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# Identification Of Lipolysis-Derived Lipid Mediators And The Activation Of A Pro-Inflammatory Cyclooxygenase Pathway, Via Cyclooxygenase-2, In Adipose Tissue

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# IDENTIFICATION OF LIPOLYSIS-DERIVED LIPID MEDIATORS AND THE ACTIVATION OF A PRO-INFLAMMATORY CYCLOOXYGENASE PATHWAY, VIA CYCLOOXYGENASE-2, IN ADIPOSE TISSUE

by

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## DEDICATION

*This dissertation is dedicated to my family who has been an amazing support system for me throughout my entire life. They always tell me that I can do anything I put my mind to and to never give up. They have instilled in me the value of a strong work ethic, a motivation to succeed, and to live life to the fullest. I am so thankful for all of their love and encouragement.*

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# CHAPTER 1: INTRODUCTION

## Obesity – A Global Epidemic

Obesity is a global epidemic and its co-morbidities; including type 2 diabetes, cardiovascular disease, and metabolic syndrome, represent today's major public health crises (1-3). Excessive food intake coupled with a sedentary lifestyle can, over time, contribute to obesity. According to the World Health Organization (WHO), individuals with a body mass index (BMI) of 30 $kg/m^2$ or more are considered obese (4). In 2014, an estimated 600 million adults were obese, which is more than double what it was in 1980 (4). This overwhelmingly demonstrates the gravity of the exponentially growing obesity epidemic. Figure 1 depicts the worldwide prevalence of obesity in adult men and women in 2014 (5,6). Most of the regions of the world where the prevalence of obesity is the highest are associated with the Western Diet, which contains foods with high levels of saturated fats, starches, and sugars (7,8). Individuals overindulging in this type of diet are more susceptible to becoming obese. The health complications faced by the majority of people who are obese suffering from co-morbidities are overbearing and contribute to an overall lower quality of life and a shortened lifespan of about 20 years compared to non-obese individuals (9). Based on 2006 data, medical costs associated with treating obesity were roughly $147 to $210 billion and accounted for more than 10% of all annual health care spending in the United States alone (10). Based on the current trend, the financial burden of the treatment of obesity and its associated diseases is expected to increase. Obesity is characterized by excess fat accumulation in the body, which often leads to health complications and morbidities. Therefore, in order to combat this complex condition, it is imperative to research the molecular mechanisms and responses in adipose tissue that are occurring in obesity.

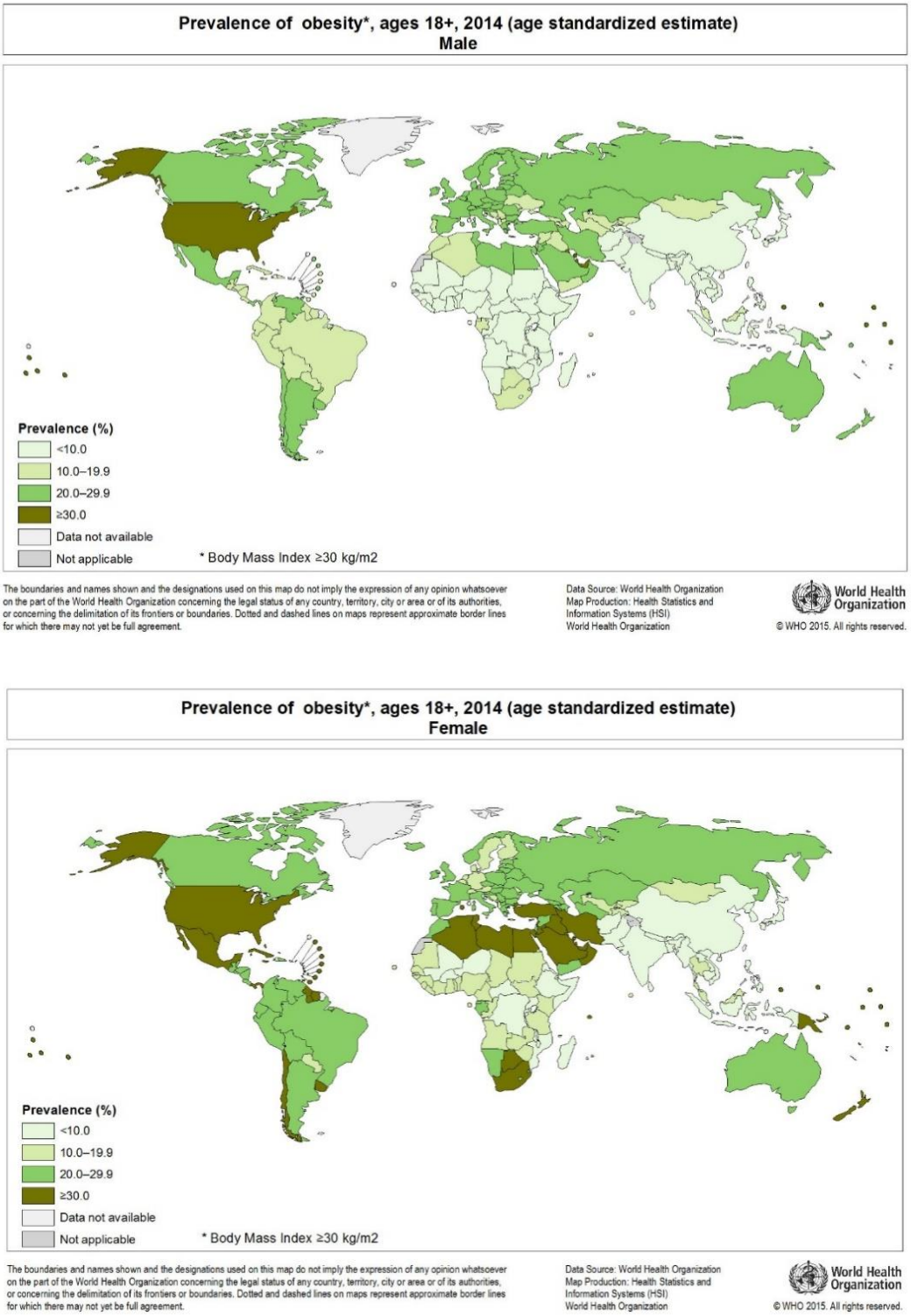


**Figure 1: Worldwide prevalence of obesity in 2014 of adult men and women.**

The World Health Organization depicts the global prevalence of obesity in the year 2014 of adult men and women (5,6).

## Functions of Adipose Tissue

Adipose tissue is composed of adipocytes, or fat cells, pre-adipocytes, resident macrophages, fibroblasts, and endothelial cells (11). The two main types of adipose tissue are brown and white. Brown adipose tissue is multilocular and contains numerous mitochondria that oxidize fatty acids in order to produce heat to keep the body warm (12). Although brown adipose tissue is prevalent in newborns and infants, there is very little in human adults (12). White adipose tissue is unilocular and can be subcutaneous or visceral, which is primarily in the abdominal region. The primary function of white adipose tissue is to store fat as triglycerides for use during periods of high energetic demand and low calorie intake in a lipid droplet within the adipocytes. The white adipose tissue also serves as an endocrine organ. White adipose tissue is the focus of this study.

### *Fat deposition and Fat mobilization*

White adipose tissue is responsible for regulating energy homeostasis through the processes of lipogenesis and lipolysis. Lipogenesis is the process of storing fat as triglycerides in the lipid droplet of adipocytes. The lipid droplet has a hydrophobic core containing triglycerides and cholesterol that is contained by a monolayer of phospholipids (13). The diameter of the lipid droplet typically ranges from 20-100 μm and often occupies the majority of the cell's volume (14). A triglyceride (TG) is a molecule that has three fatty acids attached to a glycerol backbone. The fat that is stored in the lipid droplets of adipocytes is derived from fatty acids that are absorbed in the diet or from fatty acid synthesis in the liver (15). When food is digested, lipases in the small intestine breakdown the TGs into fatty acids, which are then absorbed along with sugars, proteins, and other nutrients by the small intestine. The fatty acids are converted back to TGs and packaged into a lipoprotein known as a chylomicron for transport to other parts of the body. The apolipoprotein B (ApoB) on the chylomicron activates the lipoprotein lipase (LPL) in the capillaries, which hydrolyzes the TGs into glycerol and fatty acids (16). The majority of these fatty acids are taken up by the adipocytes and re-esterified to form TGs for storage in the lipid droplet. De novo

lipogenesis involves synthesizing fatty acids from glucose in the liver. These fatty acids are converted to TGs, packaged into very low density lipoproteins (VLDLs), and released into the bloodstream. Like chylomicrons, the VLDLs possess ApoB that activates the LPL, triggering the hydrolysis of TGs into glycerol and fatty acids (16). These fatty acids are taken up by the adipocytes, re-esterified into TGs, and stored in the lipid droplet of the adipocytes.

Conversely, lipolysis is the breakdown of the triglycerides that are stored in the lipid droplets of the adipocytes into glycerol and free fatty acids. When the body is in the fasting state or when energy is in high demand, lipolysis is stimulated. A series of lipases hydrolyze the TGs into glycerol and fatty acids that are then released into the bloodstream. Upon exiting the adipocyte, the fatty acids bind to albumin and travel to different tissues, such as the liver and skeletal muscle. There, they are used as substrates to produce adenosine triphosphate (ATP), the cell's main form of energy (12,17). In addition to energy production, the released fatty acids can be used as scaffolds for cell membrane phospholipids or for the synthesis of bioactive lipid mediators (18). The molecular mechanism of lipolysis will be described in detail in the Lipolysis section of the Introduction.

The balance of lipogenesis and lipolysis and their intricate molecular signaling pathways are carefully regulated by the responses of the adipocytes to hormones. Immediately after a meal, insulin is secreted from the pancreas and stimulates lipogenesis in the body. Insulin not only stimulates the LPL, but is also involved in activating the glucose transporters in different tissues that facilitate the uptake of glucose for storage and use at a later time. In the fasting state, glucagon and catecholamines stimulate lipolysis in the adipose tissue, which causes the mobilization of nutrients and provides the body with energy.

In addition, one's overall health and energy homeostasis can be affected by the type of fatty acids consumed in the diet that are stored in the adipocytes and incorporated in cell membranes. Omega-3 and omega-6 fatty acids are considered essential fatty acids because they are unable to be synthesized in humans and must be obtained through the diet (19). The

difference between these types of fatty acids is the position of the double bond from the methyl end of the fatty acid's molecular structure. The Western diet, consisting mainly of red meat, eggs, and processed food, has high levels of omega-6 fatty acids. High levels of omega-6 fatty acids have been shown to produce pro-inflammatory effects and promote cardiovascular disease, different kinds of cancer, and rheumatoid arthritis (18,19). On the contrary, omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that are commonly found in fish, cod liver oil, and flaxseed oil, have been shown to produce anti-inflammatory effects and help fight these diseases (20). The omega-6 to omega-3 fatty acid ratio is overwhelmingly high in the Western diet; 20-30:1 versus the ideal 1:1 ratio (21). In regards to energy homeostasis, studies suggest that omega-3 fatty acids play a role in down-regulating genes involved in lipogenesis leading to decreased fat accumulation (15,22,23). For example, in 3T3-L1 mouse adipocytes, DHA and EPA have been shown to reduce the activity of stearoyl-CoA desaturase (24,25) and EPA shown to down-regulate glucose-3-phosphate dehydrogenase activity (26). Fish oil fed rats displayed significantly lower plasma triglyceride levels and decreased LPL activity in the adipose tissue, demonstrating a favorable increase in fatty acid oxidation (15,27). Additional research has shown that DHA treatment can increase basal lipolysis in 3T3-L1 mouse adipocytes by up-regulating the adipose triglyceride lipase (ATGL), a key enzyme in the lipolysis process (24). Incorporating EPA in the differentiation process of 3T3-L1 adipocytes resulted in smaller lipid droplets and an up-regulation of hormone sensitive lipase (HSL) gene expression (25). Therefore, diet is influential in affecting lipid metabolism and energy homeostasis.

*Endocrine Organ*

Adipose tissue is not only utilized for storing triglycerides, but it also functions as an endocrine organ that produces and secretes cytokines, chemokines, and other signaling mediators, known as adipokines, into the bloodstream (12,28,29). Adipokines carry out their physiological functions in an autocrine, paracrine, or systemic manner by binding to their

respective receptors. Leptin and adiponectin represent two adipokines that are involved in energy homeostasis and glucose and lipid metabolism (29).

In 1994, Dr. Jeffrey M. Friedman's laboratory at Rockefeller University identified the first adipokine, leptin, as the missing gene in the grossly obese *ob/ob* mice (30). The name, leptin, comes from the Greek term, *leptos*, meaning thin (28,29). When leptin is secreted by the adipose tissue, it activates its receptors in the hypothalamus and in the adipose tissue to control appetite and promote fatty acid oxidation, respectively (31,32). Although leptin exhibits favorable effects at normal quantities, high levels of leptin are found in obese individuals (33,34). The increased fat accumulation in obesity leads to the desensitization of the body to leptin, disrupting the homeostasis of energy metabolism (35).

Unlike leptin, there is an inverse correlation between the levels of circulating adiponectin in the plasma and body mass (36,37). Therefore, adiponectin is high in healthy individuals and low in obese subjects. Low levels of adiponectin are associated with diseases such as diabetes mellitus, hypertension, cardiovascular diseases, and metabolic syndrome (38-40). In fact, in 2001, Yamauchi et al. discovered that adiponectin treatment lowered plasma triglycerides, increased fatty acid oxidation, and improved the insulin resistance endured by the KKAy and *db/db* mice (41). Adiponectin has also been shown to inhibit the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), a transcription factor involved in multiple inflammatory signaling pathways (42), and decrease the expression of monocyte adhesion molecules in the endothelium (43), therefore resulting in anti-inflammatory and anti-atherosclerotic effects.

Tumor necrosis factor α (TNFα), interleukin-6 (IL-6), plasminogen activator inhibitor 1 (PAI-1), and monocyte chemotactic protein 1 (MCP-1)/chemokine (C-C motif) ligand 2 (CCL2) are pro-inflammatory adipokines that contribute to the initiation and exacerbation of adipose tissue inflammation seen in obesity (28), and will be described in more detail in the next section.

**Adipose Inflammation**

Obesity involves excess fat accumulation and chronic low-grade inflammation in the adipose tissue (3,18,44). This chronic low-grade inflammation is characterized by a significant production of pro-inflammatory cytokines followed by the recruitment and activation of macrophages in the adipose tissue (45). When the caloric intake consistently exceeds the caloric expenditure, an excess amount of fat is stored in the lipid droplet causing the adipocytes to expand and possess a hypertrophied phenotype. It has been reported that the lipid droplet has the ability to expand up to 1000 times its normal volume and 10 times its diameter to accommodate the fat storage (46). The distended lipid droplet causes the nuclei of the adipocytes to be pushed to the perimeter of the cell causing physical strain and stress on the cell membrane. The hypertrophied adipocytes in obese adipose tissue produce and secrete significantly high levels of pro-inflammatory cytokines, including TNFα, IL-6, PAI-1, and MCP-1/CCL2, and very low amounts of anti-inflammatory cytokines, compared to adipose tissue seen in lean, healthy individuals (47).

