I, David Hall, hereby submit this original work as part of the requirements for the degree of Master of Science in Nutrition.

It is entitled:
The effects of protease-activated receptor 2 on atherosclerosis

Student's name: David Hall

This work and its defense approved by:

Committee chair: Abigail Peairs, Ph.D.

Committee member: Phillip Owens, Ph.D.

Committee member: Sarah Couch, Ph.D.
The effects of protease-activated receptor 2 on atherosclerosis

A thesis submitted to the
Graduate School
of the University of Cincinnati
in partial fulfillment of the
requirements for the degree of

Master of Science

in the Department of Nutritional Sciences
of the College of Allied Health

by

David Hall

B.A. University of Louisville

March 2016

Committee: Abigail Peairs, PhD (Chair)
A. Phillip Owens III, PhD
Sarah Couch, PhD, RD
Abstract

Background: Cardiovascular disease (CVD), including heart attack, stroke, and heart failure, is the leading cause of morbidity and mortality worldwide. Atherosclerosis is a progressive disease of the arterial wall that is initiated by lipid retention, oxidation, and modification, which provoke inflammation, stenosis, and eventual thrombosis. Atherosclerotic plaques are highly procoagulant due to the presence of tissue factor (TF), the primary initiator of the coagulation cascade. Moreover, >97% of the total thrombotic potential in atherosclerotic plaques is attributable to TF. TF, when combined with factor VIIa (FVIIa), not only triggers clotting but also activates protease-activated receptor 2 (PAR-2). While TF deletion results in embryonic lethality and TF inhibition is not a feasible therapeutic option, targeting TF-induced PAR-2 signaling decreases smooth muscle cell migration and secretion of inflammatory cytokines, both of which are crucial to atherosclerotic initiation and progression. The role of PAR-2 in atherosclerosis has not yet been elucidated.

Objective: To determine the effects of a high fat diet (HFD) on atherosclerotic plaque deposition and lipid profile of PAR-2 proficient (Par-2\textsuperscript{+/+}) verses PAR-2 deficient (Par-2\textsuperscript{-/-}) mice on a low-density lipoprotein deficient (Ldlr\textsuperscript{-/-}) background.

Methods: 8-12 week old male Ldlr\textsuperscript{-/-} mice that were PAR2\textsuperscript{+/+} (n=20) or PAR2\textsuperscript{-/-} (n=13) were fed a HFD for 12 weeks. Chimeric mice were generated in which male recipient Ldlr\textsuperscript{-/-}/Par2\textsuperscript{+/+} or Ldlr\textsuperscript{-/-}/Par2\textsuperscript{-/-} mice (8-12 weeks old) were irradiated with a total of 11 Gy (2 doses of 550 rads 4 hours apart) using a Cs\textsuperscript{137} irradiator. Irradiated mice were re-populated with bone marrow harvested from Ldlr\textsuperscript{-/-}/Par2\textsuperscript{+/+} (n = 17) or Ldlr\textsuperscript{-/-}/Par2\textsuperscript{-/-} (n = 16) donor mice via retro-orbitally injected cells to create four different chimeric groups. Body weight was measured at 8-12 weeks of age and at the time of sacrifice. Glucose tolerance and insulin sensitivity was assessed prior to high fat feeding. Upon sacrifice, plasma, heart,
aorta, liver, epididymal fat, and retroperitoneal fat was collected. The liver, epididymal and retroperitoneal fat was weighed. Plasma was assessed for triglycerides, total cholesterol, high-density lipoprotein, and low-density lipoprotein. Quantification of atherosclerotic plaque within the aortic sinus was conducted via cryo-sectioning techniques.

**Results:** Consumption of a high fat diet for 12 weeks trends towards (P<0.053) a decrease in atherosclerotic plaque development in PAR-2 deficient mice relative to PAR-2 proficient mice. Significant attenuation of weight gain (P<0.001), liver weight (P<0.001), epididymal (P<0.001) and retroperitoneal (P<0.001) fat was observed in Ldlr−/−PAR2−/− mice as well as PAR2−/−/BM-PAR2−/− mice (P<0.001, P<0.001, P<0.001, and P<0.001, respectively). Glucose tolerance and insulin sensitivity was not significantly different among Ldlr−/−/Par2+/+ and Ldlr−/−PAR2−/− mice. No significant change in plasma triglycerides, cholesterol, HDL, or LDL was observed among any mouse groups.

**Conclusion:** PAR-2 deficiency attenuated atherosclerotic burden and reduced weight gain and adipose deposition without affecting plasma lipids in a diet-induced atherogenic murine model.
Acknowledgements

I would first like to thank the Owens lab for all of their support and encouragement throughout my time spent working in their lab. I am forever indebted to Dr. A. Phillip Owens III, Adrien Mann, Shannon Jones, Nathan Robbins, Lisa McKinney, Erin Celesti, Carrie Lunsford, Christopher Lamm, Keith Saum and all of those within the Rubinstein lab including Dr. Jack Rubinstein, Dr. Sheryl Koch, and Mariah Worley. Their kindness and generosity will never be forgotten.

I would also like to give gratitude to my advisor, Dr. Abigail Peairs, whose sincerity and thoughtfulness brought ease to my graduate career. Her compassion for her students is apparent as is her willingness to assist her students in achieving their goals. I would also like to thank Dr. Sarah Couch, Dr. Seung-Yeon Lee, and Dr. Debra Krummel for sharing their knowledge and expertise in a way that made my graduate career truly rewarding and enjoyable.

Lastly, I would not have been able to find the strength to finish my program without the support of all of my family and friends. They stood by me during the best and worst times of graduate school and encouraged me throughout the process. I am truly blessed to have so many wonderful people in my life to support me throughout this journey. To you all, I say with my deepest sincerity, thank you.
# Table of Contents

**Chapter 1 Introduction**  
Literature Review 2  
Coagulation Cascade 2  
Extrinsic pathway mechanism 3  
Intrinsic pathway mechanism 3  
Common pathway mechanism 3  
Protease-activated Receptors and Atherosclerosis 4  
Protease-activated Receptor-2 6  
Inflammation 8  
Regulation 8  
Nutrition and inflammation 9  
Disease and inflammation 11  

**Chapter 2 Atherosclerosis**  
Response to injury hypothesis 13  
Oxidation of lipoproteins hypothesis 14  
Research question 15  
Hypothesis 15  
Methods 15  
Results 19  
Discussion 21  
Conclusion 23  

**Chapter 3 Metabolic observations**  
Obesity literature review 24  
Research question 26  
Hypothesis 26  
Methods 27  
Results 29  
Discussion 35  
Conclusion 37  

**Chapter 4 Conclusions**  
References 40
Tables and Figures

Table 1. Diet composition of chow diet (CD) and western diet (WD)

Table 2. Plasma lipid levels in PAR-2 proficient and deficient mice

Figure 1. The Coagulation Cascade

Figure 2. PAR-2 deficiency reduces atherosclerotic lesion size

Figure 3. PAR-2 deficiency reduces mean lesion area

Figure 4. PAR-2 deficiency results in significantly less weight gain during 12 week high fat diet

Figure 5. Global PAR-2 deficient mice show significant attenuation of weight gain when fed a high fat diet for 12 weeks

Figure 6. PAR-2 deficiency results in less epididymal and retroperitoneal fat depots

Figure 7. PAR-2 deficient mice have a significant decrease in liver weight

Figure 8. Global PAR-2 deficient mice show significant reduction in epididymal fat production when fed a high fat diet for 12 weeks.

