAEC induced Nrf2 nuclear localization and phase II antioxidant enzyme expression, and Protandim treatment prior to an oxidative challenge was protective against apoptosis in a Nrf2 dependent manner.

Nrf2 nuclear localization was observed following 1hour of Protandim treatment, and remained elevated for all treatment durations up to twelve hours. In cardiac myocytes we have observed increases in Nrf2 nuclear localization as quickly as 15 minutes after initiation of Protandim treatment (unpublished data). Experiments determining how long increases in Nrf2 localization remain following removal of Protandim have shown the Protandim effect is diminished as early as 15 minutes following removal. Experiments to determine how long Protandim induced increases in antioxidant enzymes persist are ongoing. These data suggest that Protandim has an immediate and robust effect on Nrf2 nuclear localization that remains for a limited time after the removal of Protandim. The transient effects of Protandim on Nrf2 may be important because chronic Nrf2 activation has been shown to be carcinogenic [28].

The components in Protandim work synergistically to activate Nrf2. HO-1 RNA was increased to 1,000 percent of control in response to 40μg/ml Protandim in MIN6 cells, and 20μg/ml Protandim in SK-N-MC cells [29]. However, when each phytochemical component of Protandim was tested individually at the low

concentration found in Protandim extract, the maximum increase in HO-1 RNA was less than 200 percent of control [29].

Subjects taking Protandim report no adverse side effects [29, 30]. It has been shown that with oral Protandim supplementation in humans circulating TBARs decrease in 5-12 days, an effect that persists with continued supplementation as measured at 30 and 120 days [30]. In addition, erythrocytes isolated from subjects who ingested Protandim for 120 days demonstrated increased SOD and catalase activity, [30]. However, in each of these studies how long these effects persisted after Protandim supplementation was stopped was not assessed. Thus, while our data are promising, studies *in vivo* will need to be performed to determine if these results are translatable.

Protandim treatment protected HCAEC from H_2O_2 induced apoptosis, an effect that was diminished but not abrogated with silencing of Nrf2 prior to Protandim treatment. There are multiple potential explanations for this observation. First, Nrf2 may have only been partially silenced. This could be due to limitations in the amount of transfection reagent the cells tolerate, and thus the amount of Nrf2 siRNA that could be delivered. Second, diminished but incomplete protection may also be due to the rapid turnover rate in Nrf2. Nrf2 is constitutively expressed and rapidly degraded [21] The amount of siRNA that can be delivered is limited by the ability of the cells to tolerate the transfection procedure, therefore the capacity of the delivered Nrf2 siRNA to silence Nrf2 may be exceeded by the continuous turnover and subsequent increases induced by Protandim. Finally, Nrf2

independent mechanisms for induction of antioxidant enzymes and protection against oxidative stress may exist; possible candidates that can increase antioxidant defenses independently of Nrf2 include p53 and sirtuins [31, 32].

Increased apoptosis as an outcome in endothelial cells has direct translation to vascular disease development and outcomes. Endothelial cell apoptosis contributes to plaque progression and rupture, and may be an independent risk factor for thrombosis [33]. Endothelial cell apoptosis can be induced by oxidized lipids as well as by ROS, and the role of endothelial cell apoptosis in atherosclerosis has been extensively reviewed [34-36].

While we observed significant increases in expression of multiple antioxidant enzymes (approximately 125 % of control for SOD1, Grx, and NQO1), the Protandim induced increase in HO-1 was over 700 % of control. Until recently, the antioxidant properties of HO-1 were thought to be through production of the ROS scavenger bilirubin, but data now suggest HO-1 may have other antioxidant qualities and be important in multiple cell types in the vasculature. Kadl *et al.* found that cell death in macrophages was exacerbated in the absence of Nrf2 or with inhibition of HO-1 activity [23]. It has also been demonstrated in macrophages that HO-1 functions as an antioxidant by decreasing heme availability, which decreases expression of the NADPH oxidase heme containing subunit $gp91^{phox}$ and subsequently decreases superoxide production [37].

Decreasing NADPH oxidase superoxide production has important implications in both macrophages and endothelial cells. Substantial evidence exists

demonstrating a role for NADPH oxidase and increased superoxide production in obesity and atherosclerosis. Compared to non-obese controls, overweight and obese subjects demonstrate increased NADPH oxidase subunit expression and augmented oxidative stress in the vascular endothelium [38]. NADPH oxidase subunit expression is elevated in lesions of coronary arteries in bypass graft patients, particularly in the vicinity of macrophages, and NADPH oxidase expression levels correlate with severity of atherosclerosis [10]. Superoxide produced by any mechanism including NADPH oxidase is rapidly converted to H_2O_2. Our experiments used H_2O_2 as an oxidative stress to induce apoptosis in HCAEC. While the concentration of H_2O_2 used in our experiments was chosen based on consistent induction of apoptosis in 30-50% of untreated cells, H_2O_2 is a relevant oxidant *in vivo* [41]. Experiments in our lab to examine other relevant stressors are ongoing

Whether HO-1 also functions as a protective antioxidant in endothelial cells through decreasing heme availability, inhibiting NADPH oxidase, and decreasing superoxide production, or via an alternative mechanism also is yet to be determined. Greater baseline HO-1 expression in aortic endothelial cells also results in decreased effects of oxidized phospholipids on inflammatory genes [39], suggesting that presence of HO-1 may delay progression of disease related phenotypic changes when cells are faced with chronic lipid and oxidative stresses. While it was beyond the scope of this study, determining whether HO-1 is essential to the Nrf2 dependent Protandim induced protection against an oxidative challenge is warranted.

Grx, SOD1, NQO1 were also significantly increased in HCAEC by Protandim, though the effect was not as large as that seen with HO-1. These are the first data indicating increases in Grx and NQO1 in response to Protandim; increases in SOD1 expression and activity have been previously reported [27, 30]. In erythrocytes isolated from human subjects following 120 days of Protandim supplementation, SOD activity was increased by 30% [30]. In an *ex vivo* preparation of human saphenous veins, Protandim treatment increased SOD activity 3-fold, HO-1 activity 7-fold, and catalase activity 12-fold [27]. Catalase has been shown to be increased by Protandim and to mediate protective effects in erythrocytes and saphenous vein preparations, as well as protect against skin cancer development in an animal model [27, 30, 40]. Our data in HCAEC show an approximate 10-fold increase in HO-1 expression, however catalase is undetectable in our model. Collectively, these data suggest that the antioxidant enzymes increase following Protandim treatment in a cell type specific manner.

Our data suggest positive effects of Protandim in healthy cells supporting the future examination of how Protandim may affect endothelial cells that have been chronically exposed to oxidative and lipid challenges. Most individuals experience transient lipid and oxidative stress in the vasculature throughout life, and it is the accumulated effects that eventually lead to overt vascular disease. Therefore it is of interest to determine if Protandim can slow or reverse disease related endothelial cell phenotypic changes using *in vivo* models of chronic oxidative stress.

Our current investigation shows for the first time that Protandim treatment in HCAEC induces Nrf2 nuclear localization, phase II antioxidant enzyme expression, and Nrf2 dependent protection from an oxidant stress. Oxidative stress has a well-established role in CAD disease initiation and progression, and our data support further research on phytochemical activation of Nrf2 and the endogenous antioxidant response as a potential therapeutic approach.

Acknowledgments

The authors thank Danielle Reuland and Laurie Biela for their previous work and technical assistance, and Dr. Manfred Diehl for his assistance with statistical analysis. Funding was provided by CSU Proteomics and Metabolomics Facility Academic Enrichment Program, and a Colorado State University CAHS mini grant.

List of Abbreviations:

HO-1, Heme oxygenase-1; Nrf 2, NF-E2-related factor 2; ROS, reactive oxygen species; SOD, superoxide dismutase; Grx, glutathione reductase; NQO1, NAD(P)H dehydrogenase [quinone] 1; Keap-1, Kelch-like ECH-associated protein 1; CAD, coronary artery disease; HCAEC, human coronary artery endothelial cells; Ptd, Protandim.

Figure Legends

Figure 1. Ptd treatment induces phase II antioxidant enzyme protein expression in HCAEC. (A) HCAEC were treated with increasing concentrations of Ptd for 12 hrs, HO-1 protein expression was measured. Concentrations of Ptd ranged from 1 μg/ml to 50 μg/ml. (B) Following determination of the appropriate concentration of Ptd, HCAEC were treated with 20μg/ml Ptd for 12hrs then protein expression of HO-1, Grx, SOD-1, and NQO1 was determined by Western blot. All bands were normalized to β-Actin as a loading control and are expressed as a percent of the control condition. *= $p<0.01$ compared to Ptd, **=$p<0.05$ compared to Ptd. Data are presented as mean $\pm$ SE.

Figure 2. Ptd induced Nrf2 expression and nuclear localization. HCAEC were treated with Ptd for 1 hr, following which immunofluorescence was used to visualize changes in Nrf2 expression and localization. Following Ptd treatment Nrf2 signal was increased and became visible in the nucleus. Increases in Ptd expression and nuclear localization were diminished within 15 min of removal of Ptd.

Figure 3. Ptd treatment protects HCAEC against an oxidative stress. HCAEC were treated with Ptd for 12 hrs, followed by H_2O_2 4hrs. (A) Treatment of HCAEC with Ptd prior to an oxidative challenge resulted in significantly fewer apoptotic nuclei as determined by TUNEL assay. (B) Approximately 35% of cells underwent apoptosis without Ptd treatment prior to an oxidative challenge while pre-treatment with Ptd resulted in approximately 5% of cells undergoing apoptosis in response to the same

oxidative challenge. *=p<0.01 compared to control (no Ptd). Data are presented as mean ± SE percent of TUNEL positive (+) nuclei.

Figure 4. Silencing of Nrf2 abrogated Ptd induced increases in HO-1 expression. (A) Cells were treated with no RNA, control RNA or Nrf2 siRNA prior to 12hrs Ptd, then HO-1 expression determined by Western blotting. (B) HO-1 expression in response to Ptd treatment increased to over 1,000 % of control in the no RNA and control RNA conditions, while increases in HO-1 expression in the Nrf2 siRNA condition were negligible. Data are presented as mean ± SE percent of control (no RNA, no Ptd) condition. * = p<0.01 # = p<0.01 compared to within RNA condition Ptd treatment

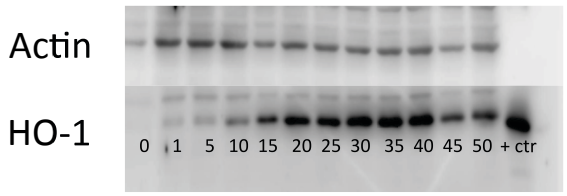
Figure 5. Silencing of Nrf2 resulted in a significant increase in the number of cells undergoing apoptosis in response to an oxidative challenge regardless of Ptd.

Figure 6. Ptd treatment following Nrf2 silencing protected HCAEC against an oxidative challenge, however, protection following Nrf2 silencing was significantly diminished compared to controls. (A) In no RNA, control RNA, and Nrf2 RNA conditions, significantly fewer cells underwent apoptosis when treated with Ptd prior to an oxidative stress. No RNA conditions and control RNA were not significantly different with or without Ptd. In Nrf2 siRNA conditions, % TUNEL positive (+) nuclei was significantly higher than no RNA and control RNA conditions

with and without Ptd. (B) The percent protection afforded by Ptd was not significantly different between no RNA and control RNA conditions. The protection afforded by Ptd in the Nrf2 siRNA condition was significantly less compared to both no RNA and control RNA conditions. Data are presented as means $\pm$ SE. *= $p<0.01$ between indicated groups, **= $p< 0.05$ between indicated groups. #=$p<0.01$ compared to within RNA condition Ctr(no Ptd), ##=$p<0.05$ compared to within RNA condition Ctr(no Ptd)

Figure 1.

A.



Increasing Ptd Concentration from 0µg/ml to 50µg/ml

B.

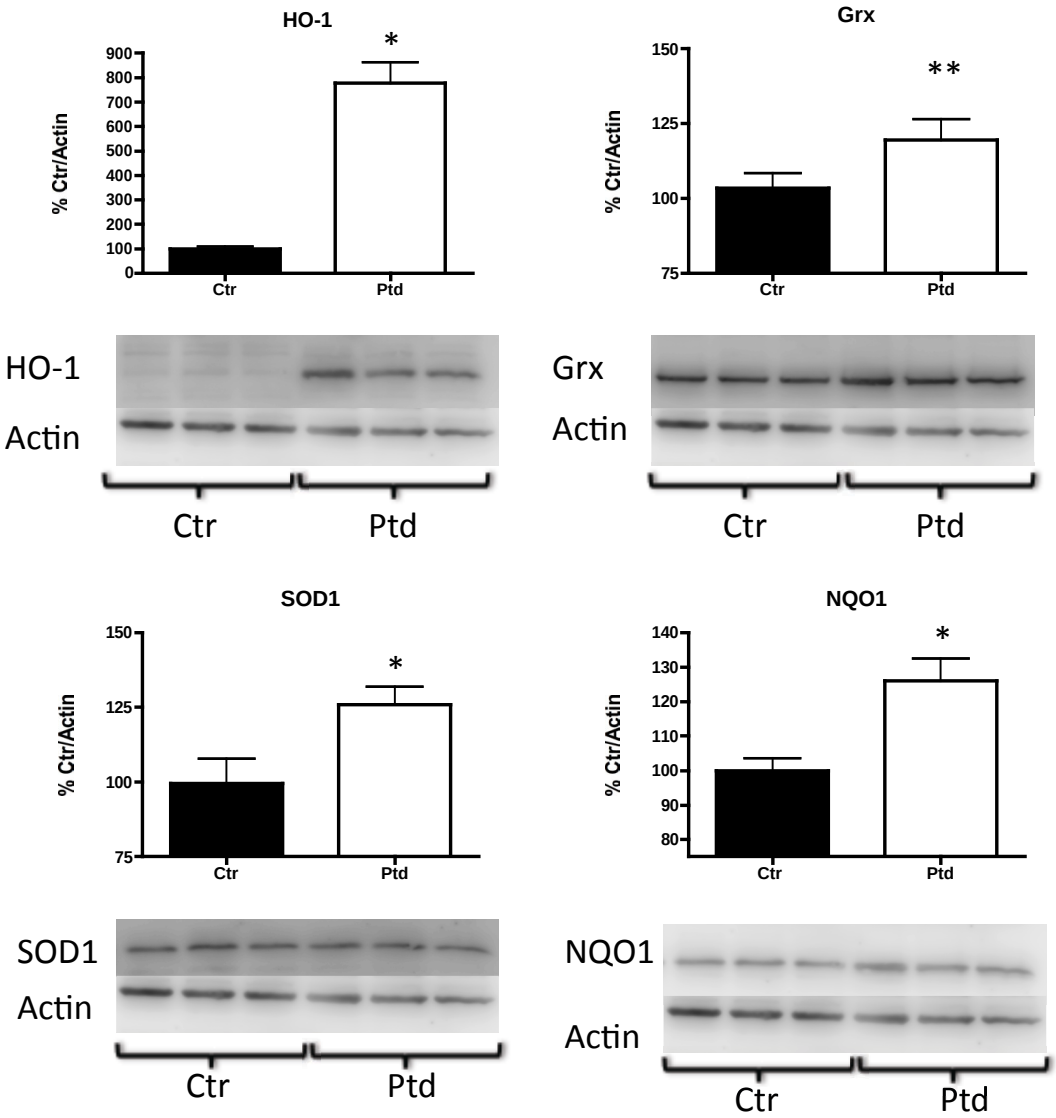


Figure 2.