*Pro-inflammatory cytokines*

TNFα was first recognized as a cytokine produced by macrophages that had antagonistic effects against tumors, thus its name, tumor necrosis factor α (48). Since then, TNFα has been shown to be highly expressed in the adipose tissue of obese subjects (49,50) and play critical roles in stimulating inflammation and insulin resistance. TNFα binds to its receptor and activates NFκB, triggering multiple inflammatory cascades (28), and impairs insulin signaling in the skeletal muscle and adipose tissue by interfering with the phosphorylation of insulin receptor substrate 1 (IRS-1) (28,51). In addition, TNFα has been linked to increased rates of atherosclerosis (52). Therefore, up-regulated TNFα expression is associated with many adverse effects in the adipose tissue and the whole body.

Another inflammatory adipokine is interleukin-6. High levels of circulating IL-6 have been linked to obesity and glucose intolerance (28,53). The overall effects of IL-6 on adiposity and

insulin resistance differ in the peripheral versus central nervous system. Peripheral administration of IL-6 has been shown to inhibit insulin signaling, thus contributing to insulin resistance, and to reduce the production of adiponectin (54,55). In contrast, central administration of IL-6 has displayed a decrease in adiposity and increased energy expenditure (56). Further research is warranted on the physiological functions and involvement of IL-6 in cell signaling pathways.

Plasminogen activator inhibitor 1 is produced and secreted from the adipose tissue and blocks the functions of urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) (28). As a result, the breakdown of fibrin is inhibited. High levels of PAI-1 are associated with obesity (57), insulin resistance (58), and myocardial infarctions (59). The oral PAI-1 antagonist, tiplaxtinin, has been shown to ameliorate insulin resistance and reduce fat accumulation, but due to the risk of toxic and adverse effects, is not used commercially (60). Consequently, PAI-1 is another adipokine that produces undesirable effects at abnormal levels.

Monocyte chemotactic protein 1 (MCP-1)/chemokine (C-C motif) ligand 2 (CCL2) is significantly increased in obesity and is responsible for recruiting monocytes and macrophages to the hypertrophied adipose tissue (61). MCP-1/CCL2 acts by binding its receptor, CCR2, expressed on the monocytes (29). In 2006, Dr. Stuart Weisberg's laboratory showed that the mice lacking CCR2 fed a high fat diet possessed improved insulin resistance, reduced body weight and macrophage accumulation, and down-regulated inflammatory genes in the adipose tissue (62). The Weisberg laboratory also administered a pharmacological CCR2 inhibitor to diet-induced obese mice and found significantly reduced macrophage accumulation in the adipose tissue (62). Therefore, MCP-1/CCL2 plays a critical role in the development of adipose inflammation.

The pro-inflammatory cytokines released from the hypertrophied adipocytes can also activate the endothelial cells in the adipose tissue (47). As a consequence, the endothelial cells produce cell adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule 1 (ICAM-1), that facilitate the attachment of monocytes to the

endothelial cell wall and their passage through the cell-cell junctions into the adipose tissue (47). As reported by Claudio Ferri et al in 1999, circulating levels of VCAM-1 and ICAM-1 are significantly higher in obese adults (63). Therefore, the activation of the endothelium contributes to inflammation in the adipose tissue.

*Macrophages*

Due to the up-regulated gene expression of MCP-1/CCL2 in hypertrophied adipose tissue, there is a mass accumulation of macrophages that are recruited to the adipose tissue in obese mice and humans (64). In fact, the amount of macrophages in the adipose tissue is directly related to an individual's BMI (64). The recruited macrophages possess a different phenotype than the macrophages that already reside in the adipose tissue. The resident macrophages are "alternatively activated" by cytokines, interleukin-4 and interleukin-13, and possess an M2 phenotype (65). The M2 macrophages express an arginase enzyme that blocks the inducible nitric oxide synthase (iNOS) and also produce anti-inflammatory cytokines (65). The recruited macrophages have an M1 phenotype and are "classically activated" by interferon gamma (INF-γ) or lipopolysaccharide (LPS) (65). The M1 macrophages produce high levels of pro-inflammatory cytokines, reactive oxygen species (ROS), and trigger iNOS reactions (65). The different macrophage phenotypes are associated with different metabolic properties. M1 macrophages induce insulin resistance by inhibiting glucose uptake in response to insulin, whereas M2 macrophages enhance insulin signaling (66). The majority of macrophages found in lean, healthy individuals possess the M2 phenotype whereas an abundance of M1 macrophages are seen in obese individuals (67). Therefore, it has been suggested that the macrophages in obese adipose tissue undergo a phenotypic conversion from M2 to the inflammatory, M1 phenotype (66,67). However, as described by Dr. Carey Lumeng et al in 2008, this switch is more likely linked to differential macrophage recruitment (66). Macrophages can produce and release pro-inflammatory cytokines that recruit more macrophages to the adipose tissue as well as activate the endothelium, promoting their own attachment and diapedesis.

*Hypoxia and ER Stress*

Due to the enlarged size of the adipocytes in obese adipose tissue, the blood flow carrying necessary oxygen and nutrients to the cells is limited. When the availability of the oxygen and nutrients do not meet the demands of the cells, localized hypoxia and necrotic cell death could occur (68). A histologic characteristic of necrotic cell death in the adipose tissue is the formation of “crown-like” structures by the macrophages (69). Most of the M1 macrophages recruited to the adipose tissue aggregate and surround the dead or dying adipocytes (66). In 2005, Dr. Saverio Cinti et al reported that the rate of adipocyte necrosis is directly related to the size of the adipocytes and takes place about 30 times more in in obese humans and mice models (69).

In addition, hypoxia induces stress on organelles such as the endoplasmic reticulum (ER) in the adipocytes. ER stress is initiated when hypoxia, toxins, excess nutrition, or energy deprivation, disrupts the ER’s ability to properly assemble proteins, causing a build-up of unfolded proteins (70). ER stress has been shown to occur in obese adipose tissue and generate high levels of ROS that cause oxidative damage to lipids, proteins, and DNA (71). Mitochondria also produce elevated amounts of ROS in obesity and together, the two organelles perpetuate the oxidative stress and damage in the cells (72). In an attempt to reestablish homeostasis, the ER activates a coordinated series of signaling pathways that serve as a protective mechanism known as the unfolded protein response (UPR) (71). The UPR is mediated by three transmembrane proteins in the ER: inositol-requiring kinase 1 (IRE-1), pancreatic ER kinase (PERK), and activating transcription factor 6 (ATF6). If the ER stress in the cell is unable to be corrected, apoptosis signaling pathways are initiated (70).

*Excessive lipolysis*

In addition, the hypertrophied adipocytes in obese adipose tissue undergo excessive lipolysis (73,74) and secrete abnormally high levels of free fatty acids into the bloodstream (75,76). The surplus of free fatty acids is in part due to the adipose tissue’s compromised sensitivity to insulin, which would normally inhibit lipolysis (23). The fatty acids released from the

visceral adipose tissue enter the portal vein that travels directly to the liver (77). When the influx of fatty acids exceeds the oxidation capacity in the liver, triglyceride synthesis increases and hepatosteatosis (fatty liver) occurs (77). Hepatosteatosis is a form of lipotoxicity, which is a condition where fatty acids infiltrate and accumulate in different organs of the body other than the adipose tissue. Hepatosteatosis can progressively worsen into non-alcoholic steatohepatitis (NASH) or non-alcoholic liver disease (NALD) or even cause permanent damage and scarring (cirrhosis) in the liver (77). Fatty liver is a common occurrence in not only obese individuals but also those who are glucose intolerant or have diabetes mellitus. The buildup of triglycerides inhibits the ability of organs such as the liver and skeletal muscle to respond to insulin and take in glucose, therefore contributing to insulin resistance (18). Overtime, the beta cells in the pancreas will "burnout" from constantly producing and secreting insulin, which will result in diabetes mellitus.

Free fatty acids can also activate toll-like receptor 4 (TLR4) that is expressed in adipocytes and macrophages, resulting in the production of pro-inflammatory cytokines (78). The expression of TLR4 has been shown to be up-regulated in the adipose tissue of obese mice (61,78). TLR4 activation stimulates pathways that include c-jun N-terminal kinase (JNK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), which often lead to inflammation and insulin resistance. JNK can be activated by a variety of stimuli including pro-inflammatory cytokines, fatty acids, ROS, and oxidative stress (70,72). In obese animals, JNK activity has been shown to be significantly increased in the adipose tissue (79). JNK and IKKβ are serine/threonine kinases that, when activated, can interfere with the phosphorylation of the insulin receptor substrate 1 (IRS-1), thus contributing to insulin resistance. Therefore, excessive fatty acids that are released from recurrent lipolysis contribute not only to adipose tissue inflammation, but also lipotoxicity and adipocyte dysfunction.

*Exacerbated cycle*

The culmination of the hypertrophied adipocytes, pro-inflammatory cytokines, ER and oxidative stress, the elevated free fatty acids from excessive lipolysis, the up-regulated cell adhesion molecules in the endothelium, and the recruitment and activation of macrophages exacerbates inflammation in the adipose tissue. All of these factors are inter-related and cause adipose inflammation, insulin resistance, and a disruption in the ability of the adipose tissue to function properly. The involvement of the signaling molecules and mediators in chronic obesity can be recaptured by the acute activation of the beta-3 adrenergic receptor-mediated lipolysis in mouse adipocytes, which is discussed in the next section of the Introduction. Therefore, the activation of the beta-3 adrenergic receptor-mediated lipolysis provides a tractable model for studying adipose inflammation.

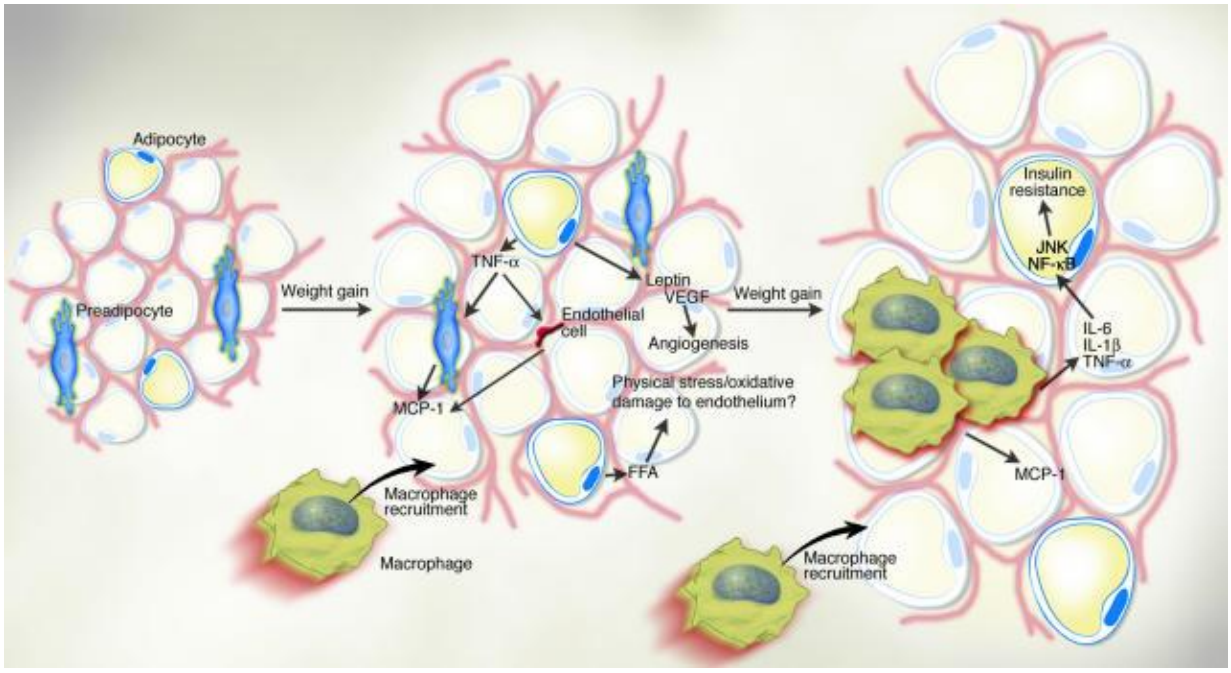


**Figure 2: The progression of inflammation in adipose tissue.**

As described by Wellen et al in the Journal of Clinical Investigation, persistent overeating causes large amounts of fat to be stored in the adipocytes causing them to become hypertrophied. The enlarged adipocytes secrete pro-inflammatory cytokines and chemokines, such as TNFα and MCP-1, which recruit macrophages to the adipose tissue. In addition, lipolysis occurs in excess in inflamed adipose tissue and the surplus free fatty acids could lead to oxidative stress. The macrophages, hypertrophied adipocytes, and the endothelium produce and secrete more pro-inflammatory cytokines that recruit more macrophages to the adipose tissue, which exacerbates the inflammation and interferes with the normal physiological functions performed by the adipose tissue (80).

**Lipolysis**

Lipolysis is initiated through the stimulation by catecholamines of the beta-adrenergic receptors (β1-3) that are expressed in the adipocytes (81). The beta-adrenergic receptors are G-protein coupled receptors that are associated with the Gs protein, which activates the adenylyl cyclase. In the adipose tissue, the β1 and β2-adrenergic receptors are mostly expressed and active in humans, while the β3-adrenergic receptor is predominantly expressed and active in rodents (82).

In order to study the signaling molecules and mediators involved in lipolysis, I used the well-studied, β3-adrenergic receptor (ADRB3)/hormone sensitive lipase (HSL)-signaling model in mouse adipocytes (83,84). The two chemical agonists that were used to stimulate the ADRB3 were isoproterenol (ISO) and CL 316-243 (CL). ISO is a non-selective beta-adrenergic receptor agonist that was used in the *in vitro* experiments with the 3T3-L1 mouse adipocyte cell line. CL is an agonist selective for the ADRB3 and was used in the *in vivo* experiments with the C57BL/6 mice. Following the stimulation of the ADRB3, hormone sensitive lipase, a key enzyme in the lipolytic process, is activated. BAY 59-9435 (BAY) is a selective HSL antagonist that was used in the *in vitro* and *in vivo* experiments (83).

When the ADRB3 is stimulated with ISO or CL, its associated Gs protein activates the adenylyl cyclase. Adenylyl cyclase converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). The increased levels of cAMP act as a second messenger to activate protein kinase A (PKA), which phosphorylates hormone sensitive lipase (HSL) and perilipin-1 (Plin1), a lipid droplet surface protein. In the basal state, comparative gene identification-58 (CGI-58), the co-activator of adipose triglyceride lipase (ATGL), is bound to the non-phosphorylated Plin1 (85). The phosphorylation of Plin1 causes CGI-58 to dissociate, allowing CGI-58 to interact with ATGL, maximizing ATGL's activity (86). The phosphorylation of HSL causes HSL to translocate from the cytosol to the surface of the lipid droplets containing Plin1 (86). The breakdown of a triglyceride molecule by a series of lipases takes place on the surface of the lipid

droplet as follows. A triglyceride is hydrolyzed by ATGL, resulting in one free fatty acid and diglyceride. Diglyceride is hydrolyzed by HSL, resulting in one free fatty acid and monoglyceride. Monoglyceride is hydrolyzed by monoglyceride lipase (MGL), resulting in one free fatty acid and glycerol. The majority of the glycerol and fatty acids are released into the bloodstream where they bind to albumin and travel to the liver and skeletal muscle for beta oxidation in order to provide the cells with energy (12,17). When lipolysis occurs in excess, as seen in obesity, the abundance of free fatty acids in circulation leads to an accumulation of fatty acids in the liver, heart, or other organs, causing lipotoxicity. The high levels of fatty acids contribute to the severity of the inflammation in the adipose tissue.