Figure 9. Global PAR-2 deficient mice show significant reduction in retroperitoneal fat production when fed a high fat diet for 12 weeks

Figure 10. Global PAR-2 deficient mice have a significant decrease in liver weight

Figure 11. No significant differences occur between PAR-2 proficient and PAR-2 deficient mice following IPGTT

Figure 12. No significant differences occur between PAR-2 proficient and PAR-2 deficient mice following IPITT
Chapter 1

Introduction

Modern diets high in saturated fats and refined carbohydrates contribute to the nutritional overload accompanying cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), and obesity (Lim 2013). In conjunction with an increase in sedentary lifestyle seen within the Western world, more than one-third of U.S. adults (35% or 78 million) are obese and/or overweight (Ogden 2014). Obesity may increase the chance of developing cardiovascular disease (CVD) by a number of risk factors, including raising blood cholesterol and triglyceride levels, increasing the proportion of “bad” low-density lipoprotein (LDL) cholesterol to “good” high-density lipoprotein (HDL) cholesterol, increasing blood pressure, and increasing the incidence for T2DM (Mazzone 2008). One hallmark of CVD is the development of lipid laden atherosclerotic plaques within major blood vessels. Seen as an inflammatory disease, atherosclerosis may lead to the occlusion of major blood vessels resulting in myocardial infarction (heart attack) or cerebral ischemia (stroke). Similarly, obesity is a chronic inflammatory state mediated by immune cells acting on the metabolically stressed adipose cells oversupplied with glucose and lipids (Lim 2013).

Tissue factor (TF) is a 47-kDa transmembrane cell-surface glycoprotein that serves as the primary initiator of the coagulation cascade. As the initiating protein of the extrinsic pathway of coagulation, TF is required for hemostasis, or the regulation of blood flow. Damage to the blood vessel leads to the formation of a TF: factor VIIa (FVIIa) complex that activates both FIX and FX. The prothrombinase complex (FXa:FVa) cleaves prothrombin to thrombin, which subsequently cleaves fibrinogen to fibrin monomers. Cross-linked fibrin then acts to stabilize the clot. Thrombin also activates platelets, vascular smooth muscle cells (VSMCs), monocytes, and
endothelial cells (ECs) by cleavage of protease-activated receptors (PARs). Importantly, PAR-2, which acts as a G-protein coupled receptor that mediates cellular effects of the serine protease trypsin (Aman 2010), can be activated by either a TF:FVIIa or TF:FVIIa:FXa complex (Camerer 2000). Stimulation of PAR-2 via trypsin induces cytokine release and leukocyte adhesion which perpetuates an inflammatory state (Aman 2010, Hamilton 2009). Interestingly, upregulation of PAR-2 expression results from other inflammatory stimuli such as tumor necrosis factor-α (TNF-α), interleukin-1α and 1β, and lipopolysaccharide (LPS) (Aman 2010). Thus, cyclical activation of PAR-2 expression contributes to the pathogenesis and pathophysiology of vascular disease.

When PAR-2 deficient mice are fed a high fat diet (60%) for 16-20 weeks, attenuation of weight gain and insulin resistance has been observed (Badeanlou 2012). Limiting in this discovery is the lack of a systemic lipid profile assessment. Information regarding levels of triglycerides, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and total cholesterol would enhance such an observation noted by Badeanlou et al.

**Literature Review**

**Coagulation Cascade**

Following vascular injury, physiologically, the goal of the coagulation cascade is to convert soluble plasma fibrinogen into insoluble fibrin wherein platelets, trapped red blood cells, and fibrin act to stabilize a hemostatic platelet “plug” used to heal vascular injury (Mackman 2007, Palta 2014). The traditional view of coagulation as a “waterfall-like” cascade dates back to 1964 when MacFarlane (1964) and Davie & Ratnoff (1964) proposed similar models for the formation of fibrin. Under their model(s), three metabolic pathways form the coagulation system: the extrinsic pathway, the intrinsic pathway, and the common pathway (David & Ratnoff 1964, MacFarlane 1964).
Extrinsic Pathway Mechanism

TF is a 47-kDa transmembrane cell-surface glycoprotein that serves as the primary initiator of the coagulation cascade. TF is inhibited by tissue factor pathway inhibitor (TFPI) (Broze 1995). Under basal conditions, TF is constitutively expressed by cells surrounding the vessel wall, including VSMCs and adventitial fibroblasts (Mackman 2007, Owens 2010). Damage to the vessel wall results in TF exposure to the blood, binding to circulating FVII, conversion of FVII to FVIIa, and formation of the TF:FVIIa complex. This binary complex, through mediation with calcium, then combines with FX, and results in its subsequent activation to FXa (Palta 2014) (Figure 1).

Intrinsic Pathway Mechanism

Acting in parallel to the extrinsic pathway, the intrinsic pathway begins with activation of FXII on a charged surface in a process called contact activation (Gailani 2007). This in turn activates FXI, which subsequently activates FIX (Gailani 2007, Palta 2014). Formation of a tenase complex results from the interaction of FIX and its cofactor, FVIII, also culminating in formation of FXa similar to the extrinsic pathway (Gailani 2007, Palta 2014) (Figure 1). Deficiencies in contact pathway factors results in classic haemophilia in which the body is unable to adequately form blood clots (Cawthern, 1998).

Common Pathway Mechanism

Activated FX (FXa) interacts with its cofactor, FV, calcium, tissue phospholipids, and platelet phospholipids to form the prothrombinase complex, which subsequently converts
prothrombin (FII) to thrombin (FIIa) (Owens 2010, Palta 2014). Thrombin continues to exert its effects by converting fibrinogen (FI) into insoluble fibrin (FIIa) and activating FXIII used to stabilize cross-linking fibrin polymers within the hemostatic plug (Palta 2014) (Figure 1).

![Coagulation Cascade](image)

Figure 1 The coagulation cascade (Source: MedMarket Diligence, LLC; Report #S190)

**Protease-activated Receptors and Atherosclerosis**

PARs are a 4 member family of G protein-coupled receptors that are ubiquitously expressed by vascular cells (Coughlin 2000). They play a role in hemostasis but also contribute to thrombosis and vascular disease. PARs are activated by proteolytic cleavage of their N-terminal domain, which exposes a “tethered ligand” that binds to the receptor. Thrombin activates platelets by cleaving PAR-1 and PAR-4 on human platelets, and PAR-3 and PAR-4 on mouse platelets (Coughlin 2000, Major 2003). PAR-1, in mice, is not located on platelets. PAR-2 is activated by various proteases including FVIIa and FXa (Antoniak 2011). A deficiency of either PAR-3 or PAR-4 in mice is associated with reduced thrombosis in arterial injury models (Hamilton 2004, Vandendries 2007, Weiss 2002). In addition, activation of PAR-1 and PAR-2 enhances migration of VSMCs, and expression of inflammatory mediators by ECs (MacFarlane 2001).
The generation of the enzyme thrombin is the penultimate event of the coagulation cascade and is essential in hemostasis and thrombosis (Mann 2003). Several animal studies have demonstrated use of direct thrombin inhibitors (DTIs) or genetic manipulation of thrombin can attenuate the initiation and progression of atherosclerosis (Bea 2006, Borissoff 2013, Vicente 2007). However, the platelet thrombin receptor PAR-4 (in mice) has no effect on atherosclerosis (Hamilton 2009). Interestingly, clinical findings in humans are contradictory. For example, DTIs are associated with increased risk of myocardial infarction in a large patient meta-analysis (Artang 2013), while vorapaxar (human PAR-1 antagonist and mouse PAR-4 homologue) is protective in secondary prevention trials (Cheng 2015). However, vorapaxar does not prevent cardiovascular death or myocardial infarction in primary prevention trials, and is usually only given as a triple platelet therapy with aspirin and P2Y12 inhibitors, and significantly increases the risk of bleeding (Cheng 2015). Often overlooked, PAR-1 is also localized to VSMCs and ECs. Importantly, thrombin activation of PAR-1 promotes the migration and proliferation of VSMCs, cytokine and chemokine production, vascular calcification, and cellular apoptosis (Borissoff 2009, Nguyen 2001, Papadaki 1998, Schini-Kerth 1997). This data suggests thrombin activation of PAR-1 contributes to several aspects of atherosclerosis and requires further analysis.