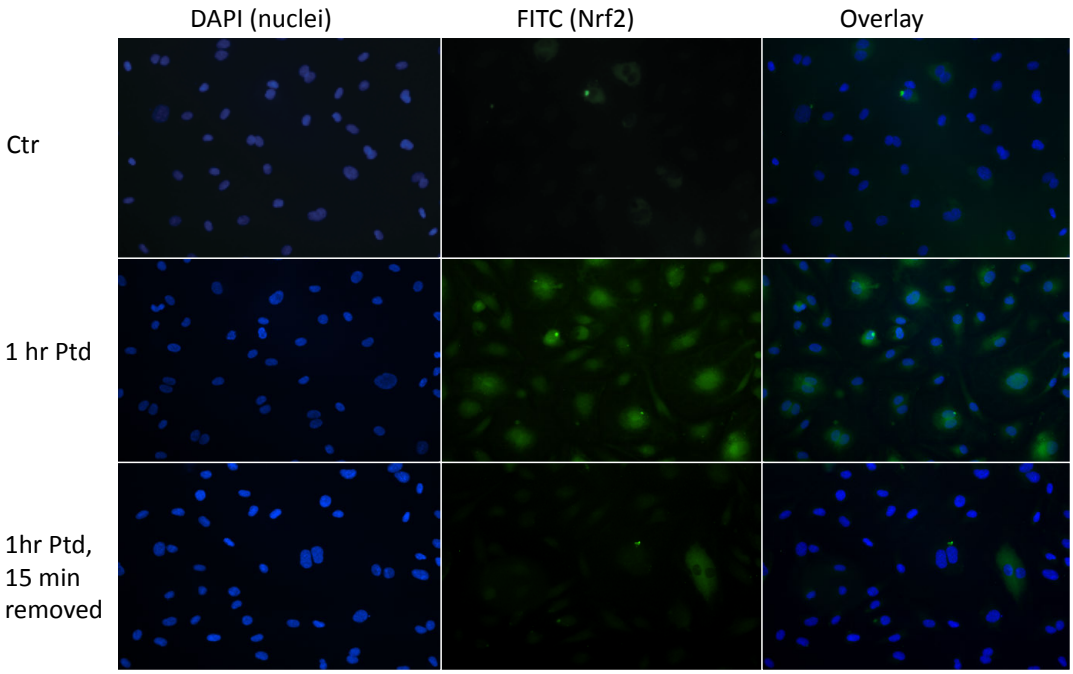
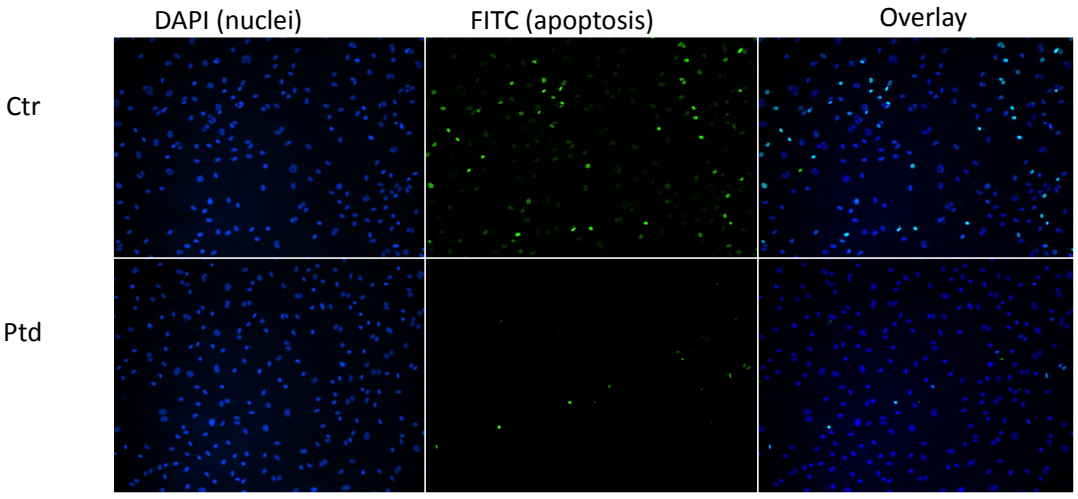

DAPI (nuclei)
FITC (Nrf2)
Overlay
Ctr
1 hr Ptd
1hr Ptd, 15 min removed

Figure 3.

A.


DAPI (nuclei)
FITC (apoptosis)
Overlay
Ctr
Ptd

B.

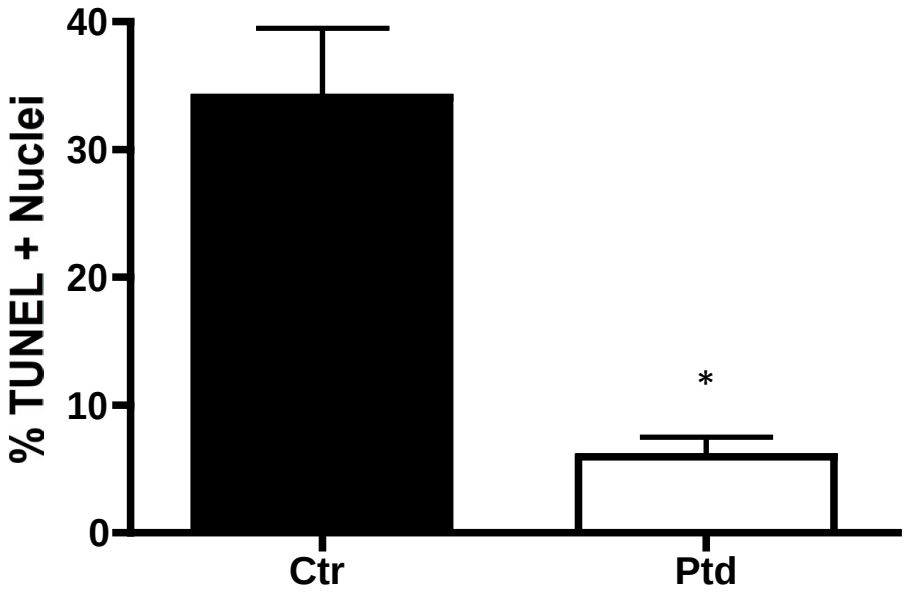
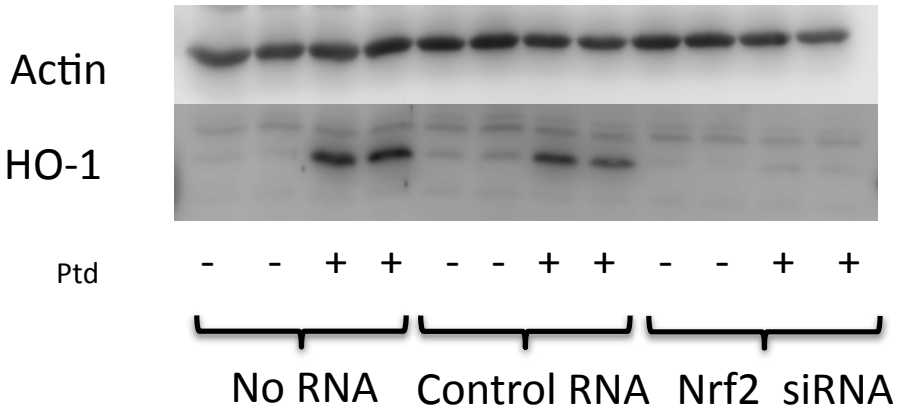

% TUNEL + Nuclei
40
30
20
10
0
*
Ctr
Ptd

Figure 4.

A.


Actin
HO-1
Ptd
- - + + - - + + - - + +
No RNA
Control RNA
Nrf2 siRNA

B.

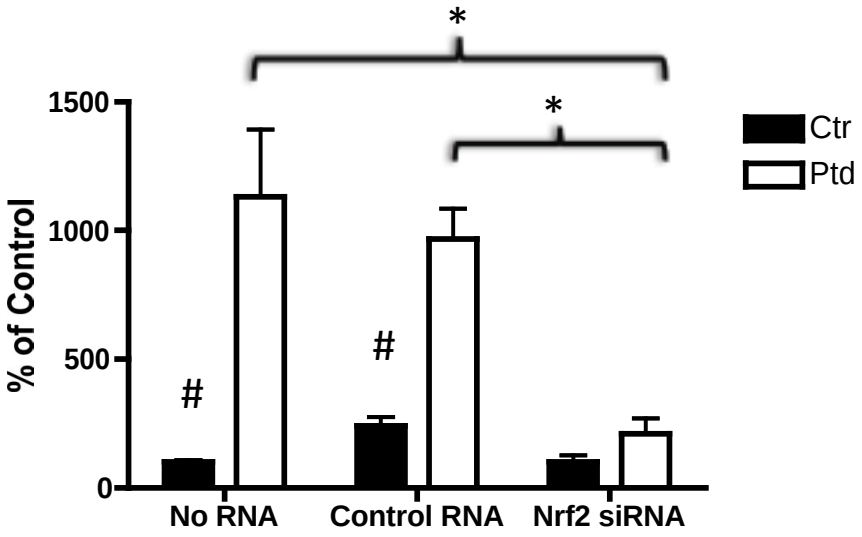

*
*
1500
1000
500
0
% of Control
#
#
Ctr
Ptd
No RNA
Control RNA
Nrf2 siRNA

Figure 5.

A.

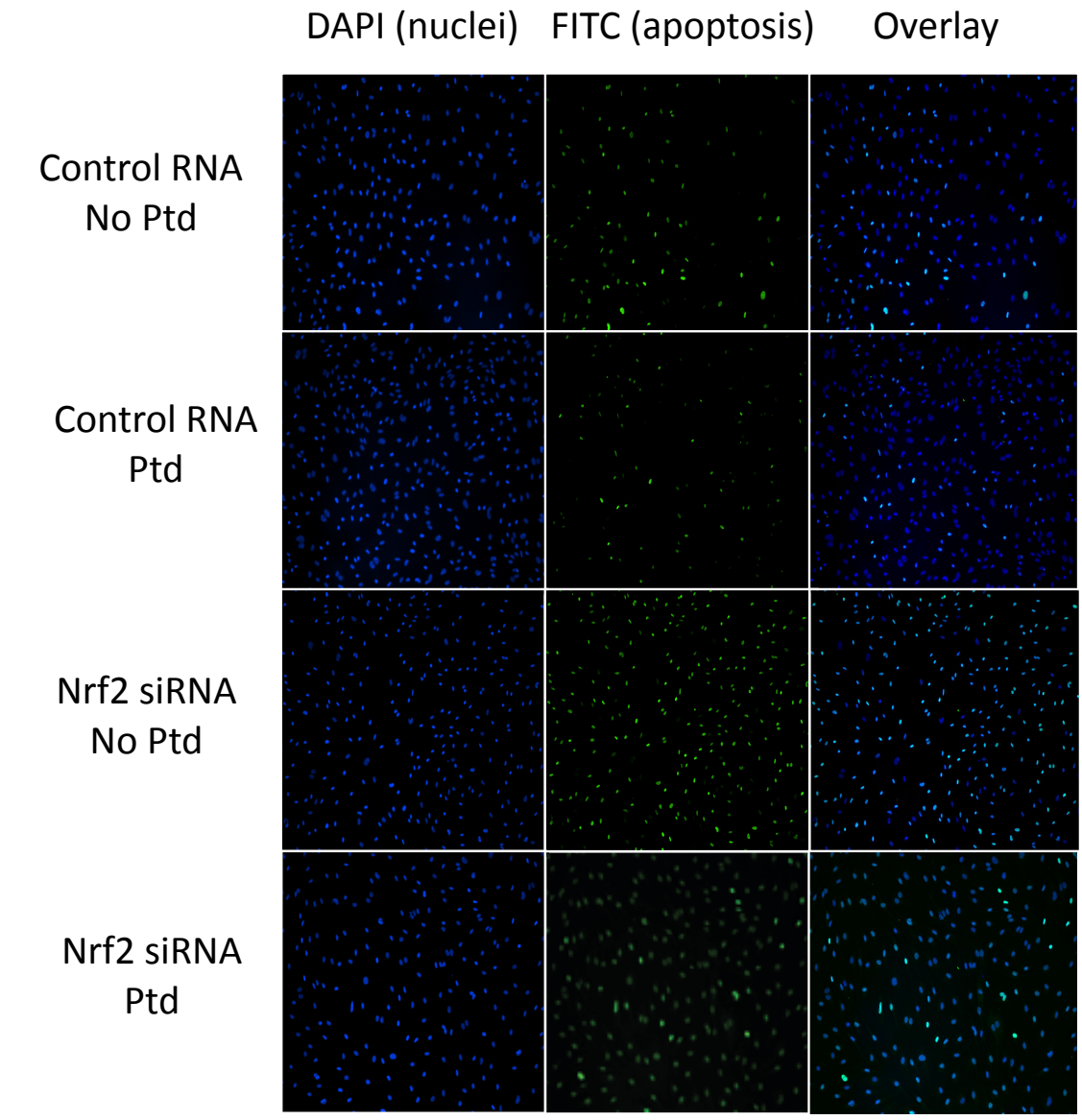
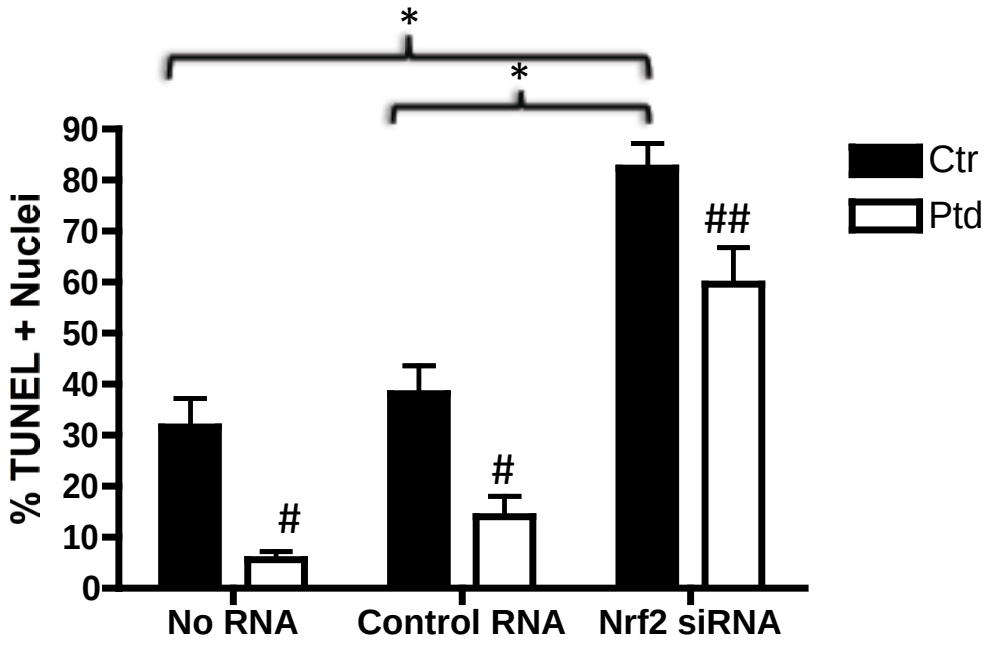
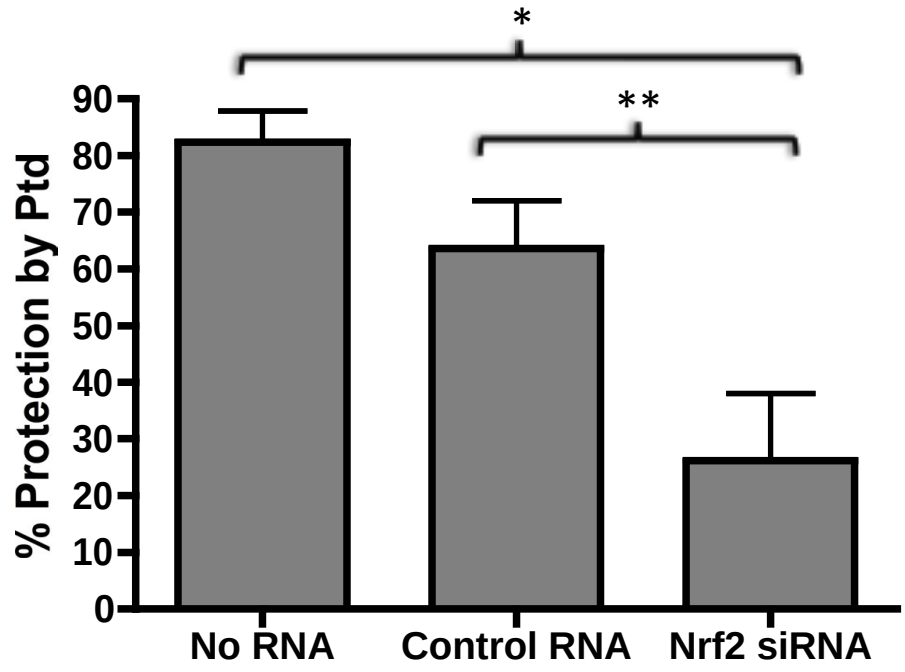

DAPI (nuclei)
FITC (apoptosis)
Overlay
Control RNA No Ptd
Control RNA Ptd
Nrf2 siRNA No Ptd
Nrf2 siRNA Ptd

Figure 6.