Mutations and genetic defects in the molecular players of lipolysis result in debilitating diseases. Mice lacking the *PNPLA2 (ATGL)* gene have been shown to exhibit significant triglyceride accumulation in multiple organs including the heart and die prematurely due to heart failure (87). On the contrary, transgenic mice overexpressing the *PNPLA2* gene display increased rates of lipolysis and fatty acid oxidation in the adipose tissue (88). Humans with a defect in the *PNPLA2* gene suffer from Neutral Lipid Storage Disease with Myopathy (NLSDM). Similar to the clinical manifestations in *PNPLA2* null mice, humans with NLSDM have triglyceride accumulations throughout the body, an enlarged liver, and suffer from cardiovascular diseases (89). Humans with a mutated CGI-58 gene suffer from Chanarin-Dorfman Syndrome. Those affected have triglyceride accumulations throughout the body due to the weakened ATGL activity, mild myopathy, and ichthyosis (89).

Hormone sensitive lipase knock out mice have an accumulation of diglycerides due to the disruption in the lipolytic process. Although they have hypertrophied adipocytes containing an increased number of macrophages, they are resistant to high fat diet-induced obesity and instead have elevated thermogenesis in their more pronounced brown adipose tissue (90). In humans, a mutation in the *LIPE* gene that encodes the HSL protein causes symptoms that are less prominent than those experienced in NLSDM (91). Even though those affected are not obese, they develop

partial lipodystrophy as they age and are diabetic (91). Furthermore, they have a fatty liver, a high plasma triglyceride count, and down-regulated genes involved in lipogenesis and lipid synthesis (91). Therefore, when lipolytic genes are mutated or defective, the energy homeostasis and lipid metabolism processes are disrupted. The clinical manifestations of these genetic disorders demonstrate the importance of these genes in maintaining overall health.

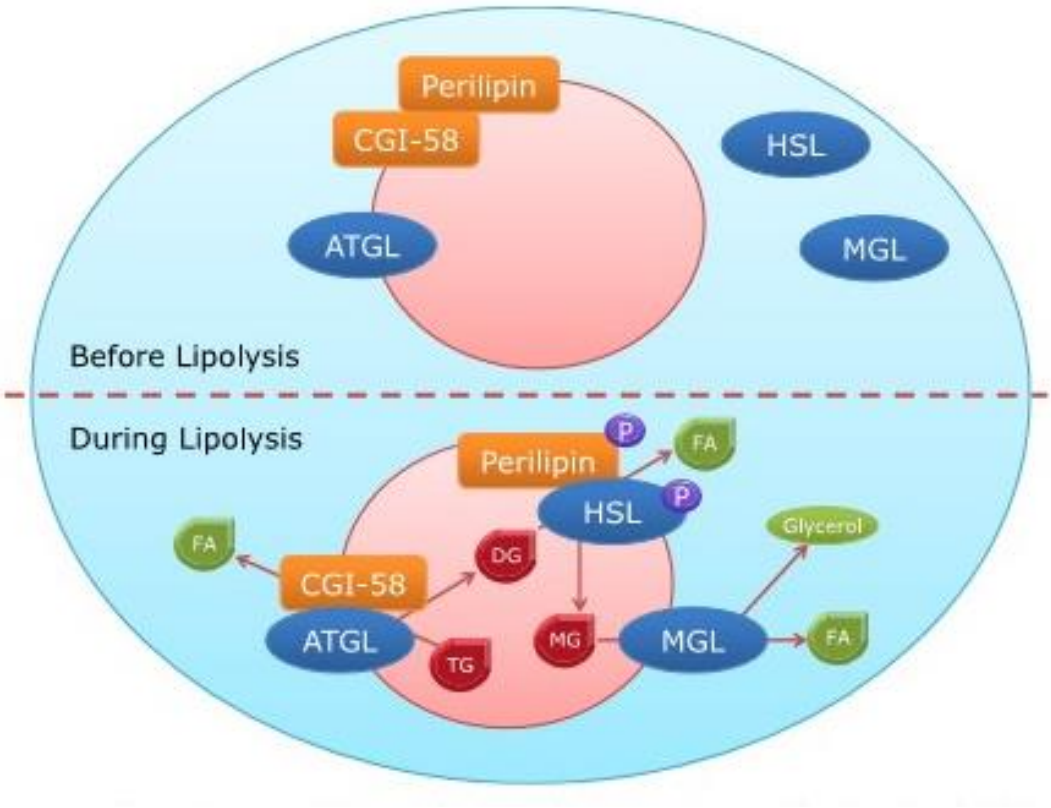


**Figure 3: Mechanism of lipolysis in adipocytes.**

As described in Luglio et al in the Journal of Clinical Biochemistry and Nutrition, in the basal state, perilipin is bound to CGI-58, the coactivator of adipose triglyceride lipase (ATGL), on the surface of the lipid droplet in the adipocytes. Upon phosphorylation of perilipin by protein kinase A (PKA), perilipin dissociates from CGI-58 which allows CGI-58 to associate with ATGL and maximize its activity. Hormone sensitive lipase (HSL) is also phosphorylated by PKA and translocates from the cytosol to the surface of the lipid droplet in close association with perilipin. The triglycerides are hydrolyzed by a series of lipases; ATGL, HSL, and monoglyceride lipase (MGL), into free fatty acids and glycerol (92).

**Eicosanoids**

The involvement of lipids in adipose tissue inflammation is of interest to scientists in the lipidomic field. Eicosanoids are a class of lipids derived from omega-3 and omega-6 fatty acids that have numerous physiological functions that contribute to homeostasis or the pathogenesis of many diseases, including cancer, inflammation, asthma, and autoimmune diseases (93). The name, eicosanoid, is derived from the Greek word, *eicosa*, meaning twenty, because most of the eicosanoids have 20 carbons in their molecular structure (94). The specific eicosanoids and the amounts produced are dependent on the type of cell and tissue. When a cell is exposed to an external stimuli, such as growth factors, hormones, or cytokines, phospholipase A2 (PLA2) and phosopholipase C (PLC) are activated (93). PLA2 cleaves membrane phospholipids at the sn-2 position releasing arachidonic acid, EPA, and DHA which are then used as substrates for the biosynthesis of eicosanoids (18). PLC cleaves the membrane phospholipid releasing diacylglyceride. Free fatty acids are readily metabolized into lipid mediators and exert their effects by binding to their respective receptors. The types of dietary fatty acids incorporated into the cell membranes dictate the types of eicosanoids produced and their physiological properties. The omega-3 fatty acids, EPA and DHA, and their derivatives exert anti-inflammatory effects, while metabolites from the omega-6 fatty acid, arachidonic acid, are mostly pro-inflammatory, with the exception of the lipoxins.

Arachidonic acid is metabolized by three main enzymatic pathways: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (see Figure 4). The COX enzyme produces prostaglandins, prostacyclins, and thromboxanes. The COX enzyme and its derivatives will be described in detail in the next section. The LOX enzymes produce hydroperoxyeicosatetraenoic acids (HPETEs), which are reduced by peroxidases to the corresponding hydroxyeicosatetraenoic acids (HETEs). Leukotrienes and lipoxins are also metabolites of the LOX enzymatic family. The cytochrome P450, or epoxygenase, enzymes produce epoxyeicosatrienoic acids (EETs) and HETEs.

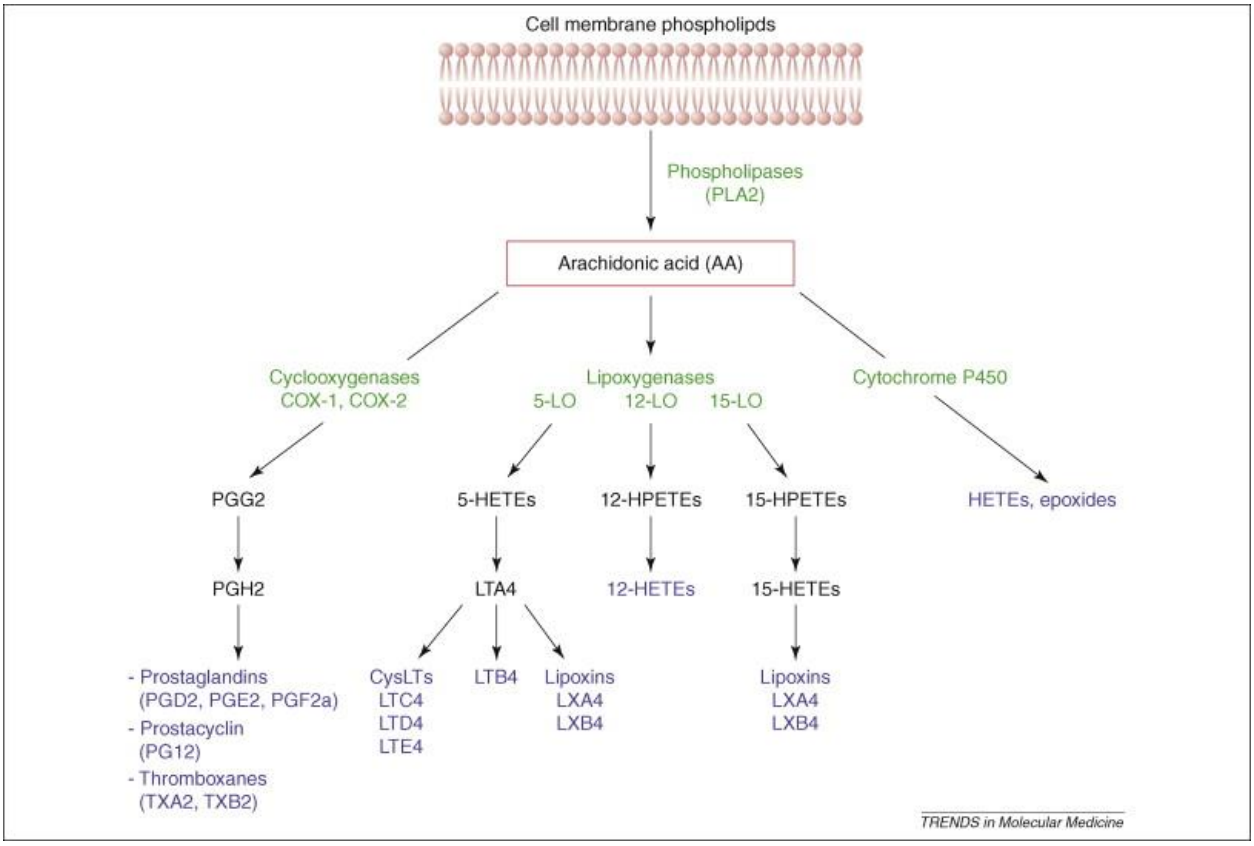


**Figure 4: Arachidonic acid metabolism and the generation of eicosanoids.**

As described in Harizi et al in Trends in Molecular Medicine, upon exposure of an external stimulus, phospholipase A2 (PLA2) is activated and cleaves the cell membrane phospholipids, releasing arachidonic acid. Arachidonic acid is metabolized by three main families of enzymes: cyclooxygenase (COX), responsible for producing prostaglandins, prostacyclins, and thromboxanes; lipoxygenase (LOX), which produces HPETEs, that are reduced to HETEs, leukotrienes, and lipoxins; and cytochrome P450 enzyme, which generates EETs and HETEs (93).

**Cyclooxygenase enzyme**

The cyclooxygenase (COX) enzyme, also known as prostaglandin H synthase, was first discovered using extracts of seminal vesicles from sheep (95). Extensive research revealed that the COX enzyme has two isoforms, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). In humans, the amino acid sequences of COX-1 and COX-2 are approximately 60% identical (96). The crystal structures of COX-1 and COX-2 also appear similar, however, COX-2 has a larger active site than COX-1 (97). The COX-2 gene has a TATA box and NFκB as an inducible enhancer element, which are not present in COX-1 (97). COX-1 is considered a "housekeeping" gene and is constitutively expressed in the majority of tissues in the body, whereas the COX-2 isoform is barely detected in most tissues and is an inducible gene that is stimulated by pro-inflammatory cytokines or mitogens. Both isoforms use their cyclooxygenase activity to transform arachidonic acid to prostaglandin G2 (PGG2), which is then rapidly converted to prostaglandin H2 (PGH2) by the COX enzyme's peroxidase activity (98). PGH2 is converted into different species of prostanoids, which include prostaglandins (PGD2, PGE2, PGF2α, d15-PGJ2 (dehydrated form of PGD2)), prostacyclin (PGI2), and thromboxane (TXA2) by specific enzymes. The most abundant prostaglandin is PGE2. Each of these bioactive metabolites have different chemical structures, varying functions, and mediate multiple cell signaling pathways.

The prostanoids derived from COX-1 are predominantly involved in regulating homeostasis and normal physiological functions in the body (99). For instance, PGE2 and PGI2 are involved in protecting the gastrointestinal and renal tracts, and the tone of the vasculature is influenced by the vasodilator actions of PGE2 and the vasoconstriction of PGF2α (99). The levels of prostacyclin (PGI2) and TXA2 dictate platelet function, as the former inhibits platelet aggregation and the latter triggers it (99). Prostaglandins have also been shown to be involved in regulating the sleep/wake cycle in the brain and inducing uterine contractions during labor (99,100). Moreover, 15d-PGJ2 is a potent PPAR-gamma agonist and mediates cell injury (101). Therefore, prostanoids have roles in regulating homeostatic functions in the body.

The prostanoids produced by the COX-2 isoform are predominantly involved in regulating inflammation in the body (99). COX-2 has been shown to be significantly up-regulated in pathological diseases such as rheumatoid arthritis and colon cancer (102,103). As a result, PGE2 is abundantly present and is hypothesized to play a role in the development of these diseases. COX-2 expression is also associated with the presence of the amyloid β peptide in Alzheimer's disease (104). In addition, myocardial infarctions and strokes are more likely to occur when there are disproportionate levels of PGI2 and TXA2 (105). Therefore, the dysregulation of prostaglandins by the COX enzyme contributes to the pathogenesis of inflammatory diseases and cancers.

In 1898, the first non-steroidal anti-inflammatory drug (NSAID), aspirin, was put on the market for its anti-inflammatory effects. It wasn't until the early 1970s that scientists discovered that the mechanism of aspirin was the inhibition of the COX enzyme and its prostaglandin derivatives (106). Non-selective NSAIDs inhibit the cyclooxygenase activity of COX-1 and COX-2 by binding to the active site. NSAIDs are blood thinners and exert antipyretic, analgesic, and anti-inflammatory effects (107). They have a protective effect from gastric and colorectal cancer, and even Alzeheimer's disease (96,108). The main adverse side effects associated with NSAIDs are gastric bleeding and ulcerations. This led to the development of a new class of selective NSAIDs that specifically inhibit COX-2 activity, known as the "coxibs". In 1999, celecoxib and rofecoxib were commercially introduced as the first selective COX-2 NSAIDs (109). Celecoxib and rofecoxib were prescribed to treat patients with arthritis and painful joints and to prevent the development of colon cancer (100,110). The GI and renal side effects seen in the non-selective NSAIDs were considerably improved, however, the platelet aggregation associated with this class of drugs created a significant cardiovascular risk; so much so that rofecoxib was removed from the market (105). Nevertheless, medications that block the activity of the COX enzyme, particularly the COX-2 isoform, in order to reduce inflammation are taken by millions of people around the world on a daily basis (108).