The TF:FVIIa complex not only triggers clotting but also activates the protease-activated receptor 2 (PAR-2), which results in proinflammatory signaling (Antoniak 2011, Borissoff 2011). TF induction of proinflammatory cytokines and chemokines can result in leukocyte recruitment to the atherosclerotic lesion, thus enhancing the progression of atherosclerosis (Demetz 2012, Mackman 2004, Mackman 2007, Tremoli 1999). VSMCs play a key role in atherosclerosis both in early and in late stages (Doran 2008). In early stages, VSMCs migrate
from the media to the intima where they are trapped and proliferate to contribute to the development of fatty streaks. More advanced atherosclerotic lesions contain VSMCs that have a higher proliferative index and a greater synthetic capacity for extracellular matrix, particularly collagen, proteases, and cytokines (Campbell 1994, Owens 2004, Worth 2001). Similar to macrophages, late-stage VSMCs can express a variety of receptors for lipid uptake and can form foam-like cells, thereby participating in the accumulation of plaque lipid. Importantly, the presence of TF contributes to VSMC migration in vitro and in vivo (Pyo 2004), which appears to be due to the activation of the PAR-2 signaling pathway (Marutsuka 2002). Further, TF:FVIIa activation of PAR-2 results in the secretion of the inflammatory cytokine IL-6 and chemokine IL-8, which further the atherogenic immune phenotype (Demetz 2010). In addition, ligation of the CD40 receptor, implicated in the atherogenic immune process, on VSMCs and monocyte/macrophages can augment the expression of TF protein and activity in the atherosclerotic lesion (Mach 1997, Schonbeck 2000). Together, TF activation of PAR-2 contributes to cytokine and chemokine production and SMC migration which may initiate and propagate atherosclerosis.

**Protease Activator Receptor-2**

The effects of serine proteases thrombin and trypsin signal through PARs (Aman 2010, Lim 2013, Rothmeier & Ruf 2011). Although mice have four metabolically different PARs, PAR-2 activation relies upon trypsin, coagulation factors VIIa and Xa, and additional transmembrane proteases (Rothmeier & Ruf 2011). Mast cell tryptase is also a potential PAR-2 agonist (Steinhoff 2000). Tryptase stimulates vasodilation, immune cell diapedesis including

PAR-2 stimulates G protein activity for recruitment of β arrestins for cytoskeletal scaffolding and suppression of phosphatidylinositol 3 kinase (PI3K) dependent protein kinase B (Akt) phosphorylation (Badeanlou 2012). PAR-2 activation may increase the pro-inflammatory response seen in atherosclerotic plaques through generation of TF-dependent coagulation proteases (Tilley 2006). Additionally, PAR-1 and PAR-2 stimulation contributes to the pathogenesis of vascular disease via release of cytokines, adhesion of leukocytes, and exocytosis of Weibe-Palade bodies in endothelial cells (Aman 2010, Hamilton 2009).

Animal studies have shown that mice lacking PAR-2 have attenuation of weight gain (Badeanlou 2012) and insulin resistance (Badeanlou 2012) when fed a high fat diet. In addition, oxidative stress in rat aortas lead to upregulation of PAR-2 in endothelial cells suggesting that such strain may enhance the endothelium-dependent relaxation response seen in PAR-2 agonists (Aman 2010).

Humans also possess PAR-2 throughout the body (D’Andrea 1998). Although examination of PAR-2 within the human population is sparse, D’Andrea et al (1998) found tissue-specific cellular localization of PAR-2 within human tissues via immunohistochemical techniques. The receptor is localized to the smooth muscle vasculature (Molino 1998), endothelial and epithelial cells, the gastrointestinal tract, and astrocytes/neurons within the central nervous system (D’Andrea 1998).

Clinical studies are assessing the pathological role of PAR-2 within varying body systems. Steinhoff et al (2003) examined the role of PAR-2 in pruritus (i.e., itch) and found that patients presenting with atopic dermatitis given endogenous PAR-2 agonists had enhanced and
prolonged itch sensations relative to the control group. Steinhoff et al (2003) conclude that a link may exist between PAR-2 signaling and the inflammatory and sensory phenomena present within atopic dermatitis patients via neurogenic mechanisms. Additionally, Molino et al (1998) found PAR-2 expression within human aorta and coronary artery smooth muscle cells, intestinal smooth muscle cells, as well as throughout the arterial vasculature.

Although lacking in a definitive mechanism, research is beginning to suggest the role of mast cell tryptase as a potential PAR-2 agonist (Steinhoff 2000). Tryptase stimulates vasodilation, immune cell diapedesis including neutrophil infiltration of tissue, and secretion of pro-inflammatory cytokines (He & Peng 1997, He & Walls 1997, Steinhoff 2000).

**Inflammation**

Inflammation is the body’s defense mechanism against infection and tissue injury (Calder 2009). Under normal physiological conditions the inflammatory response may be characterized by redness, swelling, pain, and warming sensations as a result of chemical mediator (i.e., interleukins, cytokines, tumor necrosis factor-alpha) release. The inflammatory process assists in pathogen destruction as well as repairing damaged tissue. Once the pathogen and/or tissue is neutralized, the body halts production of pro-inflammatory chemical mediators and returns to a homeostatic state (Calder 2009).

**Regulation**

Nuclear factor-κB (NF-κB) is a redox-sensitive, dimeric transcription factor that increases transcription of pro-inflammatory cytokines, chemokines, growth factors, and adhesion molecules (Hanada & Yoshimura 2002). Found within the cytoplasm in its inactive form (subunits p100 and p105), NF-κB becomes active (subunits p50 and p52) upon exposure to the
presence of pathogens and/or reactive oxygen species (ROS), lipopolysaccharides (LPS), and
tumor necrosis factor-alpha (TNF-α) wherein it translocates to the nucleus and binds to the
a mechanism enhances transcription and subsequent translation of pro-inflammatory proteins
(Calder 2009).

Nutrition and inflammation

Specific nutritional components (i.e., antioxidants and fatty acids) are linked to systemic
inflammation (Galland 2010, Seifreid 2007). Alongside chronic inflammation, the body produces
excess reactive oxygen species (ROS) (i.e. hydrogen peroxide and superoxide radicals) that
contribute to damage within healthy tissue (Seifreid 2007). When the body does not obtain
adequate antioxidant (i.e., vitamins C, and E, etc.) levels, an imbalance develops and results in
pathologic ROS activation of redox-sensitive transcription factors (i.e. NF-κB), which
subsequently induce transcription of proteins used to perpetuate the inflammatory response via
the immune system (Calder 2009, Seifreid 2007). As a result, the body fails to self-regulate the
inflammatory response while increasing immune system activity in hopes of neutralizing oxidant
production.

Consumption of antioxidant rich foods may help reduce chronic inflammation by
neutralizing pathologic ROS. By providing the body with adequate antioxidants, individuals
reduce the activation of redox-sensitive transcription factors (i.e. NF-κB). Antioxidant rich foods
include colorful fruits and vegetables, whole grains, green tea, dark chocolate, nuts and seeds.
Sufficient consumption of these foods increases circulating antioxidant levels, which may
ultimately reduce the transcription of proteins used to perpetuate the inflammatory response
(Conner 1996).
Dietary fat exerts varying effects on cellular response based upon the type of fatty acids consumed. Saturated fats— lipid molecules composed of carbon atoms attached via single bonds and “saturated” with hydrogen atoms— increase rigidity of cell membranes and, overall, promote a pro-inflammatory state. Although numerous saturated fats have been studied, palmitic and myristic acid have been found to be the most hypercholesterolemic (Fernandez 2005, Zock 1994). Common sources of saturated fats include: cream, butter, cheese, whole milk, red meat, as well as processed foods such as pizza, ice cream, and fried foods. Evidence suggests that saturated fatty acids have the greatest negative effect on plasma LDL cholesterol concentrations (Fernandez 2005, Hu 2001). Similarly, omega-6 fatty acids are generally thought to promote a state of inflammation as a result of eicosanoid— predominately arachidonic acid— activity (Calder 2011). Omega-3 fatty acids, however, exhibit anti-inflammatory properties as a result of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) activity (Fernandez 2005). EPA and DHA reduce vasoconstriction and exhibit anti-coagulative properties (Calder 2009). Traditional western diets tend to be high in the pro-inflammatory saturated and omega-6 fatty acids and low in the anti-inflammatory omega-3 fatty acids (Calder 2011, Zock 1994).