A.


*
*
90
80
70
60
50
40
30
20
10
0
% TUNEL + Nuclei
#
#
##
Ctr
Ptd
No RNA
Control RNA
Nrf2 siRNA

B.


*
**
90
80
70
60
50
40
30
20
10
0
% Protection by Ptd
No RNA
Control RNA
Nrf2 siRNA

References

1. Walter, M.F., et al., Serum levels of thiobarbituric acid reactive substances predict cardiovascular events in patients with stable coronary artery disease: a longitudinal analysis of the PREVENT study. J Am Coll Cardiol, 2004. 44(10): p. 1996-2002.

2. Robertson, R.P., Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. J Biol Chem, 2004. 279(41): p. 42351-4.

3. Rottkamp, C.A., et al., Oxidative stress, antioxidants, and Alzheimer disease. Alzheimer Dis Assoc Disord, 2000. 14 Suppl 1: p. S62-6.

4. Harrison, D., et al., Role of oxidative stress in atherosclerosis. Am J Cardiol, 2003. 91(3A): p. 7A-11A.

5. Carr, A.C., M.R. McCall, and B. Frei, Oxidation of LDL by myeloperoxidase and reactive nitrogen species: reaction pathways and antioxidant protection. Arterioscler Thromb Vasc Biol, 2000. 20(7): p. 1716-23.

6. Judkins, C.P., et al., Direct evidence of a role for Nox2 in superoxide production, reduced nitric oxide bioavailability, and early atherosclerotic plaque formation in ApoE-/- mice. Am J Physiol Heart Circ Physiol. 298(1): p. H24-32.

7. Lee, S., et al., A role for NADPH oxidase 4 in the activation of vascular endothelial cells by oxidized phospholipids. Free Radic Biol Med, 2009. 47(2): p. 145-51.

8. Rouhanizadeh, M., et al., Oxidized-1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine induces vascular endothelial superoxide production: implication of NADPH oxidase. Free Radic Biol Med, 2005. 39(11): p. 1512-22.

9. Landmesser, U., et al., Vascular extracellular superoxide dismutase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Circulation, 2000. 101(19): p. 2264-70.

10. Sorescu, D., et al., Superoxide production and expression of nox family proteins in human atherosclerosis. Circulation, 2002. 105(12): p. 1429-35.

11. Vendrov, A.E., et al., Atherosclerosis is attenuated by limiting superoxide generation in both macrophages and vessel wall cells. Arterioscler Thromb Vasc Biol, 2007. 27(12): p. 2714-21.

12. Bjelakovic, G., et al., Mortality in Randomized Trials of Antioxidant Supplements for Primary and Secondary Prevention. JAMA: The Journal of the American Medical Association, 2007. 297(8): p. 842-857.

13. Podmore, I.D., et al., Vitamin C exhibits pro-oxidant properties. Nature, 1998. 392(6676): p. 559-559.

14. Clarke, R. and J. Armitage, Antioxidant vitamins and risk of cardiovascular disease. Review of large-scale randomised trials. Cardiovasc Drugs Ther, 2002. 16(5): p. 411-5.

15. Giudice, A., C. Arra, and M.C. Turco, Review of molecular mechanisms involved in the activation of the Nrf2-ARE signaling pathway by chemopreventive agents. Methods Mol Biol. 647: p. 37-74.

16. Lyakhovich, V.V., et al., Active defense under oxidative stress. The antioxidant responsive element. Biochemistry (Mosc), 2006. 71(9): p. 962-74.

17. Itoh, K., et al., Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev, 1999. 13(1): p. 76-86.

18. Apopa, P.L., X. He, and Q. Ma, Phosphorylation of Nrf2 in the transcription activation domain by casein kinase 2 (CK2) is critical for the nuclear translocation and transcription activation function of Nrf2 in IMR-32 neuroblastoma cells. J Biochem Mol Toxicol, 2008. 22(1): p. 63-76.

19. Huang, H.C., T. Nguyen, and C.B. Pickett, Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. J Biol Chem, 2002. 277(45): p. 42769-74.

20. Cullinan, S.B., et al., Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival. Mol Cell Biol, 2003. 23(20): p. 7198-209.

21. Surh, Y.-J., J.K. Kundu, and H.-K. Na, Nrf2 as a Master Redox Switch in Turning on the Cellular Signaling Involved in the Induction of Cytoprotective Genes by Some Chemopreventive Phytochemicals. Planta Med, 2008. 74(EFirst): p. 1526,1539.

22. He, M., et al., Induction of HO-1 and redox signaling in endothelial cells by advanced glycation end products: a role for Nrf2 in vascular protection in diabetes. Nutr Metab Cardiovasc Dis. 21(4): p. 277-85.

23. Kadl, A., et al., Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. Circ Res. 107(6): p. 737-46.

24. Warabi, E., et al., Shear stress stabilizes NF-E2-related factor 2 and induces antioxidant genes in endothelial cells: role of reactive oxygen/nitrogen species. Free Radic Biol Med, 2007. 42(2): p. 260-9.

25. Jyrkkanen, H.K., et al., Nrf2 regulates antioxidant gene expression evoked by oxidized phospholipids in endothelial cells and murine arteries in vivo. Circ Res, 2008. 103(1): p. e1-9.

26. Ungvari, Z.I., et al., Adaptive induction of NF-E2-Related Factor-2-driven antioxidant genes in endothelial cells in response to hyperglycemia. Am J Physiol Heart Circ Physiol.

27. Joddar, B., et al., Protandim attenuates intimal hyperplasia in human saphenous veins cultured ex vivo via a catalase-dependent pathway. Free Radic Biol Med. 50(6): p. 700-9.

28. Padmanabhan, B., et al., Structural basis for defects of Keap1 activity provoked by its point mutations in lung cancer. Mol Cell, 2006. 21(5): p. 689-700.

29. Velmurugan, K., et al., Synergistic induction of heme oxygenase-1 by the components of the antioxidant supplement Protandim. Free Radic Biol Med, 2009. 46(3): p. 430-40.

30. Nelson, S.K., et al., The induction of human superoxide dismutase and catalase in vivo: a fundamentally new approach to antioxidant therapy. Free Radic Biol Med, 2006. 40(2): p. 341-7.

31. Hussain, S.P., et al., p53-induced up-regulation of MnSOD and GPx but not catalase increases oxidative stress and apoptosis. Cancer Res, 2004. 64(7): p. 2350-6.

32. Pfluger, P.T., et al., Sirt1 protects against high-fat diet-induced metabolic damage. Proc Natl Acad Sci U S A, 2008. 105(28): p. 9793-8.

33. Xu, F., et al., Endothelial cell apoptosis is responsible for the formation of coronary thrombotic atherosclerotic plaques. Tohoku J Exp Med, 2009. 218(1): p. 25-33.

34. Hulsmans, M. and P. Holvoet, The vicious circle between oxidative stress and inflammation in atherosclerosis. J Cell Mol Med. 14(1-2): p. 70-8.

35. Frey, R.S., M. Ushio-Fukai, and A.B. Malik, NADPH oxidase-dependent signaling in endothelial cells: role in physiology and pathophysiology. Antioxid Redox Signal, 2009. 11(4): p. 791-810.

36. Stoneman, V.E. and M.R. Bennett, Role of apoptosis in atherosclerosis and its therapeutic implications. Clin Sci (Lond), 2004. 107(4): p. 343-54.

37. Taille, C., et al., Induction of heme oxygenase-1 inhibits NAD(P)H oxidase activity by down-regulating cytochrome b558 expression via the reduction of heme availability. J Biol Chem, 2004. 279(27): p. 28681-8.

38. Silver, A.E., et al., Overweight and obese humans demonstrate increased vascular endothelial NAD(P)H oxidase-p47(phox) expression and evidence of endothelial oxidative stress. Circulation, 2007. 115(5): p. 627-37.

39. Romanoski, C.E., et al., Network for activation of human endothelial cells by oxidized phospholipids: a critical role of heme oxygenase 1. Circ Res. 109(5): p. e27-41.

40. Liu, J., et al., Protandim, a fundamentally new antioxidant approach in chemoprevention using mouse two-stage skin carcinogenesis as a model. PLoS One, 2009. 4(4): p. e5284.

41. Brandes, R., Schroder, K., Differential vascular functions of Nox family NADPH oxidases. Curr Opin Lipidol, 2008 19(5): p. 513-518

CHAPTER IV – MANUSCRIPT III

LIPOPROTEIN-ASSOCIATED PHOSPHOLIPASE A(2) IS EXPRESSED IN HUMAN ADIPOSE TISSUE AND CORRELATES WITH HBA1C IN OBESITY

Lipoprotein-associated Phospholipase A(2) is expressed in human adipose tissue and correlates with HbA1C

Elise L Donovan[1], Tracy L Nelson[1], Stefan M Pettine[2], John E Hokanson[3], *Karyn L Hamilton[1], and *Benjamin F Miller[1]:

[1]Department of Health and Exercise Science, Colorado State University, Fort Collins, Colorado. [2]Bariatric Center of the Rockies, Fort Collins, Colorado. [3]Department of Epidemiology, University of Colorado Anschutz Medical Campus, Denver, Colorado.

*Co-Principal Investigators

Corresponding Author:

Benjamin F. Miller

220 Moby B Complex 1582

Colorado State University

Fort Collins, CO 80523

970-491-3291 (phone)

benjamin.f.miller@colostate.edu

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) catalyses the hydrolysis of platelet activating factor (PAF) and oxidized phospholipids. Lp-PLA_2 activity is elevated in plasma of type 2 diabetics and potentially predicts diabetes. The purpose of this study was to examine Lp-PLA_2 protein in adipose and skeletal muscle from obese subjects and lean controls, and determine how adipose tissue Lp-PLA_2 relates to plasma Lp-PLA_2 activity and HbA1C. We found unmodified and glycosylated Lp-PLA_2 in adipose tissue and skeletal muscle from lean and obese subjects. In adipose tissue Lp-PLA_2 co-localized predominantly with macrophages. Adipose tissue total and glycosylated Lp-PLA_2 were not different between lean and obese groups (p= 0.084, and p=0.1872). Correlations between obese subject adipose tissue total Lp-PLA_2 and HbA1C, and obese subject adipose tissue glycosylated Lp-PLA_2 and HbA1C trended towards significant (r=-0.517, p=0. 126) and (r=0.491, p=0.125), respectively. When these correlations were re-calculated excluding subjects taking glycemic control medication, the correlation between adipose tissue total Lp-PLA_2 and HbA1C became significant (r=-0.834, p=0.039), but the correlation between adipose tissue glycosylated Lp-PLA_2 and HbA1C was weakened and became negative (r=-0.306, p= 0.556). The presence of Lp-PLA_2 in adipose tissue, along with associations between adipose Lp-PLA_2 and HbA1C in obese subjects, suggests future research should examine a role for Lp-PLA_2 in tissue insulin resistance.

Keywords: Lipoprotein-associated phospholipase A_2 (Lp-PLA_2), adipose, obesity, diabetes, macrophages

Introduction

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is a macrophage-derived member of the platelet activating factor acetylhydrolase family that catalyses the hydrolysis of platelet activating factor (PAF) to inactive lyso-PAF and a free fatty acid. Lp-PLA_2 can be glycosylated but only a single study has examined the function of glycosylation suggesting it may be related to Lp-PLA_2 association with HDL versus LDL cholesterol in circulation [1]. Lp-PLA_2 is unique compared to other PAF acetylhydrolases because it also has affinity for short chain and oxidized phospholipids. Hydrolysis of oxidized phospholipids by Lp-PLA_2 results in potentially inflammatory lysophospholipids and oxidized fatty acids [2-5]. The role of Lp-PLA_2 has been studied more in relation to hydrolysis of oxidized phospholipids than PAF, because other PAF-acetylhydrolases also degrade PAF, while only Lp-PLA_2 has affinity for oxidized phospholipids.

Lp-PLA_2 is now recognized as a biomarker of cardiovascular disease (CVD) risk [6]. While some data indicate that increasing Lp-PLA_2 can protect against atherosclerotic lesion development [7, 8], most data associate increased plasma Lp-PLA_2 activity with CAD risk. Kolodgie *et al.* examined coronary arteries from patients that experienced sudden coronary death patients and found that in early plaques, Lp-PLA_2 was barely detectable. However, in advanced and ruptured plaques, Lp-PLA_2 was strongly expressed and co-localized with macrophages in necrotic cores [9]. Research continues to determine if Lp-PLA_2 is truly a risk factor

for CAD as opposed to just a biomarker, with drugs to inhibit Lp-PLA_2 activity in CAD patients are in clinical trials [10, 11].

Most epidemiological data show that Lp-PLA_2 is elevated in patients with the metabolic syndrome [12, 13]. Lp-PLA_2 activity is positively correlated with LDL cholesterol and negatively correlated with HDL cholesterol [14]. Data examining the relationship between fat distribution and Lp-PLA_2 are inconsistent. Pou *et al.* found no relationship between Lp-PLA_2 and subcutaneous or visceral adipose volume [15], but Detopoulou *et al*. found that plasma Lp-PLA_2 activity is associated with trunk adiposity [16]. Further, Lp-PLA_2 activity is elevated in diabetics [17], further Lp-PLA_2 activity may be predictive of diabetes regardless of BMI (in review).

Inflammatory mediators and excess fatty acids in the adipose tissue are known to play a role in insulin resistance [18-21]. The effects of lysophospholipids and oxidized fatty acids derived from Lp-PLA_2 hydrolysis of oxidized phospholipids on adipose tissue are not known, though these products can induce insulin resistance in skeletal muscle [22]. Lp-PLA_2 is predominantly macrophage derived, and a hallmark of obesity is macrophage infiltration into the adipose tissue in response to inflammatory mediators and necrotic adipocytes [23, 24]. Lp-PLA_2 protein has been detected in rodent adipocytes, but the same has not been investigated in human adipocytes or whole adipose tissue [25, 26]. Based on these collective data it is reasonable to hypothesize that Lp-PLA_2 is expressed in human adipose tissue, and may play a role in tissue insulin resistance and development of diabetes.

The purpose of this study was to: 1) examine Lp-PLA_2 expression in adipose and skeletal muscle from morbidly obese subjects and lean controls, and 2) determine how adipose tissue Lp-PLA_2 expression relates to plasma Lp-PLA_2 activity, markers of glucose homeostasis and circulating inflammatory markers. We hypothesized that Lp-PLA_2 would be expressed in human adipose but not skeletal muscle, and that expression would be greater in adipose tissue from morbidly obese subjects compared to that from lean subjects. In addition, we hypothesized that visceral adipose tissue Lp-PLA_2 protein content would correlate with circulating Lp-PLA_2 activity, markers of inflammation and markers of glucose homeostasis.