**Significance and Objective**

Although the roles of cytokines and other proteins in adipose tissue inflammation and lipolysis have been well studied, little is known about the involvement of bioactive lipid mediators. The identity of the specific bioactive lipids that are generated from adipocyte lipolysis are unknown and the physiological functions and roles they may play in pro-inflammatory signaling pathways in the adipose tissue are unclear. Research in this area would not only expand our knowledge in the lipidomic field in regards to adipose biology, but also reveal new signaling mechanisms that occur in adipose inflammation. A lipidomic approach would potentially produce novel findings and avenues for therapeutic targets for improving obesity and adipose inflammation in the future. The therapeutic strategies could include enhancing the activity of enzymes or specific bioactive lipids that exert anti-inflammatory effects or inhibiting those that produce adverse effects.

The objective of my doctoral research project was to characterize the adipose lipolysis-derived eicosanoid lipid mediators through the acquisitions and analyses of lipidomic profiles using the latest LC-MS/MS methods. Further investigation led to the discovery of a signaling pathway involving the lipolysis-triggered activation of COX-2 and its contribution to adipose inflammation.

## CHAPTER 2: METHODS AND MATERIALS

### Reagents

The beta adrenergic receptor agonist, isoproterenol (ISO) (Sigma), was dissolved in $H_2O$. The selective hormone sensitive lipase inhibitor, BAY 59-9435 (BAY), was chemically synthesized as described (111) and dissolved in 0.5% methylcellulose. The selective beta-3 adrenergic receptor agonist, CL 316-243 (CL) (Sigma), was dissolved in $H_2O$. The cyclooxygenase-2 inhibitor, celecoxib (Sigma), was dissolved in DMSO. The JNK inhibitor (SP-600125, Calbiochem), p38 inhibitor (SB 203580, Calibochem) and NFκB inhibitor (BAY 11-7082, Calbiochem) were dissolved in DMSO. The palmitic acid (Sigma) was dissolved in 0.4% fatty acid free fetal bovine serum (FBS). Antibodies against COX-1, COX-2, phospho-JNK, JNK, phospho-IκBα, IκBα, phospho-HSL, and HSL were from Cell Signaling. The polyclonal rabbit anti-F4/F80 and anti-GAPDH were purchased from Abcam and Santa Cruz, respectively. Other reagents, unless specified, were from Sigma.

### Cell culture

3T3-L1 and 3T3-L1-CAR cells were cultured and differentiated as previously described (84,112). Briefly, 3T3-L1 cells were cultured to about 80% confluency in DMEM medium with 10% bovine calf serum (BCS) and 1% penicillin/streptomycin antibiotic. Differentiation involved incubating the cells in DMEM medium (containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin) with 1 µg/ml insulin, 0.5 mM methylisobutylxanthine (MIX), and 0.25 µM dexamethasone (DEX) for three days. Next, the cells were incubated with DMEM medium (containing 10% FBS and 1% penicillin/streptomycin) with 1 µg/ml insulin for 48 hours. Two days post-differentiation, cells were cultured overnight in serum-free DMEM. Subsequently, media were replaced with phenol red free plain DMEM. Cells were treated with 10 µM of ISO or PBS control for 3 hours at 37ºC. Alternatively, cells were pretreated with a selective HSL inhibitor (BAY 59-9435, 10 µM), JNK inhibitor (SP-600125, 10 µM), p38 inhibitor (SB 203580, 10 µM), NFκB inhibitor (BAY 11-7082, 10µM), or cyclooxygenase-2 inhibitor (celecoxib, 5 µM) for 1 hour,

followed by stimulation with ISO (10 µM) or PBS control for an additional 3 hours. Cell pellets and culture media were collected and processed for biochemical analysis and lipid quantification by LC-MS/MS methods, respectively, as described below. Transduction of 3T3-L1-CAR cells with adenoviral particles was performed essentially as previously described (112).

**Sample preparation for LC-MS/MS analysis of lipid mediators**

Samples (0.85 ml) were spiked with 5 ng each (in 150 µl methanol) of 15(S)-HETE-d8, 14(15)-EpETrE-d8, Resolvin D2-d5, Leukotriene B4-d4, and Prostaglandin E1-d4 as internal standards for recovery and quantitation and mixed thoroughly. The samples were then extracted for PUFA metabolites using C18 extraction columns as described earlier (113-115). Briefly, the internal standard spiked samples were applied to conditioned C18 cartridges, washed with 15% methanol in water followed by hexane and dried under vacuum. The cartridges were eluted with 0.5 ml methanol. The eluate was dried under a gentle stream of nitrogen. The residue was dissolved in 50 µl methanol-25 mM aqueous ammonium acetate (1:1) and subjected to LC-MS analysis.

**LC-MS/MS quantification**

HPLC was performed on a Prominence XR system (Shimadzu) using Luna C18 (3µ, 2.1x150 mm) column. The mobile phase consisted of a gradient between A: methanol-water-acetonitrile (10:85:5 v/v) and B: methanol-water-acetonitrile (90:5:5 v/v), both containing 0.1% ammonium acetate. The gradient program with respect to the composition of B was as follows: 0-1 min, 50%; 1-8 min, 50-80%; 8-15 min, 80-95%; and 15-17 min, 95%. The flow rate was 0.2 ml/min. The HPLC eluate was directly introduced to ESI source of QTRAP5500 mass analyzer (ABSCIEX) in the negative ion mode with following conditions: Curtain gas: 35 psi, GS1: 35 psi, GS2: 65 psi, Temperature: 600 ˚C, Ion Spray Voltage: -1500 V, Collision gas: low, Declustering Potential: -60 V, and Entrance Potential: -7 V. The eluate was monitored by Multiple Reaction Monitoring method to detect unique molecular ion – daughter ion combinations for each of the lipid mediators using a scheduled MRM around the expected retention time for each compound.

Optimized Collisional Energies (18 – 35 eV) and Collision Cell Exit Potentials (7 – 10 V) were used for each MRM transition. Spectra of each peak detected in the scheduled MRM were recorded using Enhanced Product Ion scan to confirm the structural identity. The data was collected using Analyst 1.6.2 software and the MRM transition chromatograms were quantitated by MultiQuant software (both from ABSCIEX). The internal standard signals in each chromatogram were used for normalization, recovery, as well as relative quantitation of each analyte.

**Animal studies**

All animal procedures were performed according to the NIH and institutional guidelines, and were approved by the Wayne State University Animal Use and Care Committee. C57BL/6 mice (8 weeks old male, Jackson Laboratory) were used in this study. To examine the role of ADRB3/HSL signaling in the regulation of COX-2 expression, mice were intraperitoneally (*i.p.*) injected with the selective HSL inhibitor, BAY 59-9435 (30 mg/kg), celecoxib (100 mg/kg body weight), or vehicle controls as previous described (83,116,117). One hour later, mice were *i.p.* injected with 10 nmol of CL 316-243 or saline for an additional 3 hours (83,117). The mice were euthanized and the epididymal white adipose tissue (EWAT) pads were collected and processed for biochemical and immunohistochemical analysis as described below.

**Real-time PCR**

Total RNA was isolated from cultured cells using Trizol and was reversely transcribed with an oligo-dT primer (Promega) by M-MLV Reverse Transcriptase (Promega) for first strand cDNA synthesis. Total RNA was isolated from the EWAT using liquid nitrogen and a mortar and pestle to grind the tissue into a powder and then Trizol was added. Then, the RNA was reversely transcribed with an oligo-dT primer (Promega) by M-MLV Reverse Transcriptase (Promega) for first strand cDNA synthesis. For real-time PCR quantitation, 50 ng of reversely transcribed cDNAs were amplified with the ABI 7500 system (Applied Biosystems) in the presence of SYBR Green master mix. The PCR primer pairs used were: mouse PTGS1 (COX-1): sense, 5’ – ACA AAA

GAA CCC AGT GTC CA – 3', anti-sense, 5' – AGA ACT GTG GTG GTT TCC AA – 3'; mouse PTGS2 (COX-2): sense, 5' – TGA TCG AAG ACT ACG TGC AA – 3', anti-sense, 5' – GTG AGT CCA TGT TCC AGG AG – 3'; mouse CCL2: sense, 5' – CAC AGT TGC CGG CTG GAG CAT – 3'; anti-sense, 5' – GCT TCT TTG GGA CAC CTG CTG C – 3'; mouse IL-6: sense, 5' – ATG CTG GTG ACA ACC ACG GCC – 3', anti-sense, 5' – AAG CCT CCG ACT TGT GAA GTG G – 3'; mouse Mac-2: sense, 5' – AGG AGA GGG AAT GAT GTT GCC – 3', anti-sense, 5' – GGT TTG CCA CTC TCA AAG GG – 3'; mouse GAPDH: sense, 5' – CAC CTT CGA TGC CGG GGC TG – 3', anti-sense, 5' – GGC CAT GAG GTC CAC CAC CC – 3'; and rat COX-2: sense, 5' – CGT GGT GAA TGT ATG AGC – 3', anti-sense, 5' – CGA AGG AAG GGA ATG TTG – 3'. The qPCR reaction was performed by using a universal PCR Master Mix (Applied Biosystems) according to manufacturer's instructions. Relative quantification (RQ) was calculated using the SDS software (Applied Biosystems) based on the equation $RQ= 2^{-\Delta\Delta Ct}$ where Ct is the threshold cycle to detect fluorescence. Ct values were normalized to the internal GAPDH standard.

**Western blot analysis**

The protein extraction procedure and western blot analysis were performed as described (118). The cells were collected in ice-cold PBS using cell scrapers followed by centrifugation (250 × *g*, 5 min). The cell extracts were prepared in RIPA buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Calbiochem) and underwent constant agitation at 4 °C for 30 minutes. The epididymal white adipose tissue (EWAT) samples from mice were reconstituted in RIPA buffer containing protease inhibitors and sonicated until the tissue dissolved into the solution. The EWAT samples were then constantly agitated at 4 °C for 30 minutes. After centrifugation of the samples at 15,000 × *g* for 20 minutes, the supernatant was collected and the protein concentration was measured using a bicinchoninic acid protein assay kit with BSA as a standard. 50 µg of protein extracts were dissolved in 2× Laemmli sample buffer, heated at 95 °C for 5 minutes, and resolved on a 10% SDS-PAGE gel. After electrophoresis, gels were transferred

to nitrocellulose membranes. Subsequently, membranes were blocked in 5% non-fat dry milk (Lab Scientific) in TBST buffer (20 mM Tris–HCl, pH 7.4, 500 mM NaCl and 0.05% Tween-20). Membranes were washed and incubated with the indicated primary antibodies (1:1000 dilution) on a rotary shaker at 4 °C overnight. The blots were then incubated with peroxidase-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature, and developed with enhanced chemiluminescent reagent (Thermo Scientific).

**Immunofluorescence Staining**

Immunofluorescent staining was performed as described (112). After differentiated 3T3-L1 cells were treated with or without isoproterenol (10 μM) for 15 or 30 minutes, they were fixed with 4% paraformaldehyde at room temperature for 30 minutes. The cells were then permeabilized with 0.05% Triton X-100 and blocked with 1% bovine serum albumin for 30 minutes. Next, the cells were incubated with an NFκB primary antibody (1:100) followed by an FITC-conjugated secondary antibody (1:500). Fluorescence images were captured by the Leica TCS SP5 confocal system (Leica, Wetzlar, Germany). The nuclei were visualized by DAPI staining.

**Immunohistochemical staining**

The immunohistochemical staining procedure followed the protocol from the Vector Laboratories Vectastain Universal Elite ABC Kit (Anti-mouse IgG/Rabbit IgG, Cat. No. PK-6200). Briefly, mouse EWAT tissues were fixed in 10% formalin followed by paraffin embedding. Antigen retrieval was performed on the paraffin sections (5 μm) in citrate buffer (10 mM citric acid, 0.05% Tween-20, pH 6.0) at 90ºC for 10 min, and then deparaffinized by incubating the slides in xylene followed by a graded series of ethanol and then water. Endogenous peroxidase activity was quenched with 0.3% $H_2O_2$ for 5 minutes. After washes, sections were incubated with blocking serum (normal horse serum) for 20 minutes. Subsequently, samples were incubated with anti-F4/80 (1:200 dilution in PBS) at 4ºC overnight. The slides were then washed with PBS and incubated with the diluted biotinylated secondary antibody for 30 minutes. After washing with

PBS, Vectastain ABC Reagent was applied to the slides for 30 minutes. After washing with PBS, DAB substrate reagent was added to the slides for 10 minutes and then washed several times with water. Slides were examined and analyzed using the Leica inverted microscope and the image acquisitions were from the SPOT Pursuit monochrome digital camera.

**Transduction of 3T3-L1-CAR Cells**

Differentiated 3T3-L1-CAR cells were serum starved overnight in serum-free DMEM medium and then transduced with or without a multiplicity of 200 of the replicative-deficient adenoviral particles carrying the COX-2 vector (provided by Dr. Andrey Sorokin, Medical College of Wisconsin) for 1, 2, or 3 hours. 3T3-L1-CAR cells were collected for RNA isolation and qPCR quantification.

**Statistical analysis**

Results are shown as mean ± SD. Differences between various treatments were analyzed by ANOVA. Statistical significance was measured by Student's *t* – test. $p$ value < 0.01 is considered highly significant and $p < 0.05$ is considered statistically significant.

## CHAPTER 3: RESULTS

### Lipidomic characterization of eicosanoids generated by adipocyte ADRB3/HSL-mediated lipolysis.

The low grade chronic inflammation in the adipose tissue of obese individuals is in part due to excessive adipocyte lipolysis, which generates numerous lipid mediators. The identity of these lipolysis-produced lipid mediators is not known and their specific roles in regulating pro-inflammatory signaling in the adipose tissue are poorly understood. The resulting lipidomic profiles will fill a gap that has not yet been characterized, enhance our knowledge in the adipose tissue biology field, and provide new opportunities for future studies investigating the patho-physiological roles of the lipid mediators in adipose inflammation and the associated metabolic diseases.

In order to identify the bioactive lipid mediators generated by lipolysis, differentiated 3T3-L1 mouse adipocytes were treated with isoproterenol (ISO), a nonselective beta-adrenergic receptor agonist, or vehicle for three hours, in order to stimulate maximal lipolysis (84). The cell culture media was collected and analyzed for the presence of eicosanoids using the LC-MS/MS. From this experiment, I was able to acquire a lipidomic profile of the eicosanoids that were generated from adipocyte lipolysis. The lipidomic analysis was performed by the Wayne State University Lipidomics Core Facility following published methods (114,119,120). Out of more than 150 fatty acyl lipids detected, the levels of approximately 63 different eicosanoids, including metabolites derived from cyclooxygenase, lipoxygenase, and cytochrome P450 enzymes, were significantly elevated in the media of adipocytes treated with ISO. As a result, for the first time, a comprehensive lipidomic profile of the eicosanoids generated by ADRB3-stimulated lipolysis in adipocytes has been documented.