According to the 2013 American Heart Association and American College of Cardiology guidelines on lifestyle management to reduce cardiovascular disease risk, individuals should aim for a dietary pattern that reduces saturated fat intake to no more than 5-6% of total calories (Eckel 2014). In addition, for every 1% of energy provided by saturated fat that is replaced by 1% carbohydrate, monounsaturated fatty acids (MUFA), or polyunsaturated fatty acids (PUFA), studies have shown a decrease in LDL cholesterol by 1.2, 1.3, and 1.8 mg/dL respectively (Eckel 2014). MUFA consumption lowers total cholesterol predominately by lowering LDL cholesterol levels (Kris-Etherton 1999). PUFA intake, however, is often identified by the ratio of omega-6 to
omega-3’s, with an ideal ratio of 4:1 (Caspar-Bauguil 2010). Common sources of MUFA include olive and canola oil, avocado, walnuts, almonds, and peanut butter. PUFAs are found in, but not limited to, soybean oil, walnuts, fatty fish (i.e., salmon, tuna, halibut, etc.), flaxseed, and eggs.

*Disease and inflammation*

Pathological inflammation occurs as the body begins to lose the ability to regulate the inflammatory response. Continual stimulation by pro-inflammatory mediators causes the immune system to release phagocytic cells—immune cells responsible for “engulfing” harmful particles, bacteria, and dead/dying cells—to respond to the perceived threat. Phagocytes arrive at the site of inflammation and release cytokines and other pro-inflammatory proteins as a means of defense. This produces a positive feedback loop wherein the body continually responds to signs of inflammation. Chronic inflammation results in excessive damage to host tissues and contributes to perpetuation of disease (Calder 2009). Atherosclerosis (Hamilton 2009, Pingel 2014) and obesity (Galic 2010, Greenberg 2006, Lijnen 2012, Stapleton 2008) are likely the result of chronic inflammation.
Chapter 2 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease characterized by infiltrates of lipids, platelets, VSMCs, monocyte/macrophages (foam cells), T and B lymphocytes, and deposition of extracellular matrix in the vessel walls of large arteries (Hamilton 2009, Libby 2002, Pingel 2014, Ross 1999). Rupture of atherosclerotic plaques may lead to occlusion of major arteries within the heart and/or brain resulting in myocardial infarction or cerebral ischemia (Tilley 2006). Acquired CVD – diseases of the cardiovascular system that develop after birth – still remains the leading cause of death in the United States (Lui 2014).

Evidence supporting the notion that atherosclerosis is an inflammatory, as well as autoimmune, disease has concurrently evolved at both the basic and clinical level (Libby 2002). Animal and human studies have found leukocytes—immune cells responsible for maintaining host defenses and inflammation—and platelets localized in early atherosclerotic lesions (Ross 1999, Libby 2002). Branch sites within the arterial tree are particularly prone to develop atheroma due to disturbed blood flow via reduced laminar shear stress (Libby 2002). As a result, local production of endothelial-derived nitric oxide (NO)—a potent vasodilator that demonstrates anti-inflammatory properties—decreases. Under normal conditions, NO limits expression of vascular cell adhesion molecule-1 (VCAM-1) that binds white blood cells (i.e., monocytes and T lymphocytes) during early atherogenesis (Ross 1999, Libby 2002). As a response to endothelial damage, leukocytes and platelets accumulate at the site of injury as the endothelium manifests procoagulant rather than anticoagulant properties. During the procoagulant state, the endothelium releases vasoactive molecules, growth factors, and cytokines that perpetuate the inflammatory state (Ross 1999).
The earliest type of inflammatory lesion is the fatty streak (Ross 1999, Libby 2002). Fatty streaks refer to the accumulation of foam cells—an accretion of monocyte-derived macrophages and T lymphocytes—just beneath the endothelium (referred to as subendothelial or intimal layer) within blood vessels. Typically, many individuals develop fatty streaks before the age of twenty—though their presence has been noted in some developing embryos (Strong 1997)—that last throughout the lifetime (Ross 1999). Although these streaks have the potential to develop into atherosclerotic plaques, their presence can be reversed if the individual follows a nutritionally sound dietary pattern (i.e. DASH, TLC, etc.), remains physically active, and learns to effectively manage stress.

Two hypotheses have been proposed regarding the perpetuation of atherosclerotic plaques, the response-to-injury hypothesis and the oxidation of lipoprotein hypothesis (Ross 1999, Libby 2002, Korporeal 2005).

Response to injury hypothesis

Under the response to injury hypothesis, vascular damage typically occurs in medium and large sized muscular and elastic arteries (Ross 1999). Once a blood vessel is damaged, low-density lipoproteins (LDL) and intermediate-density lipoproteins (IDL) particles along with chylomicron remnants enter the subendothelial layer. As a response to the damage, the immune system directs macrophages and T-helper cells to the damaged tissue. T-helper cells release various cytokines that induce an inflammatory response. Under a hypercholesterolemic condition, perpetuation of the inflammatory state may result due to the continual exposure of the injury site to LDL particles (Ross 1999). However, through exploration of LDL particle contact
at the site of endothelial injury, researchers have questioned whether oxidation of lipoproteins is the main contributor to the inflammatory state seen within atherogenesis.

**Oxidation of lipoproteins hypothesis**

Oxidized LDL (oxLDL) is low density lipoprotein (LDL) that has been oxidatively modified. Proponents of the ox-LDL hypothesis suggest that under the hypercholesterolemic state, more substrate is available in the blood for oxidation as a result of the inflammatory process where oxidants are frequently released by immune and endothelial cells. The more oxidized LDL present within the subendothelial layer, the greater the level of foam cell formation via macrophage transformation. Anatomically, the accumulation of foam cells forms fatty streaks along the blood vessel. As more and more foam cells accumulate, atherosclerotic plaques may develop thus impeding blood flow through the vessel and damaging surrounding tissue (Libby 2002 & Korporaal 2005). Continual endothelial stress may also promote production of proteoglycans via arterial smooth muscle cells (SMCs) that can bind and retain lipoprotein particles thus perpetuating the amount of available substrate for oxidative modification at the lesion site (Libby 2002 & Korporaal 2005).

Researchers have examined the role of oxLDL in atherogenesis with mixed results (Parthasarathy 2010). Inconsistent findings between *in vitro* and *in vivo* studies have raised questions regarding the role of LDL oxidation in atherogenesis (Parthasarathy 2010). For example, LDL particles treated with lipoxygenase may differ in their amounts of phospholipids, cholesterol ester, and hydroperoxides whereas photooxidation in the presence of dye produces different products than traditional oxidized lipoproteins (Parthasarathy 2010). Additionally,
clinical studies have found that the types of modified lipids and proteins within human atheroma do not correspond with the compounds found in vitro (Libby 2002).

Research Question

Does the PAR-2 receptor have a significant role in atherogenesis?

Hypothesis

The PAR-2 receptor will decrease atherosclerotic plaque size within a diet-induced atherogenic murine model.

Methods

In vivo Atherosclerosis Studies

Animals and diets

All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill (UNC-CH) and the University of Cincinnati and in accordance with the Eighth Edition of the Guide for the Care and Use of Laboratory Animals. Male Ldlr<sup>−/−</sup> mice (8-12 weeks of age) were originally obtained from The Jackson Laboratory (stock number 002207) and bred in-house at UNC-CH. Par-2<sup>−/−</sup> mice were originally obtained from Johnson and Johnson Pharmaceuticals Research Division, as described previously by Damiano (1999). Ldlr<sup>−/−</sup>/Par2<sup>+/+</sup> and Ldlr<sup>−/−</sup>/Par2<sup>−/−</sup> cousin littermate mice were generated by interbreeding Ldlr<sup>−/−</sup>/Par2<sup>−/−</sup> mice, which were created by breeding Ldlr<sup>−/−</sup>/Par2<sup>−/−</sup> onto the Jackson Ldlr<sup>−/−</sup> strain.