Methods

Ethics approval

The Institutional Review Boards of Colorado State University and Poudre Valley Hospital approved this protocol (CSU protocol #- 05-116H, PVH protocol #-07-874). Each volunteer was informed of the potential risks and written consent was obtained prior to enrollment. The study followed the guidelines set forth by the Declaration of Helsinki.

Study overview

A total of 15 morbidly obese gastric bypass patients, and 13 lean controls age 18-60 were recruited to participate through the Northern Colorado Surgical Associates (NCSA) of the Bariatric Center of the Rockies. Lean controls were patients undergoing an abdominal surgery of comparable invasiveness to gastric bypass surgery. Bypass patients were required to have a body mass index (BMI) > 40 kg/m^2, while controls were required to have a BMI < 35 kg/m^2. Subjects completed medical and exercise questionnaires prior to undergoing a venipuncture blood draw following a 12-hour fast. Blood was collected in vacutainer tubes containing EDTA, 0.5ml were aliquoted for HbA1C analysis, and the remainder was centrifuged (1200g, 15 min, 4°C). Plasma was collected and stored at -80°C until assayed. Whole blood samples were sent to the University of Colorado Denver Clinical Translational Research Center for analysis of hemoglobin A1C (HbA1C) using a DCA Vantage analyzer (Siemens, Deerfield, IL). During surgery, omental

adipose and abdominal muscle were collected, frozen in liquid nitrogen, and stored at -80°C until assayed.

Plasma Lp-PLA$_2$ activity and TNF-α

Plasma Lp-PLA2 concentration was measured by an enzyme linked immunosorbent assay (ELISA) and activity determined by a colorimetric activity method, both performed by diaDexus, Inc (South San Francisco, CA) (mass, intra-assay coefficient of variation (CV)% <0.1%; activity, intra-assay CV% <2%). Plasma TNF-α was determined using a high sensitivity sandwich ELISA (R & D Systems, Minneapolis, MN). The lower limit of detection was 0.5pg/ml, average coefficient of variation was 3.96%.

Western blot analyses of adipose and muscle

Adipose tissue was weighed and homogenized in RIPA buffer (50mM Tris, 0.15M NaCl, 1% Na deoxycholic acid, 1mM EGTA, 1% NP40). The homogenate was centrifuged for 10 min at 10,000 x g at 4°C. The supernatant was collected from between the fat cake and pellet, transferred to a second tube, and centrifuged again for 3 min at 10,000 x g at 4°C. The resulting supernatant was collected, sonicated, and protein concentrations were determined using a BCA assay. Abdominal muscle was weighed, homogenized in RIPA buffer, and centrifuged at 800 x g for 10 min. The supernatant was collected and centrifuged at 1500 x g for 5 min. Again the supernatant was collected, sonicated and protein concentrations determined using a BCA assay. Adipose and muscle homogenates were diluted to equal concentrations with Laemmli sample buffer. A total of 20 μg protein from each sample was

separated on 10% polyacrylamide gels at 100-125 volts, and transferred to nitrocellulose membranes (BioRad, Hercules, CA) for 1 hr at 50 volts. Membranes were blocked for 1 hr in Superblock (Thermo Scientific, Rockford, IL), then incubated with primary antibody against Lp-PLA_2 at a dilution of 1-200 (Cayman Chemical, Ann Arbor, MI) followed by the appropriate HRP-conjugated secondary antibody. Membranes were developed by chemiluminescence using SuperSignal West Dura substrate (Thermo Scientific, Rockford, IL), with digital images obtained using the Biospectrum UVP system. All signals were normalized to β-actin obtained from the same blot.

Adipose tissue immunohistochemistry

Adipose tissue was fixed for 24 hrs in 10% neutral buffered formalin, paraffin embedded, sliced, and mounted. Slides were dewaxed by washing 2 x 5 min in xylene, 2 x 5 min in 100% ethanol, 2 x 5 min in 95% ethanol, 5 min in diH_2O, and 10 min in TBS with 0.5% Tween 20. Slides were blocked in 5% BSA with 0.5% goat serum in PBS, then incubated overnight in primary antibodies against Lp-PLA_2 1:50 (Cayman Chemical), and F4/80 1:100 (Santa Cruz Biotech, Santa Cruz, CA). Slides were then washed 3 x 5 min and incubated with appropriate FITC conjugated and Texas Red conjugated secondary antibodies. Slides were again washed 3 x 5 min each, mounted with DAPI containing mounting medium for identification of cell nuclei, then visualized by fluorescence microscopy (Nikon TE2000).

Statistics

Lean and obese groups, group means were compared using un-paired t-tests. Comparisons between tissue parameters within subjects were done using paired t-tests. Relationships between all factors within and between groups were analyzed using Pearson's correlations. Significance was set *a priori* at $p \leq 0.05$.

Results

Subject characteristics

BMI was significantly higher in the obese group (49.9 ± 11.3) compared to lean group (25.8 ± 4.4) ($p<0.01$), and HbA1C was significantly greater in the obese group (6.41 ± 0.35) compared to lean group (5.15 ± 0.10) ($p<0.01$) (Figure 1). There were no significant differences between the lean and obese groups for plasma Lp-PLA_2 activity ($p=0.40$) or TNF-α ($p=0.20$)

Tissue Lp-PLA_2

To determine if Lp-PLA_2 was present in adipose tissue, we performed western blotting on visceral adipose tissue homogenates. We found that Lp-PLA_2 is present in adipose tissue from lean and obese subjects, though total adipose Lp-PLA_2 was not significantly different between groups ($p= 0.084$)(Figure 2A). We observed a band corresponding to unmodified Lp-PLA_2 at approximately 45kDa, as well as bands corresponding to glycosylated Lp-PLA_2 at approximately 56kDa as shown previously [1](Figure 2A). For further analyses we considered both total Lp-PLA_2 and the ratio of glycosylated to unmodified Lp-PLA2. The ratio of glycosylated to unmodified was also not different in adipose between lean and obese ($p=0.1872$). Lp-PLA_2 was also present in whole muscle homogenates from a subset of subjects, though there was no difference between lean and obese total Lp-PLA_2 ($p=0.3893$) or glycosylated Lp-PLA_2 ($p=0.7552$) (Figure 2B). We compared total Lp-PLA_2 pair wise in adipose and muscle in lean and obese subjects combined, as well as in obese subjects analyzed alone. Total Lp-PLA_2 and glycosylated Lp-PLA_2 were significantly

higher in adipose compared to muscle in all subjects (<0.0001), and (p=0.01), respectively (Figure 2C).

Adipose Lp-PLA$_2$, plasma Lp-PLA$_2$, and glucose homeostasis

We derived multiple correlations between all measured factors in obese subjects. A significant correlation was found between age and adipose glycosylated Lp-PLA$_2$ (r=0.648, p=0.012). Other relationships trending towards significance (r>0.4 or p<0.2) were found between HbA1C and adipose total Lp-PLA$_2$ (r=-0.517, p=0. 126), age and HbA1C (r=0.408, p=0.213), and HbA1C and adipose glycosylated Lp-PLA$_2$, (r=0.491, p=0.125). Scatter plots for each correlation are shown in Figure 3.

The correlations were re-calculated in obese subjects with data from subjects taking glycemic control medication excluded. Data from the remaining obese subjects revealed a significant correlation between HbA1C and adipose total Lp-PLA$_2$ (r=-0.834, p=0.039). Trends toward significance(r>0.4 or p<0.2) were found between age and glycosylated Lp-PLA$_2$ (r=0.647, p=0.060), age and HbA1C (r=0.572, p=0.236). With subjects taking glycemic control medication excluded, the correlation between HbA1C and adipose Lp-PLA$_2$ glycosylation became negative and non-significant (r=-0.306, p= 0.556). Scatter plots for each correlation are shown in Figure 4.

The same correlations were then examined in lean subjects. The correlation between HbA1C and total adipose Lp-PLA$_2$ (r=0.674, p=0.067) approached significance. This positive correlation was in contrast to the negative correlation

measured in obese subjects. The relationship between plasma TNF-α and total adipose Lp-PLA_2 in lean subjects also trended towards significance (r=0.529, p=0.094). Scatter plots are shown in Figure 5. We did not observe a correlation between plasma Lp-PLA_2 activity and adipose Lp-PLA_2 in any group of subjects.

Lp-PLA_2 adipose tissue immunohistochemistry

Because Lp-PLA_2 was present in total adipose tissue homogenates, and we observed relationships between adipose Lp-PLA_2 and the circulating factors described above, we performed immunohistochemical analysis (n=8/group) to determine whether adipose tissue Lp-PLA_2 is localized within resident macrophages, adipocytes, or both. We found that Lp-PLA_2 is found almost exclusively in macrophages. A representative image is presented in Figure 6 showing a field at 30x magnification and a region within that field at 60x.

Discussion

Because it has recently been demonstrated that circulating $LpPLA_2$ is predictive of diabetes (in review), and obesity is an established risk factor for diabetes, we sought to characterize Lp-PLA_2 in human visceral adipose tissue and determine if tissue Lp-PLA_2 was related to HbA1C. The major findings of this study were that Lp-PLA_2 is expressed in human visceral adipose tissue, and adipose tissue Lp-PLA_2 is predominantly localized in macrophages. We also measured a non-significant negative correlation between HbA1C and adipose tissue total Lp-PLA_2 in obese subjects, but a positive non-significant correlation between HbA1C and glycosylated Lp-PLA_2 in adipose tissue. When subjects taking glycemic control medication were removed from our analysis of obese subjects, the negative relationship between HbA1C and adipose total Lp-PLA_2 became significant. However, the correlation between HbA1C and adipose glycosylated Lp-PLA_2 was weakened and became negative.

To our knowledge, this is the first time that Lp-PLA_2 has been characterized in human adipose tissue. While we hypothesized that Lp-PLA_2 would be greater in adipose tissue from obese subjects compared to lean, there was in fact no difference. This finding is paradoxical. We would expect Lp-PLA_2 to be greater in adipose tissue from obese subjects because we also found that Lp-PLA_2 was localized within macrophages resident in adipose tissue, and macrophage infiltration of adipose is a hallmark of obesity [23]. However, we did not quantify differences in adipose resident macrophages between our lean and obese subjects. It may be that the

absolute amount of Lp-PLA_2 is less important than post-translational modifications and activity. Indeed, we found a positive association between HbA1C and glycosylated adipose tissue Lp-PLA_2 in obese subjects, though this did not reach significance. This relationship was significantly weakened when subjects taking glycemic control medications were removed from the analysis. While glycosylation of Lp-PLA_2 is well established [1, 27], whether Lp-PLA_2 glycosylation occurs spontaneously or enzymatically is unknown, and the function of Lp-PLA_2 glycosylation is not well characterized. The single study that examined a role for Lp-PLA_2 glycosylation found that in macrophages glycosylation is not related to secretion of Lp-PLA_2 but it does play a role in Lp-PLA_2 association with HDL [1, 27]. We now submit that glycosylation of adipose Lp-PLA_2 may be associated with HbA1C, that this relationship is potentially affected by diabetes medications, but that total adipose Lp-PLA_2 is negatively correlated with HbA1C. Further studies should examine the function of Lp-PLA_2 and Lp-PLA_2 glycosylation in adipose tissue.

It is well established that TNF-α and other inflammatory cytokines produced by both macrophages and adipocytes impair insulin signaling in adipose as well as other tissues, contributing to systemic insulin resistance [19, 28, 29]. It is possible that Lp-PLA_2 from adipose tissue macrophages adversely affects insulin signaling as products of Lp-PLA_2 hydrolysis of oxidized phospholipids can induce inflammation and insulin resistance [22, 30, 31]. Macrophages are known to exist in different activation states in adipose tissue [32]. M1 macrophages are pro-inflammatory, secrete cytokines including TNF-α and IL-6, and produce increased quantities of reactive oxygen species. M2 macrophages produce anti-inflammatory cytokines

such as Il-10, and their role in wound healing and immunoregulation is well characterized (reviewed in [28]). Obesity is associated with increased M1 macrophages in adipose tissue and signals from M1 macrophages are related to insulin resistance [32], however it is unknown whether Lp-PLA_2 expression or activity are increased in M1 macrophages specifically. Collectively, these data support a hypothetical scenario that could in part explain the relationship between Lp-PLA_2 and diabetes. If adipose macrophage Lp-PLA_2 increases LPCs and free fatty acids that contribute to adipocyte insulin resistance, secretion of TNF-α and other cytokines that impair insulin signaling, adipose tissue Lp-PLA_2 may be part of the pathology of diabetes (Figure 7).

Finding Lp-PLA_2 in skeletal muscle, albeit at significantly lower levels than in adipose, was unexpected. A role for Lp-PLA_2 in muscle has not been examined. While we detected Lp-PLA_2 in muscle this was in a subset of subjects too small to use for examination of correlations with other measured factors. In addition, due to limited tissue availability we were unable to perform immunohistochemical analysis on muscle samples to determine localization of Lp-PLA_2. It is possible that Lp-PLA_2 also has a physiological role in skeletal muscle, but this cannot be determined by the data collected in our study.

While the data from these experiments are significant and hypothesis generating, limitations must be considered. The major limitation is a small number of subjects. In addition, conclusions must be drawn with caution because these are retrospective cross sectional data. It is of interest to determine if adipose

macrophages contribute to circulating Lp-PLA_2, however we did not see a strong association between adipose tissue Lp-PLA_2 and plasma Lp-PLA_2, which may be due to the small subject numbers.

In conclusion, we detected Lp-PLA_2 in human adipose tissue, and showed that adipose Lp-PLA_2 is localized within adipose tissue macrophages. We also found associations between HbA1C and adipose tissue Lp-PLA_2 glycosylation. Presence of Lp-PLA_2 in adipose tissue along with associations between adipose Lp-PLA_2 and HbA1C in obese subjects suggest future research should examine a role for Lp-PLA_2 in adipose tissue insulin resistance.

Figure Legends

Figure 1. BMI and HbA1C are significantly greater in obese subjects compared to lean. * = $p<0.01$ compared to obese.

Figure 2. Lp-PLA_2 is expressed in human visceral adipose and skeletal muscle. Whole tissue homogenates were probed for Lp-PLA_2 and expression levels normalized to β-actin or expressed as the ratio of glycosylated to unmodified. A- Representative western blot showing Lp-PLA_2in visceral adipose tissue. B- Representative western blot showing Lp-PLA_2 in skeletal muscle. C- Total Lp-PLA_2 and glycosylated Lp-PLA_2 are significantly greater in adipose than muscle. There were no differences in total Lp-PLA_2 of glycosylated Lp-PLA_2 within lean or obese tissues. Data are presented as mean $\pm$ SE, *= $p<0.01$.

Figure 3. Scatter plots showing the relationships found in obese subjects related to tissue Lp-PLA_2 with Pearson's correlation coefficients and p-values.

Figure 4. Scatter plots showing the relationships found in obese non-diabetic subjects related to tissue Lp-PLA_2 with Pearson's correlation coefficients and p-values.

Figure 5. Scatter plots showing the relationships found in lean subjects related to tissue Lp-PLA_2 with Pearson's correlation coefficients and p-values.

Figure 6. Representative immunofluorescent images showing that adipose Lp-PLA_2 co-localizes with resident macrophages. Green (FITC) = Lp-PLA_2, red (Texas Red)=

macrophage marker F4/80, blue (DAPI)= nuclei. The region denoted by the red box in the 30x FITC image shows the region magnified for 60x images.