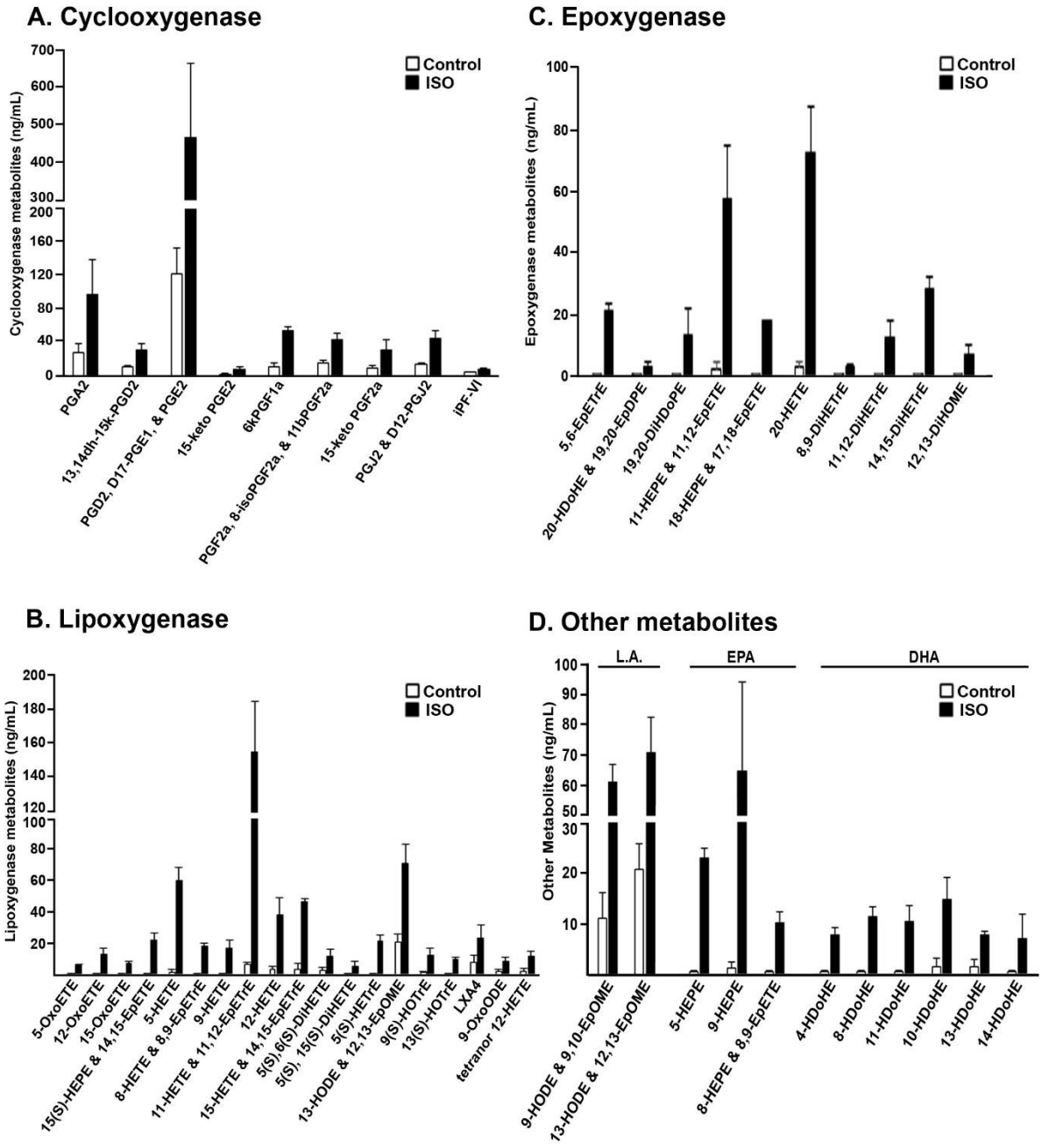


**Figure 5: Lipidomic profile of eicosanoids generated from ADRB3-stimulated lipolysis in mouse adipocytes.**

Differentiated 3T3-L1 mouse adipocytes were treated with ISO (10 µM), in order to stimulate the ADRB3, or PBS for 3 hours. The media was collected and analyzed by LC-MS/MS lipidomic methods. The lipidomic profile shows lipid metabolites of the cyclooxygenase (A), lipoxygenase (B), epoxygenase (C), and other metabolites (linoleic acid (LA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)) (D) following treatment. Data are from a representative experiment which was repeated three times with similar results. Statistical analysis show that all listed lipids are $p < 0.05$ (*t*-test) in comparing control vs. ISO.

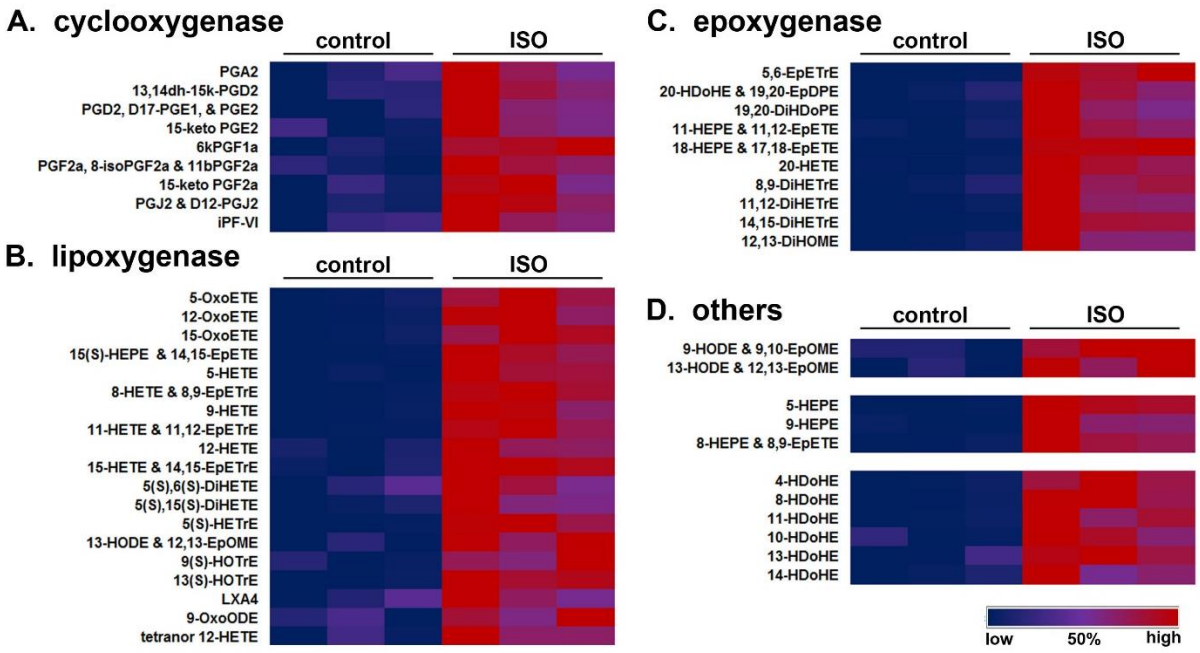


**Figure 6: Heat map of eicosanoids generated from ADRB3-stimulated lipolysis in mouse adipocytes.**

Differentiated 3T3-L1 mouse adipocytes were treated with ISO (10 µM), in order to stimulate the ADRB3, or PBS for 3 hours. The media was collected and analyzed by LC-MS/ MS lipidomic methods. The heat map shows lipid metabolites of the cyclooxygenase (A), lipoxygenase (B), epoxygenase (C), and other metabolites (linoleic acid (LA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)) (D) following treatment. Data are from a representative experiment which was repeated three times with similar results. Statistical analysis shows that all listed lipids are $p < 0.05$ (*t*-test) in comparing control vs. ISO.

| | | | | | | | Mean | | SD | | T-TEST |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control 1 | Control 2 | Control 3 | ISO 1 | ISO 2 | ISO 3 | control | ISO | control | ISO | Ctrl vs ISO |
| | (ng/ ml) | | | | | | (ng/ ml) | | (ng/ ml) | | |
| **A. Cyclooxygenase metabolites** | | | | | | | | | | | |
| **PGA2** | 18.71 | 27.77 | 37.71 | 142.29 | 92.29 | 57.87 | 28.06 | 97.48 | 9.50 | 42.45 | 2.53E-02 |
| **13,14dh-15k-PGD2** | 7.07 | 11.01 | 10.75 | 38.55 | 30.09 | 23.26 | 9.61 | 30.63 | 2.20 | 7.66 | 5.13E-03 |
| **PGD2, D17-PGE1, & PGE2** | 104.32 | 105.35 | 158.99 | 695.02 | 382.20 | 328.84 | 122.88 | 468.68 | 31.27 | 197.82 | 2.02E-02 |
| **15-keto PGE2** | 2.25 | 0.60 | 0.92 | 10.27 | 5.84 | 4.58 | 1.26 | 6.90 | 0.87 | 2.99 | 1.74E-02 |
| **6kPGF1a** | 8.14 | 14.76 | 9.89 | 50.76 | 53.22 | 58.34 | 10.93 | 54.11 | 3.43 | 3.87 | 6.65E-05 |
| **PGF2a, 8-isoPGF2a & 11bPGF2a** | 18.70 | 14.79 | 12.57 | 51.11 | 42.23 | 36.42 | 15.35 | 43.25 | 3.10 | 7.40 | 1.92E-03 |
| **15-keto PGF2a** | 7.55 | 11.23 | 8.62 | 35.85 | 39.25 | 18.04 | 9.13 | 31.05 | 1.89 | 11.39 | 1.52E-02 |
| **PGJ2 & D12-PGJ2** | 11.43 | 14.90 | 13.11 | 51.22 | 48.82 | 34.50 | 13.15 | 44.85 | 1.73 | 9.04 | 1.98E-03 |
| **iPF-VI** | 2.37 | 3.48 | 3.70 | 8.67 | 6.51 | 5.79 | 3.19 | 6.99 | 0.71 | 1.50 | 8.28E-03 |
| **B. Lipoxygenase metabolites** | | | | | | | | | | | |
| **5-OxoETE** | 0.50 | 0.60 | 0.92 | 5.37 | 6.69 | 5.09 | 0.67 | 5.72 | 0.22 | 0.85 | 2.90E-04 |
| **12-OxoETE** | 0.50 | 0.60 | 0.92 | 15.20 | 15.54 | 8.96 | 0.67 | 13.23 | 0.22 | 3.71 | 2.12E-03 |
| **15-OxoETE** | 0.50 | 0.60 | 0.92 | 6.14 | 8.51 | 7.51 | 0.67 | 7.39 | 0.22 | 1.19 | 3.27E-04 |
| **15(S)-HEPE & 14,15-EpETE** | 0.50 | 0.60 | 0.92 | 26.79 | 22.59 | 18.08 | 0.67 | 22.49 | 0.22 | 4.35 | 4.87E-04 |
| **5-HETE** | 0.50 | 3.27 | 0.92 | 70.39 | 55.90 | 54.54 | 1.56 | 60.28 | 1.49 | 8.78 | 1.68E-04 |
| **8-HETE & 8,9-EpETrE** | 0.50 | 0.60 | 0.92 | 18.67 | 19.86 | 16.41 | 0.67 | 18.31 | 0.22 | 1.75 | 3.29E-05 |
| **9-HETE** | 0.50 | 0.60 | 0.92 | 20.66 | 19.78 | 11.20 | 0.67 | 17.22 | 0.22 | 5.23 | 2.70E-03 |
| **11-HETE & 11,12-EpETrE** | 5.54 | 7.31 | 7.91 | 165.85 | 180.25 | 123.00 | 6.92 | 156.37 | 1.24 | 29.78 | 4.84E-04 |
| **12-HETE** | 4.46 | 0.60 | 4.78 | 51.24 | 32.73 | 31.04 | 3.28 | 38.33 | 2.33 | 11.21 | 3.03E-03 |
| **15-HETE & 14,15-EpETrE** | 2.94 | 0.60 | 7.46 | 47.98 | 47.95 | 43.93 | 3.67 | 46.62 | 3.48 | 2.33 | 2.96E-05 |
| **5(S),6(S)-DiHETE** | 0.50 | 2.49 | 4.98 | 16.32 | 12.41 | 7.34 | 2.66 | 12.02 | 2.24 | 4.50 | 1.61E-02 |
| **5(S),15(S)-DiHETE** | 0.50 | 0.60 | 0.92 | 8.95 | 3.63 | 3.22 | 0.67 | 5.27 | 0.22 | 3.19 | 3.39E-02 |
| **5(S)-HETrE** | 0.50 | 0.60 | 0.92 | 23.81 | 24.30 | 17.34 | 0.67 | 21.81 | 0.22 | 3.89 | 3.56E-04 |
| **13-HODE & 12,13-EpOME** | 17.70 | 26.74 | 18.26 | 78.53 | 57.75 | 79.03 | 20.90 | 71.77 | 5.06 | 12.14 | 1.29E-03 |
| **9(S)-HOTrE** | 2.27 | 0.60 | 0.92 | 10.91 | 8.27 | 17.28 | 1.26 | 12.15 | 0.88 | 4.63 | 8.06E-03 |
| **13(S)-HOTrE** | 0.50 | 0.60 | 0.92 | 11.03 | 9.18 | 10.06 | 0.67 | 10.09 | 0.22 | 0.92 | 3.37E-05 |
| **LXA4** | 4.52 | 7.54 | 12.46 | 32.32 | 21.93 | 16.41 | 8.17 | 23.55 | 4.01 | 8.08 | 2.09E-02 |
| **9-OxoODE** | 2.09 | 3.28 | 0.92 | 8.86 | 5.63 | 11.14 | 2.10 | 8.54 | 1.18 | 2.77 | 1.04E-02 |
| **tetranor 12-HETE** | 0.50 | 4.55 | 0.92 | 15.19 | 10.21 | 10.23 | 1.99 | 11.88 | 2.23 | 2.87 | 4.60E-03 |
| **C. Epoxygenase metabolites** | | | | | | | | | | | |
| **5,6-EpETrE** | 0.50 | 0.60 | 0.92 | 22.31 | 19.56 | 23.44 | 0.67 | 21.77 | 0.22 | 1.99 | 2.66E-05 |
| **20-HDoHE & 19,20-EpDPE** | 0.50 | 0.60 | 0.92 | 4.66 | 3.63 | 2.66 | 0.67 | 3.65 | 0.22 | 1.00 | 3.64E-03 |
| **19,20-DiHDoPE** | 0.50 | 0.60 | 0.92 | 23.00 | 11.17 | 6.32 | 0.67 | 13.50 | 0.22 | 8.58 | 3.04E-02 |
| **11-HEPE & 11,12-EpETE** | 2.59 | 0.60 | 4.97 | 76.91 | 53.92 | 42.72 | 2.72 | 57.85 | 2.18 | 17.43 | 2.78E-03 |
| **18-HEPE & 17,18-EpETE** | 0.50 | 0.60 | 0.92 | 18.21 | 18.15 | 18.89 | 0.67 | 18.42 | 0.22 | 0.41 | 1.53E-07 |
| **20-HETE** | 3.48 | 2.36 | 4.96 | 88.10 | 72.01 | 59.19 | 3.60 | 73.10 | 1.30 | 14.49 | 5.82E-04 |
| **8,9-DiHETrE** | 0.50 | 0.60 | 0.92 | 3.99 | 2.79 | 3.14 | 0.67 | 3.31 | 0.22 | 0.61 | 1.10E-03 |
| **11,12-DiHETrE** | 0.50 | 0.60 | 0.92 | 19.29 | 10.47 | 9.68 | 0.67 | 13.15 | 0.22 | 5.33 | 7.76E-03 |
| **14,15-DiHETrE** | 0.50 | 0.60 | 0.92 | 33.51 | 27.08 | 26.31 | 0.67 | 28.97 | 0.22 | 3.95 | 1.23E-04 |
| **12,13-DiHOME** | 0.50 | 0.60 | 0.92 | 10.98 | 5.37 | 5.31 | 0.67 | 7.22 | 0.22 | 3.26 | 1.27E-02 |
| **D. Others** | | | | | | | | | | | |
| **D.1. Metabolites of LA** | | | | | | | | | | | |
| **9-HODE & 9,10-EpOME** | 14.48 | 13.84 | 5.52 | 54.62 | 65.29 | 65.28 | 11.28 | 61.73 | 5.00 | 6.16 | 1.93E-04 |
| **13-HODE & 12,13-EpOME** | 17.70 | 26.74 | 18.26 | 78.53 | 57.75 | 79.03 | 20.90 | 71.77 | 5.06 | 12.14 | 1.29E-03 |
| **D.2. Metabolites of EPA** | | | | | | | | | | | |
| **5-HEPE** | 0.50 | 0.60 | 0.92 | 25.05 | 22.77 | 21.34 | 0.67 | 23.06 | 0.22 | 1.87 | 1.64E-05 |
| **9-HEPE** | 2.62 | 0.60 | 0.92 | 98.99 | 49.58 | 46.44 | 1.38 | 65.00 | 1.08 | 29.47 | 1.01E-02 |
| **8-HEPE & 8,9-EpETE** | 0.50 | 0.60 | 0.92 | 12.65 | 9.58 | 8.71 | 0.67 | 10.31 | 0.22 | 2.07 | 6.58E-04 |
| **D.3. Metabolites of DHA** | | | | | | | | | | | |
| **4-HDoHE** | 0.50 | 0.60 | 0.92 | 7.30 | 9.50 | 7.02 | 0.67 | 7.94 | 0.22 | 1.36 | 3.94E-04 |
| **8-HDoHE** | 0.50 | 0.60 | 0.92 | 12.46 | 12.65 | 8.97 | 0.67 | 11.36 | 0.22 | 2.07 | 4.45E-04 |
| **11-HDoHE** | 0.50 | 0.60 | 0.92 | 13.51 | 7.49 | 10.55 | 0.67 | 10.52 | 0.22 | 3.01 | 2.41E-03 |
| **10-HDoHE** | 3.40 | 0.60 | 0.92 | 18.66 | 15.68 | 10.41 | 1.64 | 14.92 | 1.53 | 4.18 | 3.33E-03 |
| **13-HDoHE** | 0.50 | 0.60 | 3.34 | 8.00 | 8.38 | 7.02 | 1.48 | 7.80 | 1.61 | 0.70 | 1.68E-03 |
| **14-HDoHE** | 0.50 | 0.60 | 0.92 | 12.64 | 3.28 | 5.57 | 0.67 | 7.16 | 0.22 | 4.88 | 4.14E-02 |