Male Ldlr<sup>−/−</sup> mice that were PAR2<sup>+/+</sup> (n=20) or PAR2<sup>−/−</sup> (n=13) were housed in standard conditions at 22 °C, with a 14-h light and a 10-h dark cycle, and ad libitum access to standard rodent chow diet (Lab Diet 13% kcal from fat rodent diet; 2015; LabDiet, St. Louis, MO)
and water. Mice were given high fat diet (Test Diet 40% kcal from fat; 2012; TestDiet, St. Louis, MO) (Table 1) at 8 to 12 weeks of age for 12 weeks. Body weight was measured upon arrival and on the day of sacrifice.

Bone Marrow Transplantation

Male recipient Ldlr\(^{-/-}\)/Par2\(^{+/+}\) or Ldlr\(^{-/-}\)/Par2\(^{-/-}\) mice (8-12 weeks old) were irradiated with a total of 11 Gy (2 doses of 550 rads 4 hours apart) using a Cs\(^{137}\) irradiator (JL Shepherd, San Fernando, CA). Irradiated mice were re-populated with bone marrow harvested from Ldlr\(^{-/-}\)/Par2\(^{+/+}\) (n = 17) or Ldlr\(^{-/-}\)/Par2\(^{-/-}\) (n = 16) donor mice via retro-orbitally injected cells (1 x 10\(^7\) cells per animal) to create four different chimeric groups. Mice were allowed to recover for 4 weeks with antibiotic (Sulfatrim) water *ad libitum*. Mice were given high fat diet (Test Diet 40% kcal from fat; 2012; TestDiet, St. Louis, MO) (Table 1) at 12 to 16 weeks of age for 12 weeks. Body weight was measured upon arrival and on the day of sacrifice.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Diet</th>
<th>CD</th>
<th>WD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate, %</td>
<td></td>
<td>56.7</td>
<td>44.4</td>
</tr>
<tr>
<td>Fat, %</td>
<td></td>
<td>13.4</td>
<td>40</td>
</tr>
<tr>
<td>Protein, %</td>
<td></td>
<td>29.8</td>
<td>15.6</td>
</tr>
<tr>
<td>Sucrose, %</td>
<td></td>
<td>3.83</td>
<td>33.6</td>
</tr>
<tr>
<td>Corn Starch, %</td>
<td></td>
<td>21.0</td>
<td>15</td>
</tr>
<tr>
<td>Cholesterol (ppm)</td>
<td></td>
<td>209</td>
<td>2,057</td>
</tr>
<tr>
<td>Omega-3 Fatty Acids, %</td>
<td></td>
<td>0.30</td>
<td>0.21</td>
</tr>
<tr>
<td>Total Saturated Fatty Acids, %</td>
<td>1.48</td>
<td>12.58</td>
<td></td>
</tr>
<tr>
<td>Total MUFA, %</td>
<td></td>
<td>1.62</td>
<td>4.6</td>
</tr>
<tr>
<td>Total PUFA, %</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Casein, %</td>
<td></td>
<td>0</td>
<td>19.5</td>
</tr>
</tbody>
</table>

Source: Lab Diet, 2015 (CD) and Test Diet, 2012 (WD)

Blood Collection

Mice were sedated with 3% isoflurane and blood was collected from the inferior vena cava into a 25-gauge x 1’ needle pre-coated with 3.8% sodium citrate. The mice were then humanely euthanized. Mouse blood was centifuged at 4,000 x g for 15 minutes to prepare
platelet poor plasma (PPP). An aliquot of blood was analyzed for complete blood count utilizing a Hemavet 950 LV veterinary multi-species hematology system (Drew Scientific), while the rest was aliquoted and stored at -80°C.

**Tissue Collection**

Post sacrifice, mice were perfused with 9% sodium chloride and the hearts and aortas were extracted. Aortas were stored in 10% formalin for 24 hours and then switched to 1x phosphate buffered saline (PBS).

**Atherosclerosis Quantification**

*Aortic Sinus Quantification*

The base of the heart was routinely processed, fixed in cryo-medium (Polyfreeze Tissue Freezing Medium Cat. # 19636), and cut into 10 µm sections using Leica CM3050 S Research Cryostat from the appearance of the aortic valves until the aortic wall disappears or is not intact anymore. Sections were placed on 8 slides following 9 serial sections. Aortic root sections were stained with Oil Red O to facilitate atherosclerotic lesion measurements as previously described (Paigen 1987, Whitman and Daugherty 2003).

**Lipid Assays**

*Triglyceride*

Total triglyceride levels in plasma were determined using L-Type TG M kits (Wako, Enzyme Color A Cat. #461-08992, Enzyme Color B Cat. #461-09092, and Lipid Callibrator Cat. #464-01601). Total triglyceride was measured as the amount of hydrogen peroxide produced by the oxidation of glycerol-3-phosphate by glycerol-3-phosphate oxidase. All enzymatic reagents were prepared following manufacturer instructions.
Cholesterol

Total plasma cholesterol was measured using Cholesterol E kits (Wako, Cat. #439-17501). Concentration of total cholesterol was measured as the amount of unesterified cholesterol within the sample. All enzymatic reagents were prepared following manufacturer instructions with reconstituted reagents stored in amber bottles at 4°C.

High density lipoprotein

High density lipoprotein levels in plasma were determined using L-Type HDL-C kits (Wako, Reagent 1 Cat. #997-72591, Reagent 2 Cat. #993-72691, and HDL-C/LDL-C Callibrator Cat. #990-28011). All enzymatic reagents were prepared following manufacturer instructions.

Low density lipoprotein

Low density lipoprotein levels in plasma were determined using L-Type LDL-C kits (Wako, Reagent 1 Cat. #999-00404, Reagent 2 Cat. #999-00504, and HDL-C/LDL-C Callibrator Cat. #990-28011). All enzymatic reagents were prepared following manufacturer instructions.

Statistical Analysis

All statistical analysis was performed using SigmaStat, now incorporated into Sigma Plot v.11 (SigmaPlot v.11.0, Systat Software, Inc., San Jose, CA). Data are represented as mean ± SEM. For two- group comparison of parametric data, a Student’s t-test was performed, while non-parametric data was analyzed with a Mann-Whitney Rank Sum. Statistical significance between multiple groups was assessed by one-way analysis of variance (ANOVA) on Ranks with a Dunn’s post hoc, One Way ANOVA with Holm Sidak Post Hoc, or Two Way ANOVA with Holm Sidak Post Hoc, when appropriate. Values of $P < 0.05$ were considered statistically significant.
Results

In vivo Atherosclerosis Studies

*Atherosclerotic sinus analysis*

Consumption of a high fat, westernized diet for 12 weeks trends towards \((P<0.053)\) a decrease in atherosclerotic plaque development in PAR-2 deficient mice relative to PAR-2 proficient mice (Figure 2). Aortic root mean lesion area trended towards a decrease in plaque accumulation within PAR-2 deficient mice relative to proficient mice (Figure 3).

![Graph showing lesion size comparison](image)

Figure 2. PAR-2 deficiency reduces atherosclerotic lesion size. Data are represented throughout the aorta root, with transitions between the sinus and ascending arch depicted as 0. Blue-filled circles, \(Ldlr^{+/−} \times Par2^{+/+}\) \((n=3)\); orange-filled circles, \(Ldlr^{−/−} \times Par2^{−/−}\) \((n=3)\). Values are mean ± SEM.
PAR-2 deficiency reduces mean lesion area. Atherosclerotic lesion size was measured in the aortic sinus of mice. Circles represent individual mice; diamonds represent the mean; error bars denote SEM.

Triglyceride levels

Total triglyceride levels were not significantly different (P=0.776) between Ldlr/−/− PAR2+/+ and Ldlr/−/− PAR2−/− mice after supplementing with a high fat diet for 12 weeks (Table 2).

Cholesterol levels

Consumption of a high fat diet for 12 weeks did not lead to any significant difference (P=0.436) in total cholesterol levels between Ldlr/−/− PAR2+/+ and Ldlr/−/− PAR2−/− mice (Table 2).

High-density lipoprotein levels

High-density lipoprotein levels were not significantly different (P=0.777) between Ldlr/−/− PAR2+/+ and Ldlr/−/− PAR2−/− mice after supplementing with a high fat diet for 12 weeks (Table 2).