Figure 7. Schematic depicting the hypothesized role of adipose tissue macrophage Lp-PLA_2 in the pathology of insulin resistance where macrophage Lp-PLA_2 in the adipose tissue and skeletal muscle increases LPCs and free fatty acids, which are one of the stimuli increasing secretion of TNF-α and other cytokines that impair local and systemic insulin signaling. Remaining question included whether Lp-PLA_2 directly or through downstream products influences insulin resistance within the respective tissue, or in other peripheral tissues, and whether tissue production of Lp-PLA_2 contributes to circulating Lp-PLA_2 activity.

Figures

Figure 1.

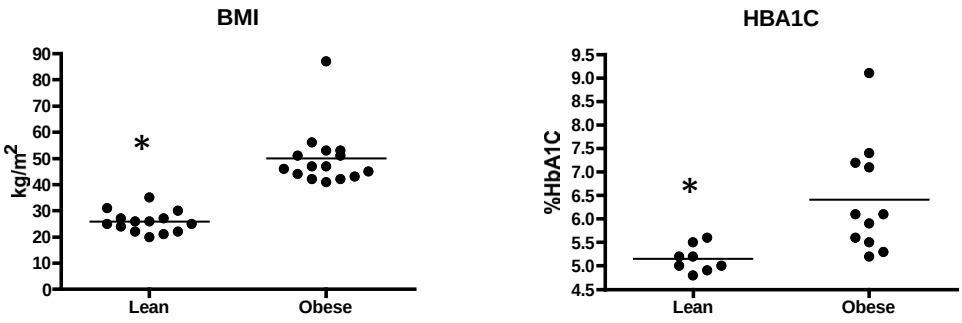


Figure 2

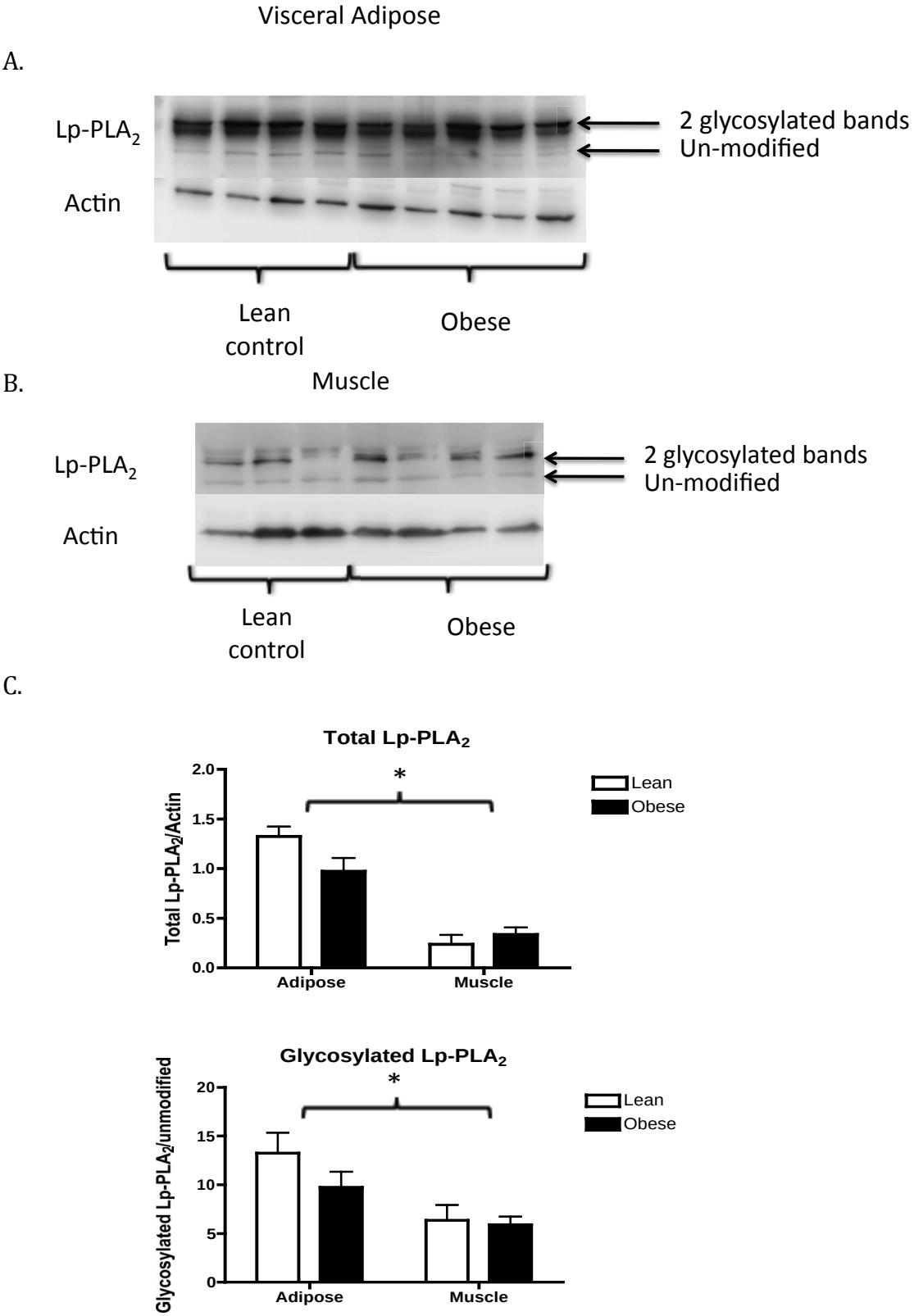
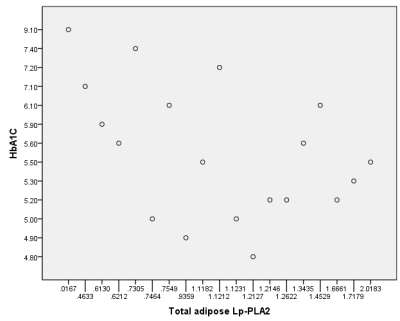
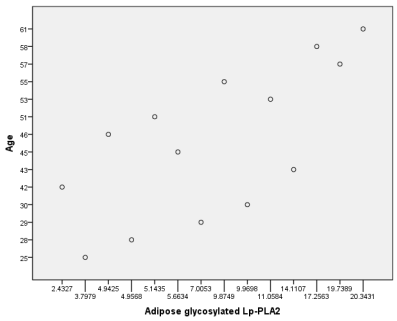

Visceral Adipose
A.
Lp-PLA2
Actin
2 glycosylated bands
Un-modified
Lean control
Obese
B.
Muscle
Lp-PLA2
Actin
2 glycosylated bands
Un-modified
Lean control
Obese
C.
Total Lp-PLA2
Total Lp-PLA2/Actin
2.0
1.5
1.0
0.5
0.0
*
Lean
Obese
Adipose
Muscle
Glycosylated Lp-PLA2
Glycosylated Lp-PLA2/unmodified
20
15
10
5
0
*
Lean
Obese
Adipose
Muscle

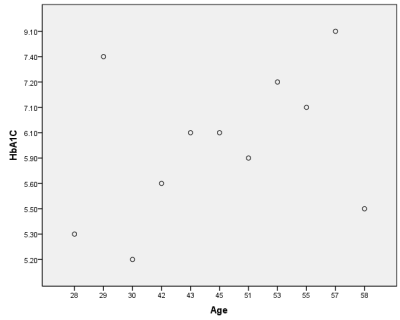
Figure 3.



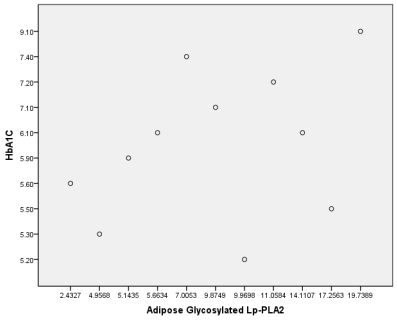
r= -0.517
p= 0.126



r= 0.648
p= 0.012

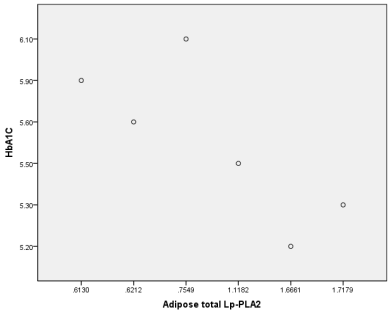


r= 0.408
p= 0.213

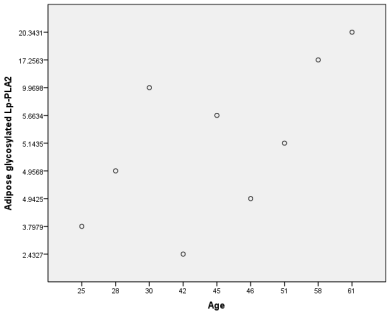


r= 0.491
p= 0.125

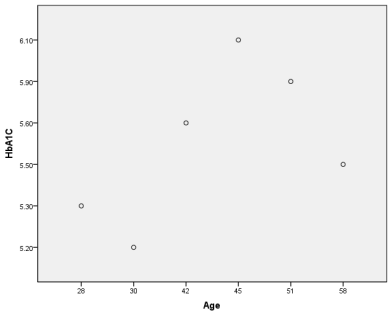
Figure 4.



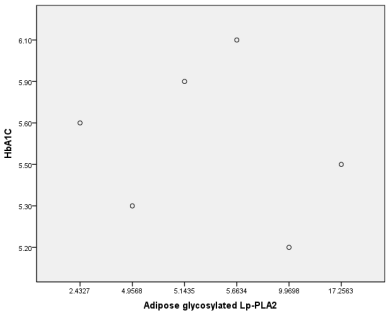
r = -0.834
p= 0.039



r = 0.647
p= 0.060

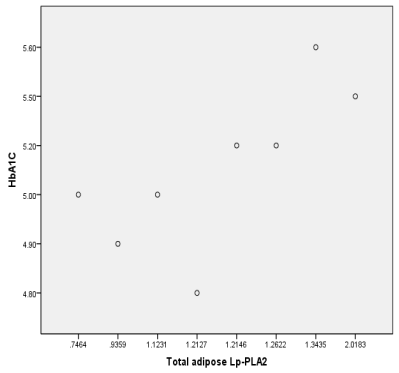


r = 0.572
p= 0.236

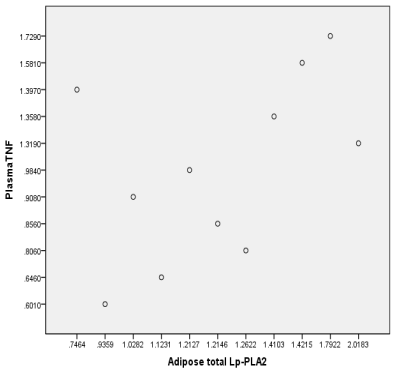


r = 0.306
p= 0.556

Figure 5.



r=0.674
p=0.067



r=0.529
p=0.094

Figure 6.

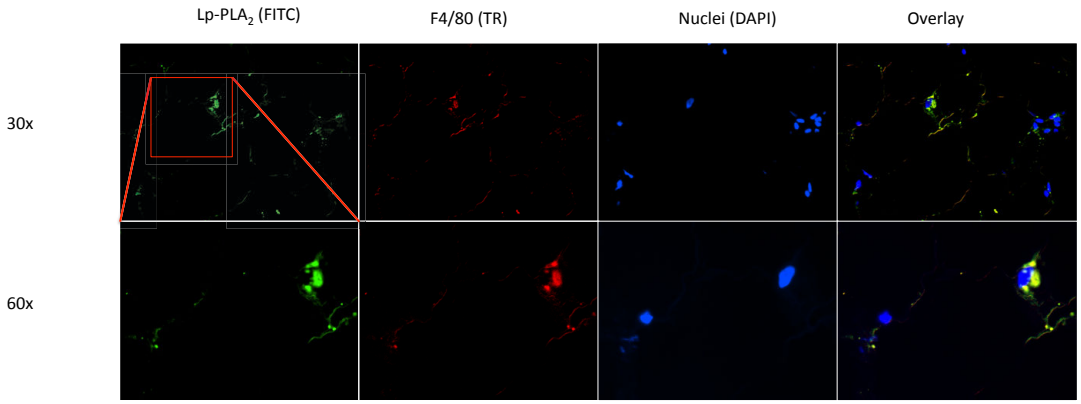
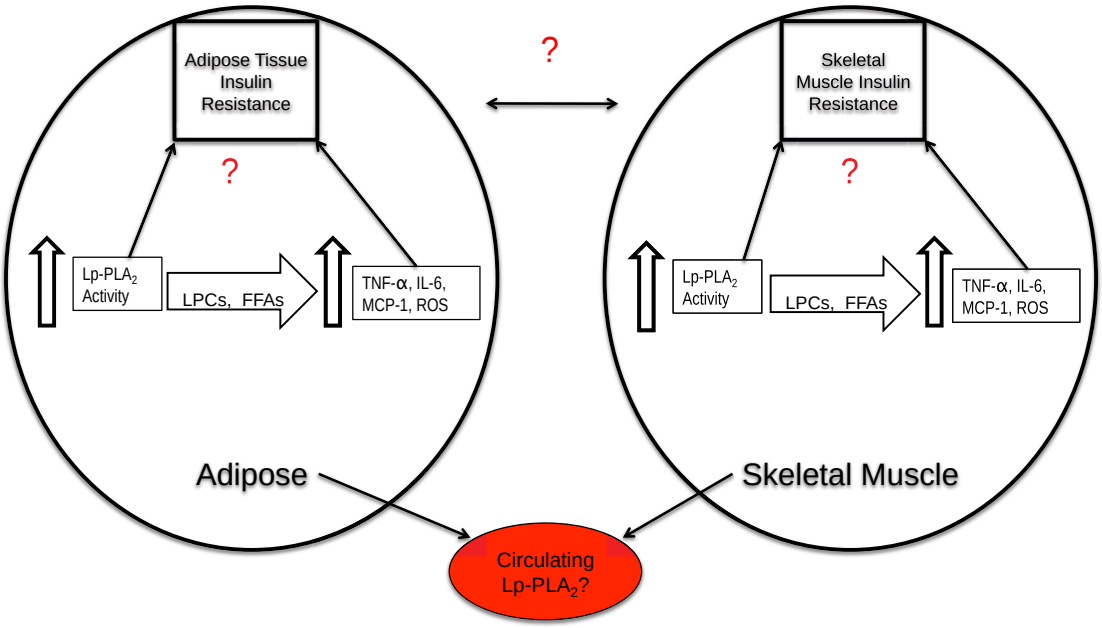


Figure 7.


Adipose Tissue Insulin Resistance
?
Lp-PLA2 Activity
LPCs, FFAs
TNF-α, IL-6, MCP-1, ROS
Adipose
?
Skeletal Muscle Insulin Resistance
?
Lp-PLA2 Activity
LPCs, FFAs
TNF-α, IL-6, MCP-1, ROS
Skeletal Muscle
Circulating Lp-PLA2?

References

1. Tselepis, A.D., et al., N-linked glycosylation of macrophage-derived PAF-AH is a major determinant of enzyme association with plasma HDL. J Lipid Res, 2001. 42(10): p. 1645-54.

2. Rosenson, R.S., M. Vracar-Grabar, and I. Helenowski, Lipoprotein associated phospholipase A2 inhibition reduces generation of oxidized fatty acids: Lp-LPA2 reduces oxidized fatty acids. Cardiovasc Drugs Ther, 2008. 22(1): p. 55-8.

3. Steinbrecher, U.P. and P.H. Pritchard, Hydrolysis of phosphatidylcholine during LDL oxidation is mediated by platelet-activating factor acetylhydrolase. J Lipid Res, 1989. 30(3): p. 305-15.

4. Tellis, C.C. and A.D. Tselepis, The role of lipoprotein-associated phospholipase A2 in atherosclerosis may depend on its lipoprotein carrier in plasma. Biochim Biophys Acta, 2009. 1791(5): p. 327-38.

5. Schmitz, G. and K. Ruebsaamen, Metabolism and atherogenic disease association of lysophosphatidylcholine. Atherosclerosis. 208(1): p. 10-8.