**Table 1: LC-MS/MS lipidomic characterization of eicosanoids produced from ADRB3-mediated lipolysis.**

Lipid metabolites of cyclooxygenase, lipoxygenase, epoxygenase, and other pathways (linoleic acid (LA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)) were quantitated by LC-MS/MS lipidomic method. Statistical analysis shows that all listed lipids are $p < 0.05$ (*t*-test) in comparing control vs. ISO.

Next, I investigated whether the elevated levels of eicosanoids from ADRB3-stimulated lipolysis were regulated by the activation of hormone sensitive lipase (HSL). HSL is a key enzyme in the lipolysis process and is responsible for two-thirds of the total fatty acids that are released during ADRB3 activation (83,84,117). Differentiated 3T3-L1 mouse adipocytes were pretreated with and without BAY 59-9435 (BAY), a selective HSL inhibitor, for one hour, followed by ISO or vehicle for three hours (84,112). The cell culture media was collected and analyzed for eicosanoids using the LC-MS/MS. The resulting lipidomic profile revealed that the ISO-induced production of approximately 43 different lipids was dramatically reduced by the BAY pretreatment. Therefore, the generation of these lipids was dependent on both the ADRB3 and HSL activation. In summary, I was able to successfully identify the eicosanoids derived from ADRB3/HSL-mediated lipolysis in adipocytes.

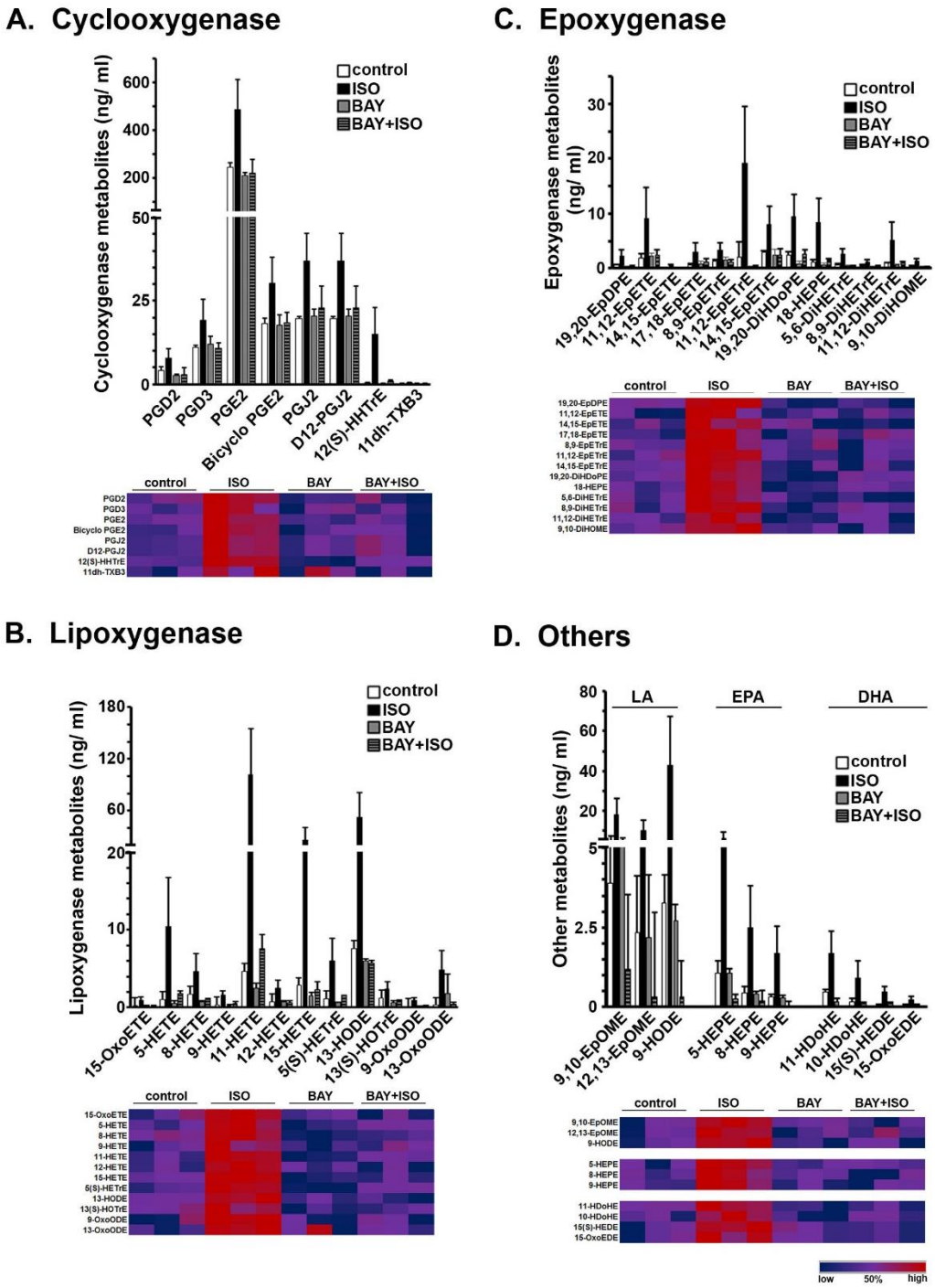


**Figure 7: Eicosanoids generated from ADRB3-stimulated lipolysis that are dependent on HSL activity in mouse adipocytes.**

Differentiated 3T3-L1 mouse adipocytes were treated with ISO (10 µM), in order to stimulate the ADRB3, or PBS for 3 hours in the presence and absence of BAY 59-9435 (BAY) (10 µM), a specific HSL inhibitor. The media was collected and analyzed by LC-MS/MS lipidomic methods. The lipidomic profiles and corresponding heat maps show lipid metabolites of the cyclooxygenase

(A), lipoxygenase (B), epoxygenase (C), and other metabolites (linoleic acid (LA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)) following treatment. Data are from a representative experiment that was repeated three times with similar results. Statistical analysis shows that all listed lipids are $p < 0.05$ (*t*-test) in comparing control vs. ISO and ISO vs. BAY + ISO. Note that the BAY treatment significantly inhibits the ISO-increased lipid species.

| | Mean | | | | SD | | | | T-TEST | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | control | ISO | BAY | BAY+ISO | control | ISO | BAY | BAY+ISO | Ctrl vs ISO | ISO vs ISO+BAY | BAY vs ISO+BAY |
| | ng/ ml | | | | | | | | | | |
| **A. Cyclooxygenase metabolites** | | | | | | | | | | | |
| **PGD2** | 4.15 | 7.73 | 2.58 | 3.00 | 1.00 | 2.98 | 0.49 | 2.08 | 7.23E-03 | 1.28E-02 | 3.76E-01 |
| **PGD3** | 11.09 | 19.17 | 11.99 | 10.80 | 0.61 | 6.35 | 2.43 | 1.54 | 4.88E-02 | 4.74E-02 | 2.56E-01 |
| **PGE2** | 245.70 | 486.61 | 210.17 | 220.10 | 19.15 | 125.84 | 12.43 | 57.34 | 7.49E-03 | 8.08E-03 | 3.92E-01 |
| **Bicyclo PGE2** | 18.23 | 30.20 | 17.83 | 18.43 | 1.51 | 7.94 | 3.04 | 3.21 | 3.42E-03 | 7.25E-03 | 4.13E-01 |
| **PGE3** | 11.09 | 19.17 | 11.99 | 10.80 | 0.61 | 6.35 | 2.43 | 1.54 | 4.88E-02 | 4.74E-02 | 2.56E-01 |
| **PGJ2** | 19.67 | 36.89 | 20.34 | 22.81 | 0.60 | 8.42 | 2.27 | 6.62 | 8.24E-03 | 3.56E-02 | 2.86E-01 |
| **D12-PGJ2** | 19.67 | 36.89 | 20.34 | 22.81 | 0.60 | 8.42 | 2.27 | 6.62 | 8.24E-03 | 3.56E-02 | 2.86E-01 |
| **12(S)-HHTrE** | 0.55 | 15.12 | 0.16 | 0.97 | 0.04 | 7.84 | 0.09 | 0.32 | 2.00E-04 | 2.33E-04 | 7.09E-03 |
| **11dh-TXB3** | 0.13 | 0.31 | 0.21 | 0.10 | 0.07 | 0.17 | 0.14 | 0.09 | 3.37E-02 | 3.02E-02 | 1.57E-01 |
| **B. Lipoxygenase metabolites** | | | | | | | | | | | |
| **15-OxoETE** | 0.29 | 0.93 | 0.22 | 0.15 | 0.21 | 0.45 | 0.05 | 0.14 | 4.21E-03 | 7.44E-04 | 2.56E-01 |
| **5-HETE** | 1.02 | 10.47 | 0.56 | 1.76 | 0.29 | 6.30 | 0.35 | 0.37 | 3.11E-03 | 4.21E-03 | 7.69E-03 |
| **8-HETE** | 1.75 | 4.66 | 0.74 | 1.02 | 0.46 | 2.30 | 0.09 | 0.11 | 3.39E-03 | 9.84E-04 | 1.37E-02 |
| **9-HETE** | 0.34 | 1.62 | 0.26 | 0.50 | 0.17 | 0.54 | 0.09 | 0.30 | 3.43E-02 | 5.21E-02 | 1.26E-01 |
| **11-HETE** | 4.67 | 101.41 | 2.47 | 7.61 | 1.46 | 53.76 | 0.65 | 1.74 | 9.29E-05 | 1.06E-04 | 4.35E-03 |
| **12-HETE** | 0.71 | 2.53 | 0.74 | 0.61 | 0.19 | 1.03 | 0.13 | 0.28 | 2.70E-04 | 4.31E-04 | 2.58E-01 |
| **15-HETE** | 2.84 | 26.30 | 1.54 | 2.31 | 0.27 | 14.47 | 0.37 | 0.99 | 2.53E-06 | 5.89E-06 | 1.38E-01 |
| **5(S)-HETrE** | 1.13 | 6.04 | 0.60 | 1.55 | 0.23 | 2.91 | 0.04 | 0.05 | 1.08E-04 | 1.22E-04 | 6.74E-06 |
| **13-HODE** | 7.63 | 52.45 | 5.98 | 5.75 | 1.22 | 28.20 | 0.29 | 0.36 | 3.04E-04 | 2.49E-04 | 2.23E-01 |
| **13(S)-HOTrE** | 1.23 | 2.41 | 0.65 | 0.83 | 0.34 | 0.94 | 0.21 | 0.15 | 4.16E-02 | 1.56E-02 | 1.41E-01 |
| **9-OxoODE** | 0.19 | 0.89 | 0.09 | 0.15 | 0.23 | 0.42 | 0.06 | 0.09 | 3.11E-03 | 1.34E-04 | 2.09E-01 |
| **13-OxoODE** | 0.31 | 4.81 | 1.76 | 0.48 | 0.13 | 2.52 | 2.52 | 0.17 | 3.75E-05 | 4.91E-05 | 2.14E-01 |
| **C. Epoxygenase metabolites** | | | | | | | | | | | |
| **19,20-EpDPE** | 0.59 | 2.28 | 0.30 | 0.33 | 0.07 | 1.12 | 0.07 | 0.09 | 9.46E-06 | 7.66E-06 | 3.44E-01 |
| **11,12-EpETE** | 1.82 | 9.11 | 2.15 | 2.43 | 0.81 | 5.61 | 0.62 | 1.04 | 3.92E-02 | 4.97E-02 | 3.54E-01 |
| **14,15-EpETE** | 0.13 | 0.56 | 0.15 | 0.06 | 0.08 | 0.19 | 0.08 | 0.02 | 1.39E-02 | 6.67E-03 | 8.42E-02 |
| **17,18-EpETE** | 0.61 | 2.96 | 0.78 | 1.15 | 0.19 | 1.72 | 0.58 | 0.58 | 1.07E-02 | 3.22E-02 | 2.39E-01 |
| **8,9-EpETrE** | 1.38 | 3.33 | 1.54 | 1.13 | 0.23 | 1.32 | 0.59 | 0.45 | 1.64E-02 | 1.38E-02 | 1.99E-01 |
| **11,12-EpETrE** | 1.98 | 19.20 | 0.33 | 0.32 | 2.80 | 10.30 | 0.07 | 0.17 | 4.40E-04 | 2.96E-05 | 4.78E-01 |
| **14,15-EpETrE** | 3.01 | 8.07 | 2.46 | 2.33 | 0.19 | 3.22 | 1.09 | 1.17 | 4.27E-03 | 4.97E-03 | 4.47E-01 |
| **19,20-DiHDoPE** | 2.44 | 9.44 | 0.80 | 2.63 | 0.56 | 3.99 | 0.45 | 0.67 | 5.59E-03 | 6.32E-03 | 8.37E-03 |
| **18-HEPE** | 1.19 | 8.33 | 0.59 | 1.45 | 0.42 | 4.49 | 0.31 | 0.31 | 6.41E-03 | 7.14E-03 | 1.41E-02 |
| **5,6-DiHETrE** | 0.61 | 2.50 | 0.41 | 0.31 | 0.24 | 1.02 | 0.18 | 0.10 | 1.34E-02 | 7.56E-03 | 2.33E-01 |
| **8,9-DiHETrE** | 0.47 | 1.12 | 0.34 | 0.48 | 0.16 | 0.43 | 0.08 | 0.08 | 1.19E-03 | 1.22E-04 | 4.23E-02 |
| **11,12-DiHETrE** | 0.96 | 5.17 | 0.60 | 0.88 | 0.17 | 3.23 | 0.11 | 0.35 | 5.91E-03 | 5.86E-03 | 1.27E-01 |
| **9,10-DiHOME** | 0.42 | 1.22 | 0.29 | 0.20 | 0.11 | 0.61 | 0.07 | 0.17 | 1.44E-03 | 9.97E-04 | 2.09E-01 |
| **D. Others** | | | | | | | | | | | |
| **D.1. Metabolites of LA** | | | | | | | | | | | |
| **9,10-EpOME** | 3.90 | 18.09 | 5.26 | 2.81 | 3.29 | 8.32 | 0.97 | 2.36 | 1.60E-03 | 5.43E-04 | 8.58E-02 |
| **12,13-EpOME** | 2.33 | 10.06 | 2.19 | 3.62 | 1.78 | 5.25 | 1.96 | 2.66 | 2.31E-03 | 1.10E-02 | 2.47E-01 |
| **9-HODE** | 3.26 | 43.01 | 2.71 | 3.38 | 0.88 | 24.41 | 0.52 | 1.13 | 1.47E-04 | 1.53E-04 | 2.05E-01 |
| **D.2. Metabolites of EPA** | | | | | | | | | | | |
| **5-HEPE** | 1.05 | 6.27 | 1.06 | 1.19 | 0.40 | 2.88 | 0.15 | 0.15 | 1.84E-03 | 1.80E-03 | 1.84E-01 |
| **8-HEPE** | 0.45 | 2.50 | 0.42 | 0.32 | 0.18 | 1.30 | 0.06 | 0.33 | 1.99E-02 | 1.80E-02 | 3.24E-01 |
| **9-HEPE** | 0.33 | 1.67 | 0.28 | 0.32 | 0.07 | 0.85 | 0.09 | 0.13 | 1.09E-02 | 1.12E-02 | 3.47E-01 |
| **D.3. Metabolites of DHA** | | | | | | | | | | | |
| **11-HDoHE** | 0.48 | 1.68 | 0.18 | 0.25 | 0.08 | 0.71 | 0.11 | 0.12 | 5.68E-03 | 3.29E-03 | 2.49E-01 |
| **10-HDoHE** | 0.17 | 0.90 | 0.09 | 0.20 | 0.09 | 0.56 | 0.06 | 0.08 | 9.45E-03 | 1.06E-02 | 5.74E-02 |
| **15(S)-HEDE** | 0.07 | 0.46 | 0.09 | 0.06 | 0.02 | 0.19 | 0.06 | 0.003 | 3.62E-04 | 2.91E-04 | 1.70E-01 |
| **15-OxoEDE** | 0.07 | 0.23 | 0.06 | 0.06 | 0.02 | 0.10 | 0.01 | 0.003 | 2.22E-02 | 1.79E-02 | 1.14E-01 |