Low-density lipoprotein levels

No significant difference (P=0.186) in low-density lipoprotein levels was observed between Ldlr/−/− PAR2+/+ and Ldlr/−/− PAR2−/− mice after supplementing with a high fat diet for 12 weeks (Table 2).
Table 2. Plasma lipid levels within PAR-2 proficient and deficient mice

<table>
<thead>
<tr>
<th></th>
<th>Triglyceride (mg/dL)</th>
<th>Cholesterol (mg/dL)</th>
<th>HDL (mg/dL)</th>
<th>LDL (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PAR2^{+/+}</strong></td>
<td>394 ± 53.4</td>
<td>1173 ± 64.4</td>
<td>194 ± 26.9</td>
<td>1035 ± 78.9</td>
</tr>
<tr>
<td>(n=20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PAR2^{-/-}</strong></td>
<td>364 ± 94.1</td>
<td>1097 ± 99.1</td>
<td>205 ± 59.7</td>
<td>800 ± 183.8</td>
</tr>
<tr>
<td>(n=13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p*-value 0.776 0.436 0.777 0.186

Data are presented as mean ± S.E.M.

**Discussion**

Although there was no significant difference in plasma triglyceride, cholesterol, HDL, and LDL levels between PAR-2 proficient and PAR-2 deficient mice, we observed a decrease in atherosclerotic burden. It is established that PAR-2 is activated by various proteases including trypsin, FVIIa and FXa, and mast cell tryptase (Antoniak 2011, Rothmeier & Ruf 2011) and that stimulation of PAR-2 enhances migration of VSMCs and expression of inflammatory mediators by ECs (MacFarlane 2001). In addition, TF:FVIIa activation of PAR-2 results in the secretion of the inflammatory cytokine IL-6 and chemokine IL-8, which further propagates the atherogenic phenotype (Demetz 2010). Our results demonstrate that mice lacking PAR-2 tended towards a decrease in atherosclerotic plaque, findings that support the established role of PAR-2 within atherogenesis.

Even though trypsin, coagulation factors VIIa and Xa, and mass cell tryptase are known to activate PAR-2 (Coughlin 2000), we hypothesize that mast cell tryptase plays a predominate role in PAR-2 activation and its downstream effects on atherogenesis. Tryptase is a serine protease secreted by activated mast cells (Molino 1997). In their peptide cleavage studies, Molino et al. (1997) found that tryptase cleaved the N-terminus of PAR-2, thus activating it,
more efficiently and at higher concentrations than did trypsin. Additionally, mast cell
degranulation occurs in areas where PAR-2 is expressed (i.e., endothelial cells, vascular smooth
muscle cells, and keratinocytes) (Molino 1997). Interestingly, mast cell deficient mice on a Ldlr
deficient background have been found to have attenuated atherosclerotic plaque development
after consuming a high fat diet for 9 weeks (Lindstedt 2007). Similarly, our results found that
PAR-2 deficient mice on a Ldlr deficient background developed less atherosclerotic plaque
relative to PAR-2 proficient mice. Taken together, these results suggest that mast cell tryptase
may activate PAR-2 during atherogenesis. Further studies are needed to assess the extent to
which mast cell tryptase and PAR-2 perpetuate the inflammatory state associated with
atherogenesis.

PAR-2 expression is not limited to mice; humans express the receptor throughout the
body within the smooth muscle vasculature, ECs, gastrointestinal tract, as well as astrocytes and
neurons within the central nervous system (D’Andrea 1998). Moreover, PAR-2 is known to play
an important role in tissue inflammation and vascular tone (Napoli 2004). Clinical trials have
found heightened PAR-2 expression within human coronary atherosclerotic lesions (Napoli
2004) as well as an upregulation of PAR-2 in situ as a result of pathological conditions such as
atherosclerosis, hypertension, and ischemia (Hamilton 2001). Our results found a decrease in
atherosclerotic burden within a murine model. Through understanding the pathological role of
PAR-2 within atherogenesis at both the basic and clinical level, future pharmacological studies
may choose to assess potential therapeutic PAR-2 antagonists for targeting vascular disease.
Conclusion

Consumption of a high fat, westernized diet for 12 weeks leads to a decrease in atherosclerotic plaque development in PAR-2 deficient mice with no significant change in triglyceride, total cholesterol, HDL, and LDL levels between PAR-2 wild type and PAR-2 knockout mice.
Chapter 3 Metabolic Observations

Obesity is chronic over nutrition resulting in an imbalance between energy intake and expenditure (Samartin 2001). Adipose tissue serves as an insulator and cushion for the body, and a storage depot for free fatty acids (FFAs) wherein it controls the release of FFAs during a fasting state (Hajer 2008). Postprandial uptake of FFAs by adipose tissue occurs after hydrolysis of triglycerides from very low-density lipoprotein-cholesterol (VLDL-c), chylomicrons and their remnants via lipoprotein lipase. Under the fasting condition, hydrolysis of adipocyte—cells of the adipose tissue—triglyceride stores occurs via hormone sensitive lipase (Hajer 2008). Regulation depends upon insulin, which serves as a potent activator of lipoprotein lipase and inhibitor of hormone sensitive lipase (Hajer 2008).

As the number of calories consumed increases past metabolic need, adipose tissue begins to hypertrophy until individual adipocytes reach “peak volume.” Once an adipocyte reaches its maximum diameter, it releases pro-inflammatory adipocytokines, interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) that induces a lipolytic state, releases non-essential fatty acids (NEFA), and creates a chemoattractant gradient wherein macrophages migrate to the site of inflammation (Galic 2010, Greenberg 2006, Lijnen 2012, Stapleton 2008). As a result of producing this dysfunctional adipose tissue—a state induced by hypersecretion of pro-inflammatory, pro-atherogenic, and pro-diabetic adipocytokines—the body enters a state of insulin resistance as evidenced by impairment in fasting glucose and glucose tolerance (Hajer 2008). Thus adipose tissue acts as an important endocrine “organ” whose effects serve a significant role in the inflammatory response (Calder 2009, Fontana 2007).

Obesity occurs with varying degrees of fat accumulation within differing adipose depots often resulting in a vast number of metabolic implications (Blüher 2009). Visceral fat
accumulation within the intraabdominal region may increase the risk for developing
dyslipidemia, diabetes, and an acceleration of atherogenesis relative to other fat depots (i.e.,
subcutaneous and peripheral) (Blüher 2009). In addition, visceral fat depots are more
lipolytically active with reduced insulin sensitivity (Blüher 2009). Excess ectopic fat (i.e.,
omentum, pericardial, perirenal, and retroperitoneal) when combined with lipid deposition in the
pancreas, liver, and muscle increases the risk of developing insulin resistance (Blüher 2009,
Rassouli 2007).

Clinical studies observing pro-inflammatory biomarkers have shown that individuals with
type 2 diabetes mellitus (Boden 2011) and obesity (Boden 2011, Samocha-Bonet 2012) express
greater levels of inflammatory and oxidative stress biomarkers. Additionally, associations have
been made between obesity indices and inflammatory markers with women expressing an
increase in C-reactive protein status, and both men and women expressing additional
inflammatory markers (Monteiro 2010). These studies suggest that excess adipose tissue,
regardless of health status (i.e., T2DM, obesity), increases circulating levels of pro-inflammatory
and oxidative stress biomarkers.

Animal studies have demonstrated improvements in levels of inflammation and insulin
found that obese mice that were calorically restricted from a high fat diet to a normal chow diet
had significantly lower expression of pro-inflammatory interleukin-6 within adipose tissue
relative to controls. Additionally, Lijnen et al (2012) observed a reduction in cholesterol and
triglyceride levels. Obese mice switched from a high-fat diet to a high carbohydrate diet were
also found to have reduced body weight as well as reduced levels of interleukin-6 and leptin,
(Lee 2010).
Interestingly, PAR-2 deficient mice are protected from weight gain and insulin resistance induced by a 60% diet-induced obesity (DIO) diet (Badeanlou 2012). Additionally, Badeanlou et al (2012) found a significant reduction in fat pad and liver weights, suggesting that PAR-2 deficiency may protect against DIO and hepatic steatosis.