6. Garza, C.A., et al., Association between lipoprotein-associated phospholipase A2 and cardiovascular disease: a systematic review. Mayo Clin Proc, 2007. 82(2): p. 159-65.

7. Theilmeier, G., et al., HDL-associated PAF-AH reduces endothelial adhesiveness in apoE-/- mice. FASEB J, 2000. 14(13): p. 2032-9.

8. Quarck, R., et al., Adenovirus-mediated gene transfer of human platelet-activating factor-acetylhydrolase prevents injury-induced neointima formation and reduces spontaneous atherosclerosis in apolipoprotein E-deficient mice. Circulation, 2001. 103(20): p. 2495-500.

9. Kolodgie, F.D., et al., Lipoprotein-associated phospholipase A2 protein expression in the natural progression of human coronary atherosclerosis. Arterioscler Thromb Vasc Biol, 2006. 26(11): p. 2523-9.

10. Serruys, P.W., et al., Effects of the direct lipoprotein-associated phospholipase A(2) inhibitor darapladib on human coronary atherosclerotic plaque. Circulation, 2008. 118(11): p. 1172-82.

11. Mohler, E.R., 3rd, et al., The effect of darapladib on plasma lipoprotein-associated phospholipase A2 activity and cardiovascular biomarkers in patients with stable coronary heart disease or coronary heart disease risk equivalent: the

results of a multicenter, randomized, double-blind, placebo-controlled study. J Am Coll Cardiol, 2008. 51(17): p. 1632-41.

12. Persson, M., et al., Elevated Lp-PLA2 levels add prognostic information to the metabolic syndrome on incidence of cardiovascular events among middle-aged nondiabetic subjects. Arterioscler Thromb Vasc Biol, 2007. 27(6): p. 1411-6.

13. Rizos, E., et al., Lipoprotein-associated PAF-acetylhydrolase activity in subjects with the metabolic syndrome. Prostaglandins Leukot Essent Fatty Acids, 2005. 72(3): p. 203-9.

14. Persson, M., et al., The epidemiology of Lp-PLA(2): distribution and correlation with cardiovascular risk factors in a population-based cohort. Atherosclerosis, 2007. 190(2): p. 388-96.

15. Pou, K.M., et al., Visceral and subcutaneous adipose tissue volumes are cross-sectionally related to markers of inflammation and oxidative stress: the Framingham Heart Study. Circulation, 2007. 116(11): p. 1234-41.

16. Detopoulou, P., et al., Lipoprotein-associated phospholipase A2 (Lp-PLA2) activity, platelet-activating factor acetylhydrolase (PAF-AH) in leukocytes and body composition in healthy adults. Lipids Health Dis, 2009. 8: p. 19.

17. Nelson, T.L., et al., Lipoprotein-associated phospholipase A(2) and future risk of subclinical disease and cardiovascular events in individuals with type 2 diabetes: the Cardiovascular Health Study. Diabetologia. 54(2): p. 329-33.

18. Karpe, F. and G.D. Tan, Adipose tissue function in the insulin-resistance syndrome. Biochem Soc Trans, 2005. 33(Pt 5): p. 1045-8.

19. Ruan, H. and H.F. Lodish, Insulin resistance in adipose tissue: direct and indirect effects of tumor necrosis factor-alpha. Cytokine Growth Factor Rev, 2003. 14(5): p. 447-55.

20. Ruan, H., et al., Profiling gene transcription in vivo reveals adipose tissue as an immediate target of tumor necrosis factor-alpha: implications for insulin resistance. Diabetes, 2002. 51(11): p. 3176-88.

21. Kennedy, A., et al., Saturated fatty acid-mediated inflammation and insulin resistance in adipose tissue: mechanisms of action and implications. J Nutr, 2009. 139(1): p. 1-4.

22. Han, M.S., et al., Lysophosphatidylcholine as an effector of fatty acid-induced insulin resistance. J Lipid Res. 52(6): p. 1234-46.

23. Weisberg, S.P., et al., Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest, 2003. 112(12): p. 1796-808.

24. Cinti, S., et al., Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res, 2005. 46(11): p. 2347-55.

25. Gountopoulou, A., et al., TNFalpha is a potent inducer of platelet-activating factor synthesis in adipocytes but not in preadipocytes. Differential regulation by PI3K. Cytokine, 2008. 41(2): p. 174-81.

26. Svetlov, S.I., et al., Secretory PAF-acetylhydrolase of the rat hepatobiliary system: characterization and partial purification. Am J Physiol, 1998. 274(5 Pt 1): p. G891-900.

27. Howard, K.M., Differential expression of platelet-activating factor acetylhydrolase in lung macrophages. Am J Physiol Lung Cell Mol Physiol, 2009. 297(6): p. L1141-50.

28. Harford, K.A., et al., Fats, inflammation and insulin resistance: insights to the role of macrophage and T-cell accumulation in adipose tissue. Proc Nutr Soc: p. 1-10.

29. Borst, S.E., et al., Neutralization of tumor necrosis factor-alpha reverses insulin resistance in skeletal muscle but not adipose tissue. Am J Physiol Endocrinol Metab, 2004. 287(5): p. E934-8.

30. Kraegen, E.W. and G.J. Cooney, Free fatty acids and skeletal muscle insulin resistance. Curr Opin Lipidol, 2008. 19(3): p. 235-41.

31. Kuniyasu, A., et al., Oxidized LDL and lysophosphatidylcholine stimulate plasminogen activator inhibitor-1 expression through reactive oxygen species generation and ERK1/2 activation in 3T3-L1 adipocytes. Biochim Biophys Acta. 1811(3): p. 153-62.

32. Lumeng, C.N., J.L. Bodzin, and A.R. Saltiel, Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest, 2007. 117(1): p. 175-84.

CHAPTER V- CONCLUSIONS

Conclusions

Because obesity is a well established independent risk factor for diabetes and coronary artery disease (CAD), it is important to identify factors associated with obesity that are responsible for disease progression, and interventions to decrease risk of developing obesity associated co-morbidities. In order to meaningfully decrease diabetes and CAD incidence, we must increase our understanding of the mechanisms responsible for the disease sequelae. Our analyses focused on obesity associated oxidative and lipid stress, and how these factors contribute to the pathophysiology of diabetes and CAD.

We first performed a lipidomic analysis of plasma from lean and obese humans to determine which lipid species are elevated in obese subjects that may contribute to vascular pathology. A group of ether-linked lipids was identified in the obese subjects, and a representative lipid from the identified group induced endothelial cell expression of cell adhesion molecules. In addition, we screened our data for oxidized phospholipids that are known to induce endothelial cell activation and did not find major differences between lean and obese subjects. Ether-linked lipids such as those identified as greater in our obese subjects may contribute to obesity associated vascular pathology, and warrant further investigation.

We also sought to characterize Lp-PLA_2 in human adipose tissue. Lp-PLA_2 hydrolyzes platelet-activating factor and oxidized phospholipids, is a risk factor for CAD and is predictive of diabetes. We found Lp-PLA_2 in human adipose in both unmodified and glycosylated forms, predominantly co-localized with macrophages. In addition, HbA1C in obese subjects was negatively associated with total adipose Lp-PAL_2 but positively with glycosylated Lp-PLA_2. These descriptive data support further investigation of the role in adipose tissue insulin resistance.

Last, we examined whether the phytochemicals in Protandim could induce Nrf2 and antioxidant enzymes in HCAEC, and protect HCAEC from an oxidative challenge. Protandim treatment induced Nrf2 nuclear localization and increased expression of HO-1, SOD1, Grx, and NQO1 in HCAEC. Protandim treatment also reduced HCAEC apoptosis in response to an oxidative challenge, and protection was significantly decreased when Nrf2 was silenced prior to Protandim treatment. These data suggest that Protandim may be an effective intervention against oxidative stress and associated vascular pathology. These results have important implications for CAD disease intervention, as exogenous antioxidant treatments have been largely ineffective.

Understanding the milieu of changes in lipids and oxidative stress is important to understanding disease pathology and designing effective interventions to treat co-morbidities associated with obesity. Collectively, our data provide insight into dyslipidemia in obesity, and the connection between dyslipidemia in obesity and CAD. In addition, our data suggest that investigation of the role of Lp-PLA_2 is warranted.

Finally, we provide insight into the mechanism and efficacy of a potential intervention against oxidative stress in vascular disease. Future investigations should further consider the role of ether-linked lipids in vascular pathology and the role of Lp-PLA_2 in obesity and diabetes. As our understanding of chronic metabolic disease characteristics and mechanisms improves, interventions will become targeted, and therefore more effective, which has significant implications for public health status and recommendations.

References

1. Ninio, E., *Phospholipid mediators in the vessel wall: involvement in atherosclerosis.* Curr Opin Clin Nutr Metab Care, 2005. **8**(2): p. 123-31.
2. Center for Disease Control and Prevention *Facts and Statistics*. 2009; Available from: http://www.cdc.gov/heartDisease/statistics.htm.
3. Poirier, P., et al., *Cardiovascular evaluation and management of severely obese patients undergoing surgery: a science advisory from the American Heart Association.* Circulation, 2009. **120**(1): p. 86-95.
4. Skilton, M.R., et al., *The effects of obesity and non-pharmacological weight loss on vascular and ventricular function and structure.* Diabetes Obes Metab, 2008. **10**(10): p. 874-84.
5. Gao, L. and G.E. Mann, *Vascular NAD(P)H oxidase activation in diabetes: a double-edged sword in redox signalling.* Cardiovasc Res, 2009. **82**(1): p. 9-20.
6. Guzik, T.J., et al., *Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase.* Circulation, 2002. **105**(14): p. 1656-62.
7. He, M., et al., *Induction of HO-1 and redox signaling in endothelial cells by advanced glycation end products: a role for Nrf2 in vascular protection in diabetes.* Nutr Metab Cardiovasc Dis. **21**(4): p. 277-85.
8. Ashraf, M.Z., N.S. Kar, and E.A. Podrez, *Oxidized phospholipids: biomarker for cardiovascular diseases.* Int J Biochem Cell Biol, 2009. **41**(6): p. 1241-4.
9. Berliner, J.A., N. Leitinger, and S. Tsimikas, *The role of oxidized phospholipids in atherosclerosis.* J Lipid Res, 2009. **50 Suppl**: p. S207-12.
10. Berliner, J.A. and A.D. Watson, *A role for oxidized phospholipids in atherosclerosis.* N Engl J Med, 2005. **353**(1): p. 9-11.
11. Lee, S., et al., *A role for NADPH oxidase 4 in the activation of vascular endothelial cells by oxidized phospholipids.* Free Radic Biol Med, 2009. **47**(2): p. 145-51.
12. Leitinger, N., *Oxidized phospholipids as triggers of inflammation in atherosclerosis.* Mol Nutr Food Res, 2005. **49**(11): p. 1063-71.
13. Rouhanizadeh, M., et al., *Oxidized-1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine induces vascular endothelial superoxide production: implication of NADPH oxidase.* Free Radic Biol Med, 2005. **39**(11): p. 1512-22.
14. Silver, A.E., et al., *Overweight and obese humans demonstrate increased vascular endothelial NAD(P)H oxidase-p47(phox) expression and evidence of endothelial oxidative stress.* Circulation, 2007. **115**(5): p. 627-37.
15. Subbanagounder, G., et al., *Determinants of bioactivity of oxidized phospholipids. Specific oxidized fatty acyl groups at the sn-2 position.* Arterioscler Thromb Vasc Biol, 2000. **20**(10): p. 2248-54.

16. Subbanagounder, G., et al., *Epoxyisoprostane and epoxycyclopentenone phospholipids regulate monocyte chemotactic protein-1 and interleukin-8 synthesis. Formation of these oxidized phospholipids in response to interleukin-1beta.* J Biol Chem, 2002. **277**(9): p. 7271-81.
17. Watson, A.D., et al., *Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo.* J Biol Chem, 1997. **272**(21): p. 13597-607.
18. Bochkov, V.N., *Inflammatory profile of oxidized phospholipids.* Thromb Haemost, 2007. **97**(3): p. 348-54.
19. Bochkov, V.N., et al., *Generation and biological activities of oxidized phospholipids.* Antioxid Redox Signal. **12**(8): p. 1009-59.
20. Niki, E., *Lipid peroxidation: physiological levels and dual biological effects.* Free Radic Biol Med, 2009. **47**(5): p. 469-84.
21. Catala, A., *Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions.* Chem Phys Lipids, 2009. **157**(1): p. 1-11.
22. Kuhn, H. and V.B. O'Donnell, *Inflammation and immune regulation by 12/15-lipoxygenases.* Prog Lipid Res, 2006. **45**(4): p. 334-56.
23. Wen, Y., et al., *Role of 12/15-lipoxygenase in the expression of MCP-1 in mouse macrophages.* Am J Physiol Heart Circ Physiol, 2008. **294**(4): p. H1933-8.
24. Back, M., et al., *5-Lipoxygenase-activating protein: a potential link between innate and adaptive immunity in atherosclerosis and adipose tissue inflammation.* Circ Res, 2007. **100**(7): p. 946-9.
25. Fruhwirth, G.O. and A. Hermetter, *Mediation of apoptosis by oxidized phospholipids.* Subcell Biochem, 2008. **49**: p. 351-67.
26. Kume, N., M.I. Cybulsky, and M.A. Gimbrone, Jr., *Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells.* J Clin Invest, 1992. **90**(3): p. 1138-44.
27. Leitinger, N., et al., *Structurally similar oxidized phospholipids differentially regulate endothelial binding of monocytes and neutrophils.* Proc Natl Acad Sci U S A, 1999. **96**(21): p. 12010-5.
28. Berliner, J.A. and N.M. Gharavi, *Endothelial cell regulation by phospholipid oxidation products.* Free Radic Biol Med, 2008. **45**(2): p. 119-23.
29. Fu, P. and K.G. Birukov, *Oxidized phospholipids in control of inflammation and endothelial barrier.* Transl Res, 2009. **153**(4): p. 166-76.
30. Gharavi, N.M., et al., *High-density lipoprotein modulates oxidized phospholipid signaling in human endothelial cells from proinflammatory to anti-inflammatory.* Arterioscler Thromb Vasc Biol, 2007. **27**(6): p. 1346-53.
31. Funk, C.D. and T. Cyrus, *12/15-lipoxygenase, oxidative modification of LDL and atherogenesis.* Trends Cardiovasc Med, 2001. **11**(3-4): p. 116-24.
32. Bolick, D.T., et al., *12/15-lipoxygenase regulates intercellular adhesion molecule-1 expression and monocyte adhesion to endothelium through activation of RhoA and nuclear factor-kappaB.* Arterioscler Thromb Vasc Biol, 2005. **25**(11): p. 2301-7.
33. Bolick, D.T., et al., *12/15 lipoxygenase mediates monocyte adhesion to aortic endothelium in apolipoprotein E-deficient mice through activation of RhoA and NF-kappaB.* Arterioscler Thromb Vasc Biol, 2006. **26**(6): p. 1260-6.