**Table 2: LC-MS/MS lipidomic characterization of eicosanoids produced from ADRB3/HSL-mediated lipolysis.**

Lipid metabolites of cyclooxygenase, lipoxygenase, epoxygenase, and other pathways (linoleic acid (LA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)) were quantitated by LC-MS/MS lipidomic method. Statistical analysis shows that all listed lipids are $p < 0.05$ (*t*-test) in comparing control vs. ISO and ISO vs. ISO + BAY.

**Lipolysis-stimulated cyclooxygenase pathway, involving cyclooxygenase-2 up-regulation, in adipocytes and adipose tissue**

The cyclooxygenase (COX) enzyme and its metabolites have been shown to be up-regulated in inflammatory states and play a role in the pathogenesis of diseases, including rheumatoid arthritis and gastric and colon cancers. As previously described, the production of COX metabolites was shown to be significantly up-regulated in response to ADRB3/HSL-mediated lipolysis in adipocytes. This result led to my investigation of the underlying molecular mechanism explaining how the initiation of lipolysis causes the increased production of COX metabolites.

Since the COX enzyme has two isoforms, COX-1 and COX-2, that both produce prostaglandins, prostacyclins, and thromboxanes from arachidonic acid, it was imperative to determine which isoform was responsible for generating the elevated levels of COX metabolites during adipocyte lipolysis. Differentiated 3T3-L1 mouse adipocytes were pretreated with and without BAY for one hour, followed by ISO or vehicle for three hours. The cells were collected and analyzed for COX-1 and COX-2 mRNA expression. As a result, the ISO significantly up-regulated the COX-2 mRNA levels. Moreover, the BAY pretreatment diminished the ISO-induced COX-2 gene expression. Therefore, the increased COX-2 mRNA expression is dependent on the activation of ADRB3 and HSL signaling in adipocytes. The COX-1 mRNA expression, however, was unaffected by the BAY or ISO treatments. The protein levels of COX-1 and COX-2 in the 3T3-L1 cell lysates were also analyzed and in parallel to the mRNA results, the COX-2 expression was significantly up-regulated in response to ISO and inhibited in conjunction with the BAY pretreatment, whereas, the COX-1 protein levels were not affected. Consequently, the data suggests that the adrenergic stimulation of lipolysis and the subsequent activation of HSL in adipocytes, leads to the up-regulation of COX-2, not COX-1, expression at the mRNA and protein levels.

In collaboration with Dr. Emilio Mottillo from Dr. James Granneman's lab at Wayne State University, I was able to validate the *in vitro* response of COX-2 expression to ADRB3 and HSL activation using C57BL/6 mice. The C57BL/6 mice were intraperitoneally (*i.p.*) injected with and without BAY for one hour, followed by CL 316-243 (CL), a specific ADRB3 agonist, for three hours. The epididymal white adipose tissue (EWAT) pads of the mice were harvested at the time of euthanasia and analyzed for COX-2 mRNA expression. Indeed, the COX-2 mRNA expression in the EWAT was significantly up-regulated in response to the CL treatment. The up-regulation of COX-2 was abrogated by the BAY pretreatment. Therefore, these results collectively suggest that COX-2 expression is induced by ADRB3/HSL-mediated lipolysis in cultured adipocytes and in the adipose tissue of mice.

In order to show that COX-2 was the isoform responsible for generating elevated levels of COX metabolites during ADRB3/HSL-stimulated lipolysis, 3T3-L1 mouse adipocytes were pretreated with and without celecoxib, a selective COX-2 inhibitor, for one hour, followed by ISO for three hours. The cell culture media was collected and analyzed for the presence of COX metabolites using the LC-MS/MS. The resulting lipidomic profile showed that celecoxib eliminated the production of the COX metabolites induced by the ISO. A study by Dr. Krishna Rao Maddipati et al, showed that the incubation time, temperature, and composition of the culture media could greatly affect the stability of lipid species identified using the multiple reaction monitoring (MRM) LC-MS/MS method (114). This study, along with the short half-lives of some of the lipids, may explain the differences in the minor lipid species that were detected in the cultured media from the various ADRB3-stimulation experiments described in this dissertation (*e.g.* 11dh-TXB3 and TXB2 in Figures 7 and 9, respectively). Therefore, the data strongly indicates that the stimulation of ADRB3/HSL lipolysis in adipocytes causes the up-regulation of COX-2 expression and activity, leading to the increased levels of COX-derived lipid mediators.

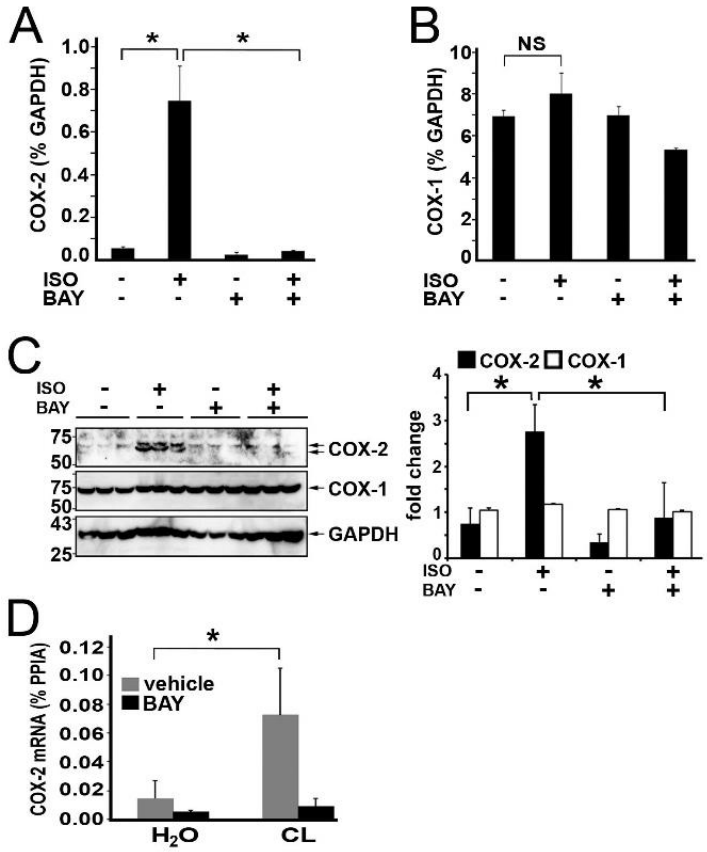


**Figure 8: Adipose ADRB3/HSL signaling pathway up-regulates cyclooxygenase-2 (COX-2), not COX-1, expression.**

Differentiated 3T3-L1 mouse adipocytes were treated with or without ISO (10 µM) for 3 hours in the presence and absence of a selective HSL inhibitor (BAY 59-9435, BAY (10 µM)). mRNA levels of COX-2 (A) and COX-1 (B) were measured by qPCR analysis and protein levels (C) were assessed by Western-blotting analysis. (D) C57BL/6 mice were intraperitoneally (*i.p.*) injected with BAY 59-9435 (BAY) (30 mg/kg) for 1 hour, followed by *i.p.* injection with CL 316-243 (10 nmol). Three hours later, the epididymal white adipose tissue (EWAT) was analyzed for COX-2 mRNA levels by qPCR analysis. Data represent mean ± SD of triplicate determinations. Each panel was repeated at least two times with similar results. (*, $p < 0.05$, *t* – test).

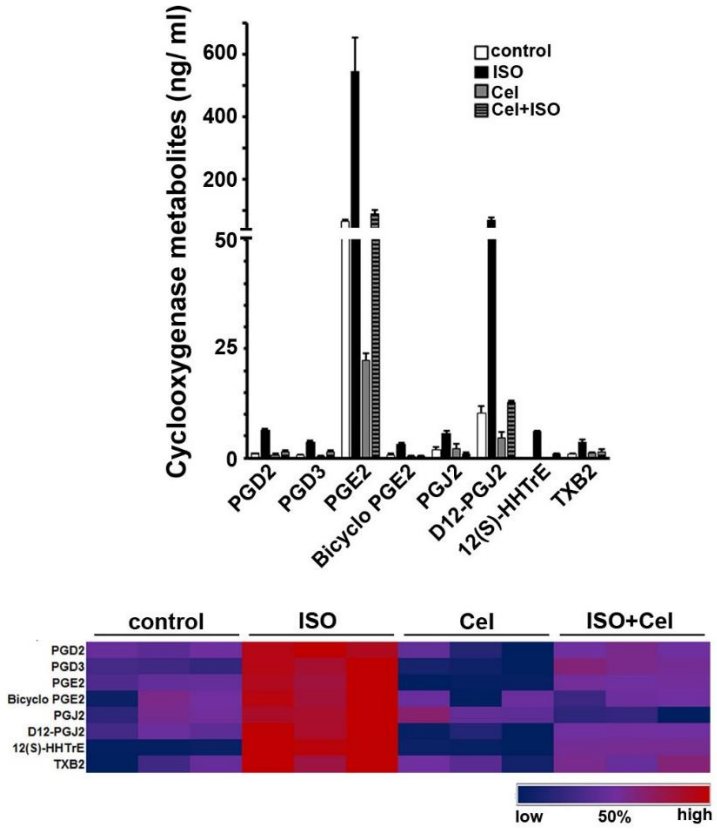


**Figure 9: Activity of COX-2 is responsible for producing elevated COX metabolites in response to ADRB3-stimulated lipolysis in adipocytes.**

Differentiated 3T3-L1 cells were pretreated with celecoxib (5 μM) or control vehicle for 1 hour, followed by stimulation with or without ISO for 3 hours. Lipid metabolites of cyclooxygenase pathway were quantitated by LC-MS/MS lipidomic method, and shown by the lipidomic profile and heat map analysis.

| | Mean | | | | SD | | | | T-TEST | |
|---|---|---|---|---|---|---|---|---|---|---|
| | control | ISO | Cel | ISO+Cel | control | ISO | Cel | ISO+Cel | Ctrl vs ISO | ISO vs ISO+ Cel |
| | ng/ ml | | | | | | | | | |
| **PGD2** | 1.05 | 6.27 | 0.62 | 1.39 | 0.07 | 0.56 | 0.37 | 0.44 | 8.61E-05 | 2.86E-04 |
| **PGD3** | 0.66 | 3.58 | 0.39 | 1.39 | 0.05 | 0.47 | 0.07 | 0.28 | 4.37E-04 | 2.33E-03 |
| **PGE2** | 64.08 | 543.49 | 22.38 | 89.64 | 5.59 | 111.57 | 1.59 | 10.99 | 1.75E-03 | 2.18E-03 |
| **Bicyclo PGE2** | 0.56 | 3.06 | 0.39 | 0.47 | 0.37 | 0.53 | 0.20 | 0.10 | 2.60E-03 | 1.15E-03 |
| **PGJ2** | 1.84 | 5.64 | 2.25 | 0.97 | 0.65 | 0.71 | 0.92 | 0.33 | 2.40E-03 | 4.88E-04 |
| **D12-PGJ2** | 10.35 | 66.55 | 4.53 | 12.81 | 1.51 | 8.82 | 1.48 | 0.38 | 4.05E-04 | 4.57E-04 |
| **12(S)-HHTrE** | 0.05 | 6.06 | 0.05 | 0.95 | 0.01 | 0.22 | 0.02 | 0.10 | 1.27E-06 | 3.43E-06 |
| **TXB2** | 0.99 | 3.65 | 1.05 | 1.53 | 0.17 | 0.67 | 0.17 | 0.40 | 2.65E-03 | 9.31E-03 |

**Table 3: LC-MS/MS lipidomic characterization of COX-2-generated lipid mediators in response to ADRB3/HSL activation in mouse adipocytes.**

Lipid metabolites of a cyclooxygenase pathway were quantitated by the LC-MS/MS lipidomic method. *P* values of all shown lipid species are less than 0.05 (*t*-test) in comparing control *vs.* ISO and ISO *vs.* ISO + celecoxib.