GPCR activity regulates obesity and reduces adipose tissue inflammation (Badeanlou 2012). The GPCR PAR-2 stimulates G protein activity for recruitment of β arrestins for cytoskeletal scaffolding and suppression of phosphatidyl inositol 3 kinase (PI3K) dependent protein kinase B (Akt) phosphorylation (Badeanlou 2012, Wang 2006). Activation of adenosine monophosphate-activated protein kinase (AMPK) by PAR-2 signaling is suppressed by β-arrestin 2 within adipocytes (Wang 2010). Additionally, TF-VIIa signaling within adipocytes initiates suppression of Akt phosphorylation via insulin and modifies the expression of Akt-regulated genes associated with weight gain (Badeanlou 2012). Regulation of glucose uptake as well as fatty acid oxidation and AMPK activation occurs as a result of adiponectin activity (Badeanlou 2012). As a result, adiponectin activation positively contributes to body weight regulation (Badeanlou 2012). Although a suggested link exists between PAR-2 and the PI3K and AMPK pathways, little is known as to whether PAR-2 influences DIO.

**Research Question**

Does feeding PAR-2 proficient and deficient mice a ‘Western’ diet for 12 weeks recapitulate the metabolic findings of Badeanlou and colleagues?

**Hypothesis**

PAR-2 deficiency will result in attenuated adipose tissue deposition and weight gain.
Methods

Animals and diets

Refer to chapter 1 methodology.

Glucose Tolerance

An intraperitoneal glucose tolerance test (IPGTT) was performed to characterize glucose tolerance of PAR-2 mice. Mice were fasted for 5 hours and a basal blood sample was collected from the tip of the tail (t= 0 minutes). Mice were then injected with 20% glucose in 1x phosphate-buffered saline (PBS) solution (2 g glucose/kg body weight). Glucose was administered with a 0.5-ml tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ) through a 27-gauge needle. Administered glucose levels ranged from 130-280 mg/dL. Blood samples were obtained at 15, 30, 60, and 90 minutes post glucose injection and analyzed for plasma glucose (FreeStyle Lite, Abbott, SKU: 9907370805).

Insulin Tolerance

An intraperitoneal insulin tolerance test (IPITT) was performed to determine the sensitivity of insulin-responsive tissues in PAR-2 mice. Mice were fasted for 5 hours and a basal blood sample was collected from the tip of the tail (t= 0 minutes). Samples were analyzed for plasma glucose using FreeStyle Lite glucometer (Abbott, SKU: 9907370805). Mice were then injected with 0.1 U/ml insulin (Humulin N, Lilly, Cat. # 0002-8315-01) in 1x dulbecco’s phosphate-buffered saline (DPBS) solution (1U insulin/kg body weight). Insulin was administered with a 0.5-ml tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ) through a 27-gauge needle. Administered insulin levels ranged from 163-280 mg/dL. Blood samples were
obtained at 15, 30, 60, and 90 minutes post insulin injection and analyzed for plasma glucose (FreeStyle Lite, Abbott, SKU: 9907370805).

**Tissue Collection**

Upon sacrifice, epididymal and retroperitoneal fat was collected and quantified as previously described by Mann et al. (2014). Briefly, the peritoneum was cut transversely below the diaphragm and then cut mid coronally to the rectum to expose the abdominal organs. The testes were located and, using sterile surgical instruments, epididymal fat depots were removed from the testes, epididymides, and vasa deferentia. The fat depots were weighed, transferred to 2 ml microcentrifuge tubes, and stored at -80°C. After removal of the epididymal fat depots, the retroperitoneal cavity was exposed and fat depots were removed around the kidneys. The retroperitoneal fat depots were weighed, transferred to 2 ml microcentrifuge tubes, and stored at -80°C. The liver was removed, weighed, and stored at -80°C. Plasma was collected and stored in 2 ml microcentrifuge tubes at -80°C for future analysis. The heart and aorta was removed and stored at -80°C for further analysis.

**Statistical Analysis**

All statistical analysis was performed using SigmaStat, now incorporated into Sigma Plot v.11 (SigmaPlot v.11.0, Systat Software, Inc., San Jose, CA). Data are represented as mean ± SEM. For two group comparison of parametric data, a Student’s t-test was performed, while non-parametric data was analyzed with a Mann-Whitney Rank Sum. Statistical significance between multiple groups was assessed by one-way analysis of variance (ANOVA) on Ranks with a Dunn’s post hoc, One Way ANOVA with Holm Sidak Post Hoc, or Two Way ANOVA with Holm Sidak Post Hoc, when appropriate. Values of $P < 0.05$ were considered statistically significant.
Results

Body weight

$Ldlr^{-/-} PAR2^{-/-}$ mice (n=13) gained significantly less weight (P<0.001) when compared with $Ldlr^{-/-} PAR2^{+/+}$ mice (n=20) over the 12 week diet duration (Figure 4). In the chimeric model, mice that were $PAR2^{+/+}/BM-PAR2^{+/+}$ (n=10) had the most significant weight gain (P<0.001) in contrast to those that were systemically and hematopoietically deficient (n=12). Significantly reduced body weight was demonstrated in $PAR2^{-/-}/BM-PAR2^{+/+}$ (n=7, P<0.001) and $PAR2^{+/+}/BM-PAR2^{-/-}$ (n=13, P<0.001) mice relative to the $PAR2^{+/+}/BM-PAR2^{+/+}$ mice (Figure 5).

Figure 4 PAR-2 deficiency results in significantly less weight gain during 12 week high fat diet (*P<0.001). Data are expressed as mean ± S.E.M.
Figure 5 Global PAR-2 deficient mice show significant attenuation of weight gain (*P<0.001) when fed a high fat diet for 12 weeks. Data are expressed as mean ± S.E.M.

**Adiposity**

Consumption of a high fat diet resulted in a significant reduction in adiposity in both epididymal (P<0.001) and retroperitoneal (P<0.001) adipose tissue within Ldlr^{−/−}PAR2^{−/−} mice relative to Ldlr^{−/−}PAR2^{+/+} mice (Figure 6). The 53% reduction in epididymal adipose tissue may be attributed to the mechanistic role of PAR-2 as a potential GPCR regulator of obesity. Liver weight was quantified and found to be significantly (P<0.001) lower in Ldlr^{−/−}PAR2^{−/−} mice when compared to Ldlr^{−/−}PAR2^{+/+} mice (Figure 8). The 33% decrease in liver weight may indicate a reduction in the amount of hepatic steatosis present within PAR-2 deficient mice.
(a) PAR-2 deficiency results in significantly less epididymal fat accumulation (*P<0.001) (b) PAR-2 deficiency results in significantly less retroperitoneal fat accumulation (*P<0.001). Data are expressed as mean ± S.E.M.

Figure 7 PAR-2 deficient mice have a significant decrease in liver weight (*P<0.001). Data are expressed as mean ± S.E.M.

In the chimeric model, mice that were PAR2+/−/BM-PAR2+/− had the most significant reduction in epididymal fat (P<0.001) in contrast to PAR2+/+ /BM-PAR2+/+ mice. Significant attenuation of epididymal fat development was demonstrated in PAR2+/− /BM-PAR2+/+ (P=0.004) and PAR2+/+ /BM-PAR2+/− (P=0.015) mice relative to the PAR2+/+ /BM-PAR2+/+ mice (Figure 8).

Similarly, mice that were PAR2+/−/BM-PAR2+/− had the most significant reduction in retroperitoneal fat (P<0.001) in contrast to PAR2+/+ /BM-PAR2+/+ mice. PAR2+/−/BM-PAR2+/+ and PAR2+/+ /BM-PAR2+/− mice showed significant attenuation of retroperitoneal fat
development (P=0.010 and P=0.010, respectively) relative to the $PAR2^{+/+}/BM-PAR2^{+/+}$ mice (Figure 9).