34. Spanbroek, R., et al., *Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis.* Proc Natl Acad Sci U S A, 2003. **100**(3): p. 1238-43.
35. Montrucchio, G., G. Alloatti, and G. Camussi, *Role of platelet-activating factor in cardiovascular pathophysiology.* Physiol Rev, 2000. **80**(4): p. 1669-99.
36. McHowat, J., et al., *Endothelial cell PAF synthesis following thrombin stimulation utilizes Ca(2+)-independent phospholipase A(2).* Biochemistry, 2001. **40**(49): p. 14921-31.
37. Howard, K.M., et al., *Lipopolysaccharide and platelet-activating factor stimulate expression of platelet-activating factor acetylhydrolase via distinct signaling pathways.* Inflamm Res. **60**(8): p. 735-44.
38. Stafforini, D.M., *Biology of platelet-activating factor acetylhydrolase (PAF-AH, lipoprotein associated phospholipase A2).* Cardiovasc Drugs Ther, 2009. **23**(1): p. 73-83.
39. McIntyre, T.M., S.M. Prescott, and D.M. Stafforini, *The emerging roles of PAF acetylhydrolase.* J Lipid Res, 2009. **50 Suppl**: p. S255-9.
40. Tellis, C.C. and A.D. Tselepis, *The role of lipoprotein-associated phospholipase A2 in atherosclerosis may depend on its lipoprotein carrier in plasma.* Biochim Biophys Acta, 2009. **1791**(5): p. 327-38.
41. Tselepis, A.D., et al., *N-linked glycosylation of macrophage-derived PAF-AH is a major determinant of enzyme association with plasma HDL.* J Lipid Res, 2001. **42**(10): p. 1645-54.
42. Rosenson, R.S., M. Vracar-Grabar, and I. Helenowski, *Lipoprotein associated phospholipase A2 inhibition reduces generation of oxidized fatty acids: Lp-LPA2 reduces oxidized fatty acids.* Cardiovasc Drugs Ther, 2008. **22**(1): p. 55-8.
43. Steinbrecher, U.P. and P.H. Pritchard, *Hydrolysis of phosphatidylcholine during LDL oxidation is mediated by platelet-activating factor acetylhydrolase.* J Lipid Res, 1989. **30**(3): p. 305-15.
44. Schmitz, G. and K. Ruebsaamen, *Metabolism and atherogenic disease association of lysophosphatidylcholine.* Atherosclerosis. **208**(1): p. 10-8.
45. Kuniyasu, A., et al., *Oxidized LDL and lysophosphatidylcholine stimulate plasminogen activator inhibitor-1 expression through reactive oxygen species generation and ERK1/2 activation in 3T3-L1 adipocytes.* Biochim Biophys Acta. **1811**(3): p. 153-62.
46. Lee, H., et al., *Lysophospholipids increase ICAM-1 expression in HUVEC through a Gi- and NF-kappaB-dependent mechanism.* Am J Physiol Cell Physiol, 2004. **287**(6): p. C1657-66.
47. Serruys, P.W., et al., *Effects of the direct lipoprotein-associated phospholipase A(2) inhibitor darapladib on human coronary atherosclerotic plaque.* Circulation, 2008. **118**(11): p. 1172-82.
48. Mohler, E.R., 3rd, et al., *The effect of darapladib on plasma lipoprotein-associated phospholipase A2 activity and cardiovascular biomarkers in patients with stable coronary heart disease or coronary heart disease risk equivalent: the results of a multicenter, randomized, double-blind, placebo-controlled study.* J Am Coll Cardiol, 2008. **51**(17): p. 1632-41.
49. Kinney, G.L., et al., *Lipoprotein-associated phospholipase A activity predicts progression of subclinical coronary atherosclerosis.* Diabetes Technol Ther. **13**(3): p. 381-7.
50. Herrmann, J., et al., *Expression of lipoprotein-associated phospholipase A(2) in carotid artery plaques predicts long-term cardiac outcome.* Eur Heart J, 2009. **30**(23): p. 2930-8.
51. Wilensky, R.L., et al., *Inhibition of lipoprotein-associated phospholipase A2 reduces complex coronary atherosclerotic plaque development.* Nat Med, 2008. **14**(10): p. 1059-66.

52. Kolodgie, F.D., et al., *Lipoprotein-associated phospholipase A2 protein expression in the natural progression of human coronary atherosclerosis.* Arterioscler Thromb Vasc Biol, 2006. **26**(11): p. 2523-9.
53. Quarck, R., et al., *Adenovirus-mediated gene transfer of human platelet-activating factor-acetylhydrolase prevents injury-induced neointima formation and reduces spontaneous atherosclerosis in apolipoprotein E-deficient mice.* Circulation, 2001. **103**(20): p. 2495-500.
54. Theilmeier, G., et al., *HDL-associated PAF-AH reduces endothelial adhesiveness in apoE-/- mice.* FASEB J, 2000. **14**(13): p. 2032-9.
55. Brilakis, E.S., et al., *Influence of race and sex on lipoprotein-associated phospholipase A2 levels: observations from the Dallas Heart Study.* Atherosclerosis, 2008. **199**(1): p. 110-5.
56. Hatoum, I.J., et al., *Dietary, lifestyle, and clinical predictors of lipoprotein-associated phospholipase A2 activity in individuals without coronary artery disease.* Am J Clin Nutr. **91**(3): p. 786-93.
57. Han, M.S., et al., *Lysophosphatidylcholine as an effector of fatty acid-induced insulin resistance.* J Lipid Res. **52**(6): p. 1234-46.
58. Gountopoulou, A., et al., *TNFalpha is a potent inducer of platelet-activating factor synthesis in adipocytes but not in preadipocytes. Differential regulation by PI3K.* Cytokine, 2008. **41**(2): p. 174-81.
59. Valko, M., et al., *Free radicals and antioxidants in normal physiological functions and human disease.* Int J Biochem Cell Biol, 2007. **39**(1): p. 44-84.
60. Strobel, N.A., et al., *Oxidative stress biomarkers as predictors of cardiovascular disease.* Int J Cardiol. **147**(2): p. 191-201.
61. Fernandez-Sanchez, A., et al., *Inflammation, oxidative stress, and obesity.* Int J Mol Sci. **12**(5): p. 3117-32.
62. Furukawa, S., et al., *Increased oxidative stress in obesity and its impact on metabolic syndrome.* J Clin Invest, 2004. **114**(12): p. 1752-61.
63. Joao Cabrera, E., et al., *Reduction in plasma levels of inflammatory and oxidative stress indicators after Roux-en-Y gastric bypass.* Obes Surg. **20**(1): p. 42-9.
64. Sugamura, K. and J.F. Keaney, Jr., *Reactive oxygen species in cardiovascular disease.* Free Radic Biol Med. **51**(5): p. 978-92.
65. Brandes, R.P. and K. Schroder, *Differential vascular functions of Nox family NADPH oxidases.* Curr Opin Lipidol, 2008. **19**(5): p. 513-8.
66. Serpillon, S., et al., *Superoxide production by NAD(P)H oxidase and mitochondria is increased in genetically obese and hyperglycemic rat heart and aorta before the development of cardiac dysfunction. The role of glucose-6-phosphate dehydrogenase-derived NADPH.* Am J Physiol Heart Circ Physiol, 2009. **297**(1): p. H153-62.
67. Hathaway, C.A., et al., *Regression of atherosclerosis in monkeys reduces vascular superoxide levels.* Circ Res, 2002. **90**(3): p. 277-83.
68. Lee, S., et al., *Ox-PAPC activation of PMET system increases expression of heme oxygenase-1 in human aortic endothelial cell.* J Lipid Res, 2009. **50**(2): p. 265-74.
69. Barry-Lane, P.A., et al., *p47phox is required for atherosclerotic lesion progression in ApoE(-/-) mice.* J Clin Invest, 2001. **108**(10): p. 1513-22.
70. Vendrov, A.E., et al., *Atherosclerosis is attenuated by limiting superoxide generation in both macrophages and vessel wall cells.* Arterioscler Thromb Vasc Biol, 2007. **27**(12): p. 2714-21.

71. Yun, M.R., et al., *5-Lipoxygenase plays an essential role in 4-HNE-enhanced ROS production in murine macrophages via activation of NADPH oxidase.* Free Radic Res. **44**(7): p. 742-50.
72. Medhora, M., et al., *20-HETE increases superoxide production and activates NAPDH oxidase in pulmonary artery endothelial cells.* Am J Physiol Lung Cell Mol Physiol, 2008. **294**(5): p. L902-11.
73. Guzik, T.J., et al., *Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors.* Circ Res, 2000. **86**(9): p. E85-90.
74. Sorescu, D., et al., *Superoxide production and expression of nox family proteins in human atherosclerosis.* Circulation, 2002. **105**(12): p. 1429-35.
75. Landmesser, U., et al., *Vascular extracellular superoxide dismutase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation.* Circulation, 2000. **101**(19): p. 2264-70.
76. Roberts, C.K., et al., *Oxidative stress and dysregulation of NAD(P)H oxidase and antioxidant enzymes in diet-induced metabolic syndrome.* Metabolism, 2006. **55**(7): p. 928-34.
77. Melissas, J., et al., *Plasma antioxidant capacity in morbidly obese patients before and after weight loss.* Obes Surg, 2006. **16**(3): p. 314-20.
78. Pories, W.J., *Bariatric surgery: risks and rewards.* J Clin Endocrinol Metab, 2008. **93**(11 Suppl 1): p. S89-96.
79. Arterburn, D., et al., *Change in predicted 10-year cardiovascular risk following laparoscopic Roux-en-Y gastric bypass surgery.* Obes Surg, 2009. **19**(2): p. 184-9.
80. Clarke, R. and J. Armitage, *Antioxidant vitamins and risk of cardiovascular disease. Review of large-scale randomised trials.* Cardiovasc Drugs Ther, 2002. **16**(5): p. 411-5.
81. Bjelakovic, G., et al., *Mortality in Randomized Trials of Antioxidant Supplements for Primary and Secondary Prevention.* JAMA: The Journal of the American Medical Association, 2007. **297**(8): p. 842-857.
82. Bleys, J., et al., *Vitamin-mineral supplementation and the progression of atherosclerosis: a meta-analysis of randomized controlled trials.* Am J Clin Nutr, 2006. **84**(4): p. 880-7; quiz 954-5.
83. Vivekananthan, D.P., et al., *Use of antioxidant vitamins for the prevention of cardiovascular disease: meta-analysis of randomised trials.* Lancet, 2003. **361**(9374): p. 2017-23.
84. Podmore, I.D., et al., *Vitamin C exhibits pro-oxidant properties.* Nature, 1998. **392**(6676): p. 559-559.
85. Giudice, A., C. Arra, and M.C. Turco, *Review of molecular mechanisms involved in the activation of the Nrf2-ARE signaling pathway by chemopreventive agents.* Methods Mol Biol. **647**: p. 37-74.
86. Lyakhovich, V.V., et al., *Active defense under oxidative stress. The antioxidant responsive element.* Biochemistry (Mosc), 2006. **71**(9): p. 962-74.
87. Itoh, K., et al., *Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain.* Genes Dev, 1999. **13**(1): p. 76-86.
88. Apopa, P.L., X. He, and Q. Ma, *Phosphorylation of Nrf2 in the transcription activation domain by casein kinase 2 (CK2) is critical for the nuclear translocation and transcription activation function of Nrf2 in IMR-32 neuroblastoma cells.* J Biochem Mol Toxicol, 2008. **22**(1): p. 63-76.

89. Huang, H.C., T. Nguyen, and C.B. Pickett, *Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription.* J Biol Chem, 2002. **277**(45): p. 42769-74.
90. Cullinan, S.B., et al., *Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival.* Mol Cell Biol, 2003. **23**(20): p. 7198-209.
91. Sun, Z., Z. Huang, and D.D. Zhang, *Phosphorylation of Nrf2 at multiple sites by MAP kinases has a limited contribution in modulating the Nrf2-dependent antioxidant response.* PLoS One, 2009. **4**(8): p. e6588.
92. Velmurugan, K., et al., *Synergistic induction of heme oxygenase-1 by the components of the antioxidant supplement Protandim.* Free Radic Biol Med, 2009. **46**(3): p. 430-40.
93. Surh, Y.-J., J.K. Kundu, and H.-K. Na, *Nrf2 as a Master Redox Switch in Turning on the Cellular Signaling Involved in the Induction of Cytoprotective Genes by Some Chemopreventive Phytochemicals.* Planta Med, 2008. **74**(EFirst): p. 1526,1539.
94. Nelson, S.K., et al., *The induction of human superoxide dismutase and catalase in vivo: a fundamentally new approach to antioxidant therapy.* Free Radic Biol Med, 2006. **40**(2): p. 341-7.
95. Joddar, B., et al., *Protandim attenuates intimal hyperplasia in human saphenous veins cultured ex vivo via a catalase-dependent pathway.* Free Radic Biol Med. **50**(6): p. 700-9.

Consent to Participate in a Research Study At Colorado State University

TITLE OF STUDY: Oxidized Phospholipids, Weight Loss, and Metabolic Function

PRINCIPAL INVESTIGATOR:

Dr. Benjamin F. Miller PhD, Assistant Professor, Department of Health and Exercise Science, Colorado State University. bfmiller@cahs.colostate.edu

CO-PRINCIPAL INVESTIGATORS:

Dr. Karyn Hamilton PhD, Assistant Professor, Department of Health and Exercise Science, Colorado State University. Karyn.Hamilton@colostate.edu

Dr. Matthew Hickey PhD, Associate Professor, Department of Health and Exercise Science, Colorado State University. Matthew.Hickey@colostate.edu

Dr. Christopher Bell PhD, Assistant Professor, Department of Health and Exercise Science, Colorado State University. Christopher.Bell@colostate.edu

Dr. Christopher Gentile, Post-doctoral Fellow, Department of Food Science and Human Nutrition, Colorado State University. CGentile@CAHS.colostate.edu

Dr. Stephan Pettine MD, Bariatric Center for the Rockies

WHY AM I BEING INVITED TO TAKE PART IN THIS RESEARCH? If you are a surgical patient of Dr. Stefan Pettine, Dr. Quaid, or Dr. Roller we are interested in you taking part in this study. WHO IS DOING THE STUDY? Dr's. Miller, Hamilton, Hickey, and Bell are professors in the Department of Health and Exercise Science at CSU, and along with Chris Gentile, a Post-doctoral Fellow and Ms. Donovan (a doctoral student in the department), are interested in the positive changes that occur in fat metabolism and insulin sensitivity following gastric bypass surgery.

WHAT IS THE PURPOSE OF THIS STUDY? **Following gastric bypass surgery, positive changes in the body's ability to regulate blood sugar and process fats occur independently of weight loss. We are interested in studying these changes and using what we learn to assist in treatment of other patients. To understand the differences and changes in gastric patients, it is necessary to compare their measurements to a group of patients not at the same level of obesity, and not undergoing a bypass but still experiencing an operation.**

WHERE IS THE STUDY GOING TO TAKE PLACE AND HOW LONG WILL IT LAST?

The study will take place at the Bariatric Center of the Rockies, and Colorado State University. All testing will coincide as closely as possible with regularly scheduled pre-operative and follow-up appointments with your physician through the one year post-operative appointment. If we are only collecting a blood sample we will do this at the doctor's office. For bypass patients, pre-operatively, and three times post-operatively we will be performing a clamp procedure (described below), and on these days, blood draws will be done concurrently with the clamp at the Colorado State University Human Performance and Clinical Research Laboratory.

WHAT WILL I BE ASKED TO DO?

If you are a bypass patient:

-We will ask you questions about your past and current health status, as well as personal information including birth date, current state of health, and any medications you are taking. In addition, we will ask you to keep a 3 day food diary before the operation.

- Consent to a liver biopsy (removal of a small sample of liver tissue), abdominal muscle biopsy, and an abdominal fat biopsy being taken during the operation. Details of the biopsies will be provided by the surgeon

-Provide venous blood samples at your pre-operative appointment, and your 5 follow-up appointments (this will take approximately 20 minutes per visit).

-Undergo a clamp to measure your insulin (hormone involved in blood sugar regulation) sensitivity at the CSU HPCRL the same day as your pre-operative appointment, and 6 week, 6, and 12 month follow-up appointments.

The "clamp": This is the name commonly given to the procedure formally known as the hyperinsulinemic euglycemic clamp. This procedure measures the ability of your body to handle sugar. We will inject sugar (glucose) into one of your veins and insulin (a naturally occurring substance produced by your body) into a different vein. We will continue to inject insulin and sugar into your veins to try to keep the concentration of sugar in your blood the same. We will measure this concentration every 5-minutes. This test lasts approximately 3 hours and we will ask you to perform it four times. The test will be administered by the research team at CSU.