Liver weight was quantified and found to be significantly (P<0.001) lower in $PAR2^{-/-}/BM-PAR2^{-/-}$ mice when compared to $PAR2^{+/+}/BM-PAR2^{+/+}$ mice. A trend in liver weight reduction was demonstrated in $PAR2^{-/-}/BM-PAR2^{+/+}$ and $PAR2^{+/+}/BM-PAR2^{-/-}$ mice relative to the $PAR2^{+/+}/BM-PAR2^{+/+}$ mice (Figure 10).

Figure 8 Global PAR-2 deficient mice show significant reduction in epididymal fat production (*P<0.001) when fed a high fat diet for 12 weeks. Data are expressed as mean ± S.E.M. **P=0.004, ***P=0.015
Figure 9 Global PAR-2 deficient mice show significant reduction in retroperitoneal fat production (*P<0.001) when fed a high fat diet for 12 weeks. Data are expressed as mean ± S.E.M. **P=0.010

Figure 10 Global PAR-2 deficient mice have a significant decrease in liver weight (*P<0.001). Data are expressed as mean ± S.E.M.
Glucose Tolerance

Blood glucose concentrations were not statistically different between \( Ldlr^{-/-}PAR2^{+/+} \) and \( Ldlr^{-/-}PAR2^{-/-} \) mice under normal chow conditions (Figure 11).

![Figure 11](image1.png)

Figure 11 No significant differences occur between PAR-2 proficient and PAR-2 deficient mice following IPGTT. Data are expressed as mean \( \pm \) S.E.M

Insulin Tolerance

Under basal conditions, blood glucose concentrations were not statistically different between \( Ldlr^{-/-}PAR2^{+/+} \) and \( Ldlr^{-/-}PAR2^{-/-} \) mice following IPITT (Figure 12).

![Figure 12](image2.png)

Figure 12 No significant differences occur between PAR-2 wild type and PAR-2 knockout mice following IPITT. Data are expressed as mean \( \pm \) S.E.M
Discussion

The decrease in weight gain observed within PAR-2 deficient mice compared to the PAR-2 proficient mice indicates a potential correlation with adipose development. We further assessed depot accumulation in the epididymal and retroperitoneal regions of PAR-2 deficient and proficient mice. Excess accumulation of adipose tissue within these regions in both mice and humans is traditionally associated with increased risk of obesity (Blüher 2009). In PAR-2 deficient mice, a decrease in epididymal and retroperitoneal depot size suggests a potential role for PAR-2 as a signaling regulator of obesity. Although our animal model was not obesity specific, we found similar results as Badeanlou et al. (2012). In their obesity study, Badeanlou et al. (2012) found that TF-PAR-2 deficient mice had a reduction in adipose depot accumulation. Additionally, our results show a reduction in liver size within PAR-2 deficient mice, which may be due to a decrease in hepatic steatosis. Badeanlou et al. (2012) also noted a decrease in hepatic steatosis in TF-PAR-2 deficient mice due to macrophage inflammation. Within humans, hepatic steatosis—often termed non-alcoholic fatty liver disease— is often a comorbid condition associated with obesity (Youssef 2002). Our observations, in conjunction with Badeanlou et al. (2012), may elucidate a potential role of PAR-2 in liver inflammation. Additional histological analysis of PAR-2 deficient and proficient samples would further validate these findings.

GPCRs have been shown to influence obesity (Haas 2009, Shi 2013) and modulate adipose tissue macrophage dependent inflammation (Badeanlou 2012, Oh 2010). For example, the G-protein estrogen receptor-30 (GPR30) has been shown to influence blood pressure regulation, vascular tone, and obesity in rats (Haas 2009). Additionally, inactivation of the melanocortin-4 receptor (MC4-R) within the brain of mice shows development of maturity onset
obesity syndrome associated with hyperinsulinemia, hyperphagia, and hyperglycemia (Huszar 1997). Reduction in inflammation within monocytic cells and primary intraperitoneal macrophages has been observed when GPCR 120 is stimulated by omega-3 fatty acids (Oh 2010). PAR-2, as a GPCR, may operate in a similar pathway as other (i.e., GPR30, MC4-R, and GPR120) GPCRs known to affect obesity development and inflammation. Our metabolic observations elucidate the role of PAR-2 as a potential regulator of obesity. Further mechanistic studies will be necessary to determine the signaling pathway associated with PAR-2 influence on diet induced obesity.

We found both systemic and hematopoietic PAR-2 deficient mice showed a significant decrease in weight gain, adipose depot accumulation, and liver size relative to PAR-2 proficient mice. Moreover, we found that PAR-2 deficient mice who received wild-type bone marrow and PAR-2 proficient mice who received deficient bone marrow showed a significant decrease in both weight gain and adipose depot accumulation relative to PAR-2 proficient mice who received wild-type bone marrow. These data are consistent with Badeanlou et al. (2012) who showed that wild-type bone marrow chimeras in proficient, TF, or PAR-2 deficient mice on a HFD had significant weight gain relative to TF-PAR-2 deficient mice. A possible explanation for these results is that PAR-2 serves an important role in adipose accumulation around gonadal and retroperitoneal tissues. This may explain why we observed a decrease in epididymal and retroperitoneal fat weight alongside weight attenuation within mice who had PAR-2 removed somatically and/or hematopoietically. Additional studies are needed to further assess if removal of the PAR-2 receptor has added effects other than a decrease in weight gain, depot accumulation, and liver size.
Under normal feeding conditions, there was no significant change in glucose tolerance or insulin sensitivity within PAR-2 deficient mice relative to PAR-2 proficient mice. Badeanlou et al. (2012) demonstrated that glucose tolerance and insulin sensitivity was improved in TF-PAR-2 deficient mice fed a 60% DIO diet. These results suggest that PAR-2 deficiency may improve glucose tolerance and insulin sensitivity under DIO conditions. Our current study found that insulin sensitivity was not affected by PAR-2 status under normal feeding conditions, however, a potential limitation may exist in our assessment of insulin sensitivity. Extended release insulin was used in our testing of PAR-2 deficient and proficient mice. A secondary analysis using fast acting insulin may be necessary to further validate these observations.

It is known that humans possess PAR-2 throughout the body (D’Andrea 1998). Although the current study was performed within a murine model, our results suggest that PAR-2 deficiency may lead to the attenuation of excess epididymal and retroperitoneal adipose depots. Within humans, an excess accumulation of fat within these depots is associated within an increase in insulin resistance, a decrease in glucose tolerance, development of T2DM, and obesity (Blüher 2009, Rassouli 2007, Shi 2013). Our findings are in agreement with Badeanlou et al. (2012) who found that TF-PAR-2 deficient mice had a significant attenuation of weight gain and reduction in epididymal fat pad weight. This suggests that PAR-2 may serve an important role in adipose tissue development. Having observed the effects of PAR-2 within an animal model, future studies may begin to assess the pharmacological role of a potential PAR-2 antagonist in the treatment of diet-induced obesity.

**Conclusion**

Consumption of a high fat, westernized diet for 12 weeks leads to attenuation of weight gain in PAR-2 deficient mice. PAR-2 deficient mice showed reduced adiposity in terms of
epididymal and retroperitoneal fat mass as well as a reduction in liver weight relative to PAR-2 proficient mice. In addition, no significant change in glucose tolerance and insulin resistance was observed between PAR-2 proficient and PAR-2 deficient mice under normal feeding conditions.
Chapter 4 Conclusions

Protease-activated receptor 2 is a G-protein coupled receptor that, when activated by tissue factor, contributes to thrombosis and vascular disease. The current study sought to investigate the effects of a ‘Western’ diet on atherosclerotic and metabolic parameters in PAR-2 proficient and deficient mice. Our results suggest that PAR-2 deficiency attenuates atherosclerotic burden and reduces weight gain and adipose deposition without affecting plasma lipids in a diet-induced atherogenic murine model.
References


82. Oh DY, Talukdar S, Bae EJ. GPR120 is an Omega-3 Fatty Acid Receptor Mediating Potent Anti-Inflammatory and Insulin Sensitizing Effects. *Cell.* 2010;142:687-698.