If you are a patient scheduled for a surgery other than gastric bypass:

-We will ask you questions about your past and current health status, as well as personal information including birth date, current state of health, and any medications you are taken.

- Consent to a blood sample, liver biopsy (removal of a small sample of liver tissue), abdominal muscle biopsy, and an abdominal fat biopsy being taken during the operation. Details of the biopsies will be provided by the surgeon

ARE THERE REASONS WHY I SHOULD NOT TAKE PART IN THIS STUDY?
If you are over age 50 or under age 18 you will be excluded from this study, and if you have uncontrolled diabetes.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?

Surgical Risks

- There are slight risks associated with tissue sampling during surgery including bleeding, and infection. These risks do not increase the risk associated with the operation were you not participating in the study. They can be addressed further by the operating physician if you have additional questions or concerns.

Research Risks

- Venous blood sampling, can cause pain, fainting, could result in bruising, and poses a slight risk of infection. General risks will be minimized by using sterile technique, and applying direct pressure to the venous sites for five minutes following removal of sampling needle. The risks associated with fainting will be minimized by conducting all blood sampling procedures with the subject in the supine position.
- The clamp procedure involves placement of a catheter (hollow plastic needle) inside a vein thus the usual risks of blood collection apply (minor discomfort, bruising, fainting and blood clot (rare)). In addition there is a risk of hypoglycemia (low blood sugar); symptoms include hunger, nervousness and shakiness, perspiration, dizziness or light-headedness, sleepiness, confusion, difficulty speaking, and feeling anxious or weak. Although hypoglycemia can happen suddenly it can usually be treated very quickly by terminating the insulin infusion and continuing the glucose infusion, returning blood sugar concentration back to normal.
- It is not possible to identify all potential risks in research procedures, but the researcher(s) have taken reasonable safeguards to minimize any known and potential, but unknown, risk*s.*

ARE THERE ANY BENEFITS FROM TAKING PART IN THIS STUDY? You will receive information regarding your changes in weight, body composition, insulin sensitivity, and

blood lipid levels periodically throughout the study period. You will also receive a diet analysis from a nutritional expert. In addition, information generated in this study may be able assist in treatment of future patients.

DO I HAVE TO TAKE PART IN THE STUDY? Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled. No decision regarding this research will impact your medical care in any way.

WHAT WILL IT COST ME TO PARTICIPATE? It will not cost you anything to participate in this study. Any treatment or medical costs that arise as a result of your participation in this study are your responsibility.

WHO WILL SEE THE INFORMATION THAT I GIVE?
We will keep private all research records that identify you, to the extent allowed by law.Your information will be combined with information from other people taking part in the study. When we write about the study to share it with other researchers, we will write about the combined information we have gathered. You will not be identified in these written materials. We may publish the results of this study; however, we will keep you name and other identifying information private.

We will make every effort to prevent anyone who is not on the research team from knowing that you gave us information, or what that information is. For example, your name will be kept separate from your research records and these two things will be stored in different places under lock and key. You should know, however, that there are some circumstances in which we may have to show your information to other people. For example, the law may require us to show your information to a court.

CAN MY TAKING PART IN THE STUDY END EARLY?
If you have difficulty tolerating the procedures or you miss multiple appointments you may be removed at any time. Also, if you begin taking medications or develop a condition that interferes with the study outcomes you may be removed. We will explain any reason why you would be removed from the study to you should such circumstances arise.

WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY?
You will receive $60.00 per visit where the clamp procedure is performed.

WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH? The Colorado Governmental Immunity Act determines and may limit Colorado State University's legal responsibility if an injury happens because of this study. Claims against the University must be filed within 180 days of the injury.

WHAT IF I HAVE QUESTIONS?

Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind now. Later, if you have questions about the study, you can contact the PhD student, Elise Donovan at 970-491-6702. If you have any questions about your rights as a volunteer in this research, contact Janell Barker, Human Research Administrator at 970-491-1655. We will give you a copy of this consent form to take with you.

Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing ___ pages.

Please initial each line indicating you understand and consent to all portions of the study.

I have read the consent form and agree to keep a 3 day food diary Yes___
No____

I have read the consent form and agree to a liver biopsy Yes___
No____

I have read the consent form and agree to a muscle biopsy Yes___
No____

I have read the consent form and agree to a fat biopsy Yes___
No____

I have read the consent form and agree to blood sampling Yes___
No____

I have read the consent form and agree to the glucose clamp procedure Yes___
No____

(If you are a control the clamp consent is not necessary)

_____ _____

Signature of person agreeing to take part in the study Date

_____

Printed name of person agreeing to take part in the study

_____ _____

Name of person providing information to participant Date

_____

Signature of Research Staff

POUDRE VALLEY HOSPITAL

POUDRE VALLEY HEALTH SYSTEM

Fort Collins, Colorado

SURGERY OR OTHER PROCEDURE: I,_____permit Dr._____/ Assistant_____(as needed) and any other doctors or assistants to assist in performing the surgery/ procedure my doctor has recommended. If an assistant is named, they may be performing one or all of the following tasks under the supervision of my primary surgeon: Opening and closing, harvesting grafts, dissecting tissue, removing tissue, implanting devices, and altering tissues. The surgery/procedure my doctor has recommended is _____

_____

THIS SURGERY OR PROCEDURE HAS BEEN RECOMMENDED BECAUSE:_____

_____

MY OTHER TREATMENT OPTIONS INCLUDE: _____

_____

I acknowledge that I have read and understand the following risks related to anesthesia. By signing this consent, I allow the use of any anesthetics, sedatives or other medications as directed by my surgeon, anesthesiologist, or certified nurse anesthetist working under the direction of an anesthesiologist, that may be necessary. I understand that the administration of anesthesia, including sedation, carries with it certain risks above and beyond those relating to the procedure itself. These risks include but are not limited to: respiratory (breathing) problems; blood pressure problems; irregular heart beat; irritability; nausea and vomiting; prolonged drowsiness; damage to teeth and/or dental work; unsteadiness; failure to achieve adequate sedation and/or possible awareness or memory of the procedure; allergic or other unexpected and possibly severe drug reactions; nerve damage; extended hospital stay and death.

I UNDERSTAND THAT:

- Any surgery or procedure and the use of anesthesia have some risks. These risks can be serious and in rare cases result in death.
- Treatment results are not guaranteed and may not cure the condition.
- The risks listed below are the more common risks but are not all the possible risks associated with this operation or procedure.
- As is deemed necessary by the surgeon of record, medical product representatives may be present in the Operating Room solely to observe the use of that supplier's surgical and/or medical equipment.

RISKS: The most common risks are bleeding, infection, nerve injury, blood clots, heart attack, allergic reactions and pneumonia. Other risks of this particular operation or procedure include: _____

_____

_____

_____

SURGERY PATIENTS:

If during my surgery the doctor finds an unsuspected medical need, I permit him/her to provide the necessary treatment(s). My doctor has fully explained the surgical procedure in words I understand. I have read and fully understand this consent form, and all my questions have been answered. **Do not sign unless you have read and thoroughly understand this form.**

Patient/Responsible party_____Date_____Time_____

Witness_____

Doctor_____

Anesthesia Provider (if other than surgeon)_____

Patient Identification

CONSENT FOR SURGERY OR OTHER PROCEDURE

65-12826
REVISED 6-06
E-219

TRANSFUSION CONSENT

BLOOD TRANSFUSIONS

I also understand that I may need to receive blood or blood products while I am in the hospital. I understand there are several options I may consider in receiving blood. I may have:

- Blood donated by myself for my own use (self-donated blood).
- Blood donated by a friend or family for my own use (directed donors).
- Blood currently available to the hospital from other donors (banked blood).
- Other sources are: _____

_____.

I UNDERSTAND SOME OF THE RISKS OF RECEIVING BLOOD OR BLOOD PRODUCTS ARE:

- getting an infectious disease (such as hepatitis or AIDS)
- bad reactions such as: fever, hives, high blood pressure, shortness of breath, and heart or kidney problems.

I UNDERSTAND THAT:

- Results of blood transfusions are not always successful and that guarantees cannot be made that the transfusion will help me.
- Losing large amounts of blood may result in death, if blood is not replaced.
- All donors are carefully screened, and all blood is tested thoroughly and properly.
- Blood donated directly for my use by friends or family has **NOT** been shown to be safer than banked blood.
- If blood I donated to be given back to myself tests positive for hepatitis, it will be discarded to protect the health care workers in the hospital.
- If there is not enough donated blood for my use (either self or directed donations), blood from the Blood Bank will be used.
- This consent to blood tranfusion(s) is effective throughout this hospitalization.

My doctor has fully explained the possibility of blood transfusion in words I can understand. I have read and fully understand this consent form, and all of my questions have been answered. **Do not sign unless you have read and thoroughly understand this form.**

Patient/
Responsible Party_____Date_____Time_____

Witness _____

Doctor _____

I refuse to receive blood or blood products for any reason. My doctor has explained the likely complications which may occur if I need blood and do not receive it. I will accept all risks associated with my refusal to receive blood and will release my doctor and Poudre Valley Hospital from any responsibility for any bad results, including my death, which may occur because I refused to accept blood or a blood product._____(Please put your initials here if refusing blood or blood products.)

Patient Identification

CONSENT FOR SURGERY
OR OTHER PROCEDURE

POUDRE VALLEY HOSPITAL

Research Authorization Form for Use and Disclosure of Health Information

**Please type your response in the gray-box area. The box will expand to accommodate your full text.*

1. **Patient Name:**
   Date of Birth:

2. **Title of Protocol:** Oxidized Phospholipids, Weight Loss and Metabolic Function

3. **General Purpose of Research: Following gastric bypass surgery, positive changes in the body's ability to regulate blood sugar and process fats occur. We are interested in studying these changes and using what we learn to assist in treatment of other patients.**

4. **What personal health information do researchers want to use? The researchers want to copy and use the portions of your medical record that they will need for their research. Research study information used and/or released may include the following:**
   - Name, address, phone number, age, sex.
   - The history and diagnosis of your disease;
   - Specific information about the treatments you received, including previous treatment(s) you may have had;
   - Information about other medical conditions that may affect your treatment;
   - Medical data may include laboratory test results, tumor and heart measurements, x-rays, CT scans, photographs of radiation therapy target areas, and pathology results;
   - Information on side effects (adverse events) you may experience, and how these were treated;
   - Long-term information about your general health status and the status of your disease;
   - Data that may be related to tissue and/or blood samples that may be collected from you
   - Codes that will identify you, such as your social security number and medical record number.

   You may request a blank copy of data forms from the study doctor or his/her research staff to learn what information might be shared.

5. **How will my personal health information be used?**
   The hospital will use your health information for research. As part of this research, they may give your information to the following sponsor/"groups" taking part in the research. The hospital may also permit staff from these sponsor/"groups" to review your original records as required by law for audit purposes.

   RELEASE/DISCLOSE TO:
   **Please type your response in the gray-box area. The box will expand to accommodate your full text.*
   Elise Donovan, PhD candidate Dept. of Health and Exercise Scienc Colorado State University

   Name/Title: Poudre Valley Health System - Institutional Review Board (IRB) - *(A group of people who review the research to protect your rights.)*
   Phone: (970) 495-7333 IRB Manager **FAX**: (970) 495-7625

Rev. 10/22/07 KWM/lw

Address: 1024 So. Lemay Avenue **City**: Fort Collins **State**: Colorado **Zip code**: 80524

6. **How will my health information be kept private?**
The research sponsor will keep all patient information private to the extent possible, even though it is not required to follow the federal privacy laws. Only researchers working with the hospital will have access to your information. The research sponsor will not release personal health information about you to others except as authorized or required by law. However, once your information is given to other organizations that are not required to follow federal privacy laws, we cannot assure that the information will remain protected.

7. **What if I do not sign this permission form?**
If you do not sign this permission form you cannot participate in the research study for which you are being considered.

8. **If I sign this form, will I automatically be in the research study?**
No, there will be further discussion and a separate consent to sign. After discussion, you may decide to take part in the research study. At that time, you will be asked to sign a specific research consent form.

9. **What happens if I want to withdraw my authorization?**
You can change your mind at any time and withdraw your permission to allow your health information to be used in the research. If this happens, you must withdraw your authorization in writing. Beginning on the date you withdraw your permission, no new health information will be used for research. However, researchers may continue to use the health information that was provided before you withdrew your authorization.

   If you sign this form and enter the research study, but later change your mind and withdraw your authorization you will be removed from the research study at that time.

   To withdraw your permission, please contact the person below. He/she will make sure your written request to withdraw your permission is processed correctly.

   Title of Contact Person: Elise Donovan
   Address: 220 Moby-B Complex Fort Collins, CO 80523
   Phone: 970-491-6702 **FAX Number:** 970-491-0445

10. **How long will this authorization last?**
If you agree by signing this form that researchers can use your personal health information, this permission has no expiration date. However, as stated above, you can change your mind and withdraw your authorization at any time.

11. **What are my rights regarding access to my personal health information?**
You have the right to refuse to sign this permission form. You have the right to review and/or copy records of your health information kept by PVHS. You do not have the right to review and/or copy records kept by the research sponsor or other researchers associated with the research study.

(Signature Page on Next Page)

AUTHORIZATION SIGNATURE PAGE

Authorization Approval and Receipt Acknowledgement:

I hereby authorize the use or disclosure of the health information described in this authorization and acknowledges receiving a signed copy of this authorization. I understand that if anyone who receives my health information is not a health care provider or a health plan, my health information may not be protected by federal privacy laws if my health information is re-disclosed by that recipient person or PVHS.

Signature: _____**Phone**: _____
Print Name: _____
Address: _____
_____
_____

Date: _____

Authorization must be signed by the patient or by the parent/legal guardian or a minor, or by the legal representative when the patient lacks decisional capacity, or if the patient is physically unable to sign but mentally understands and consents.

Basis for legal authority to sign this authorization by a personal representative:

Parent, Guardian, Etc: _____

Witness:

Signature: _____
Print Name: _____

Date: _____

Rev. 10/22/07 KWM/lw

MEDICAL AND EXERCISE HISTORY

NAME_____ GENDER_____ DATE_____

BIRTHDATE_____ AGE_____ HEIGHT_____ WEIGHT_____

ADDRESS_____

TELEPHONE_____ EMAIL_____

1. Are you Diabetic? _____

2. Are you insulin resistant_____

   Are you hyperglycemic_____

   Are you hypoglycemic_____

3. Do you exercise?_____

4. How much time per week do you spend exercising? _____ hours/week

5. Do you or have you ever smoked? _____

   If yes: How long ago?_____ For how many years?_____ How many packs/day?_____

6. Do you have a history of heart disease?_____

   Do you have a history of high blood pressure?_____

7. Have you ever had your cholesterol measured?

   1=yes

   2=no

   If yes- write the date and value (or if it was normal or abnormal)

   _____

8. Any medical complaints now (illness, injury, limitations)?

   1 = yes If yes, describe completely_____

   2 = no _____

   _____

9. Any bleeding disorders?

   1 = yes If yes, describe completely_____

   2 = no _____

   _____

10. Any major illness in the past?

    1 = yes If yes, describe completely_____

2 = no _____

_____

_____

11. Any surgery or hospitalization in the past?

1 = yes If yes, describe completely_____

2 = no _____

_____

_____

12. Are you currently taking any medications (prescription or over-the-counter: including birth control)?

1 = yes If yes, list drugs and dosages _____

2 = no _____

_____

_____

13. Are you allergic to any medications?

1 = yes If yes, list medications_____

2 = no _____

_____

_____

14. How many drinks per week do you consume on average? _____

15. Have you or anyone else in your immediate family ever been diagnosed with liver disease?

1=yes If yes, what family member(s), and what diagnosis?_____

2=no _____

_____

_____