Next, I investigated the molecular mechanism of how ADRB3-stimulated lipolysis regulates COX-2 expression. As reported by Dr. Emilio Mottillo et al, ADRB3/HSL-mediated lipolysis activates stress kinases, such as c-Jun N-terminal kinase (JNK), in mouse adipocytes and adipose tissue (84). This finding led me to investigate whether JNK had a regulatory effect on COX-2 expression. Differentiated 3T3-L1 mouse adipocytes were pretreated with and without JNK inhibitor, SP-600125, for one hour, followed by the addition of ISO for three hours. The cells were collected and analyzed for COX-2 mRNA expression. The results showed that the ISO-induced COX-2 expression was significantly inhibited by the JNK inhibitor, suggesting that COX-2 expression is dependent on JNK activation in adipocyte ADRB3-mediated lipolysis. The transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), is a well-known downstream molecular target that is activated in response to JNK activation (121-123). Furthermore, the activation of NFκB has been shown to promote COX-2 transcription (124-127). In order to determine whether NFκB is activated in ADRB3-stimulated lipolysis, differentiated 3T3-L1 mouse adipocytes were treated with and without ISO for 0, 15 and 30 minutes and immunostained with an antibody for NFκB (see Figure 10B, Red). As a result, the ISO treatment caused a progressive translocation of NFκB into the nuclei of the 3T3-L1 cells, signified by the increased NFκB staining in the nuclear regions of the cells. This result indicates that the NFκB is activated in response to beta adrenergic receptor-stimulated lipolysis in adipocytes. To further support this finding, differentiated 3T3-L1 adipocytes were pretreated with BAY 11-7082, an NFκB pharmacological inhibitor, for one hour, followed by ISO for three hours. The cells were collected and analyzed for COX-2 mRNA expression. Consequently, the ISO-induced COX-2 mRNA expression was abrogated by the NFκB inhibitor. Therefore, the data suggests that the COX-2 gene expression is induced in response to the activation of the JNK/NFκB signaling pathway.

Since JNK is known to be stimulated by fatty acids and the activation of HSL generates free fatty acids, including palmitic acid and oleic acid, I investigated whether HSL-signaling

contributes to the activation of the JNK/NFκB pathway. Differentiated 3T3-L1 mouse adipocytes were treated with palmitic acid for 0, 15, 30, and 60 minutes and the protein extracts from the cells were analyzed for the activation of JNK and NFκB, as well as the expression of COX-2. As indicated by their phosphorylation statuses in the western blot, the palmitic acid activated the JNK kinases, $p54^{JNK}$ and $p46^{JNK}$. Furthermore, the palmitic acid treatment caused the corresponding phosphorylation of IκBα, signifying the activation of NFκB, and the up-regulation of the COX-2 protein. The rapid phosphorylation of IκBα and expression of the COX-2 protein in response to palmitic acid suggest substantial amplification of signals generated by JNK activation. This data collectively indicate that the free fatty acids produced by the activation of HSL can stimulate the JNK/NFκB/COX-2 signaling pathway in adipocytes.

Moreover, when differentiated 3T3-L1 mouse adipocytes were pretreated with the pharmacological COX-2 inhibitor, celecoxib, for one hour, followed by ISO for three hours, the ISO-induced phosphorylation of IκBα was diminished in the presence of celecoxib. This result indicates that COX-2 activity is necessary for NFκB activation in ADRB3-stimulated lipolysis in adipocytes. The celecoxib did not affect the phosphorylation of HSL, thereby confirming that COX-2 is a downstream molecular target in the HSL-signaling pathway.

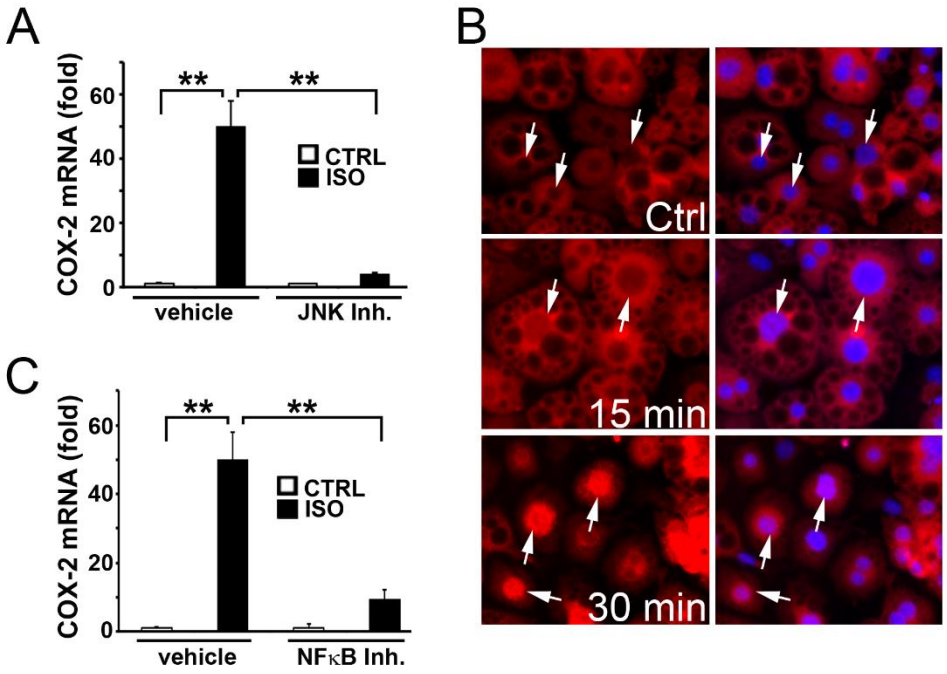


**Figure 10: ADRB3 activation-induced COX-2 up-regulation is mediated by the JNK/NFκB signaling pathway.**

(A) Differentiated 3T3-L1 cells were treated with ISO (10 µM) in the presence and absence of a JNK inhibitor (SP-600125, 10 µM). mRNA levels of COX-2 were quantitated by qPCR analysis. Data are mean ± SD (n=3; **, $p< 0.01$, *t*-test). (B) Differentiated 3T3-L1 cells were treated with ISO for 0, 15, and 30 minutes followed by immunostaining with anti-NFκB. Note that the ISO treatment progressively increases nuclear localization of NFκB (white arrows in middle and lower panels). Red = NFκB, Blue = DAPI nuclear staining. (C) 3T3-L1 cells were treated with ISO in the presence and absence of an NFκB inhibitor (BAY 11-7082, 10 µM). mRNA levels of COX-2 were quantitated by qPCR analysis. Data are mean ± SD (n=3; **, $p< 0.01$, *t*-test).

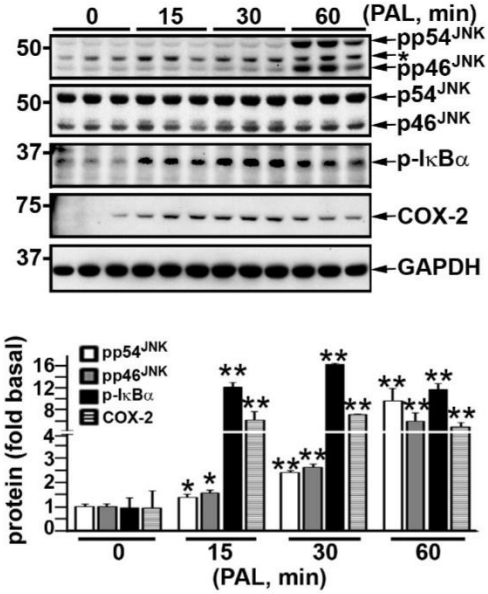


**Figure 11: Palmitic acid activates JNK and NFκB and up-regulates COX-2 expression in adipocytes.**

Differentiated 3T3-L1 cells were treated with palmitic acid (PAL, 0.5 mM) for 0, 15, 30, and 60 minutes. Cellular extracts were blotted with indicated antibodies. Lower panel, intensity of western-blotting were quantitated with NIH Image J software (normalized to GAPDH). (n=3; *, $p< 0.05$, and **, $p< 0.01$; *t*-test).

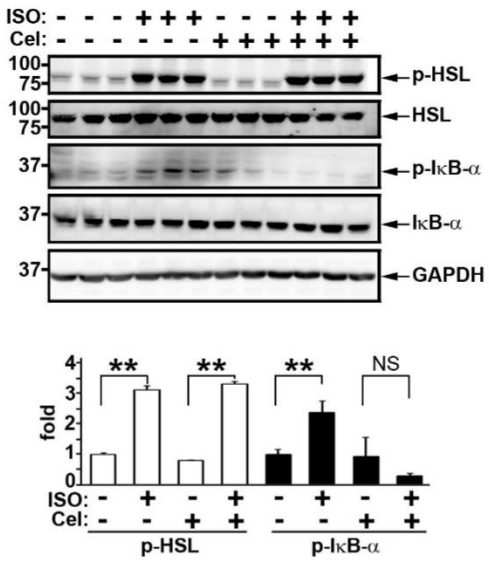


**Figure 12: COX-2 activity is required for NFκB activity and its inhibition does not affect HSL.**

Differentiated 3T3-L1 cells were treated with ISO (10 µM) in the presence and absence of a COX-2 inhibitor (Cel, celecoxib, 5 µM) for 30 minutes. Cellular extracts were blotted with indicated antibodies. Lower panel, data represent mean ± SD (n=3; **, $p< 0.01$; NS, non-statistical significance; *t*-test).

**Cyclooxygenase-2 activation regulates the recruitment and infiltration of monocytes and macrophages to the adipose tissue**

One of the hallmark characteristics of obesity and adipose inflammation is the recruitment and infiltration of monocytes and macrophages to the adipose tissue. Monocyte chemotactic protein 1 (MCP-1)/chemokine (C-C motif) ligand 2 (CCL2), a pro-inflammatory cytokine released by the adipocytes, is able to recruit the monocytes and macrophages to the adipose tissue. The adipokine, interleukin-6 has also been associated with inflammation and obesity. As previously reported by Mottillo et al, ADRB3/HSL-mediated lipolysis triggered the activation of stress kinases JNK and p38, which preceded the up-regulation of MCP-1/CCL2 and IL-6 in adipocytes (84). In order to investigate whether the lipolysis-induced activation of JNK/NFκB/COX-2 had any effect on the expression of MCP-1/CCL2 and IL-6, differentiated 3T3-L1 mouse adipocytes were pretreated with and without SP-600125 (JNK inhibitor), BAY 11-7082 (NFκB inhibitor), or celecoxib (COX-2 inhibitor) for one hour. The cells were subsequently treated with and without ISO for three hours, collected, and analyzed for MCP-1/CCL2 and IL-6 mRNA expression. The JNK inhibitor significantly suppressed the ISO-induced MCP-1/CCL2 and IL-6 gene expression, while the p38 inhibitor had no statistical effect. Both celecoxib and the NFκB inhibitor considerably reduced the MCP-1/CCL2 gene expression that was up-regulated by the ISO treatment, but did not affect the IL-6 gene expression. Therefore, the data suggests that the mRNA expression of MCP-1/CCL2 is regulated by the lipolysis-stimulated JNK/NFκB/COX-2 signaling pathway in adipocytes.

Next, I investigated whether up-regulated COX-2 expression was sufficient to induce MCP-1/CCL2 in adipocytes in the absence of lipolysis stimulation. Since the transfection efficiency with plasmid *cDNA* in 3T3-L1 mouse adipocytes is low, 3T3-L1-CAR cells were used to perform the experiment. 3T3-L1-CAR cells only differ from 3T3-L1 cells in that they possess an adenovirus receptor. Differentiated 3T3-L1-CAR cells were transduced with adenoviral particles carrying COX-2 that were generously provided by Dr. Andrey Sorokin (Medical College of

Wisconsin). After qPCR analysis of the cells, I found that the MCP-1/CCL2 expression was significantly up-regulated in the cells transduced with adenoviral COX-2 compared to the control. The IL-6 mRNA expression was not significantly affected by the 3T3-L1-CAR cells transfected with the adenoviral COX-2. Therefore, this result suggests that even in the absence of fatty acid release by ADRB3-stimulated lipolysis, COX-2 expression is sufficient in significantly inducing MCP-1/CCL2 expression, thus eliciting an inflammatory response in adipocytes.

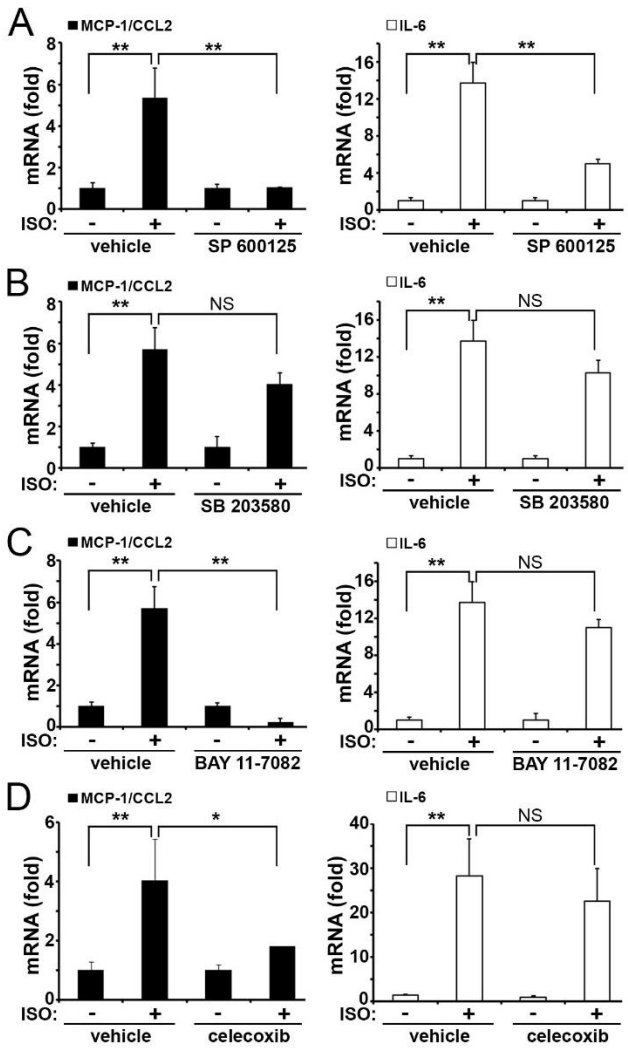


**Figure 13: Lipolysis-increased MCP-1/CCL2 expression is dependent on the JNK/NFκB/COX-2 signaling pathway.**

Differentiated 3T3-L1 cells were pretreated with or without an inhibitor of JNK (SP 600125, 10 μM) (A), p38 kinase (SB 203580, 10 μM) (B), NFκB (BAY 11-7082, 10 μM) (C), and COX-2 (celecoxib, 5 μM) (D) for 1 hour. Subsequently, cells were stimulated with or without ISO (10 μM) for an additional 3 hours. Levels of MCP-1/CCL2 (left panels) and IL-6 (right panels) were quantitated by qPCR analysis. Data represent mean ± SD of a representative experiment (n=3), which was repeated at least two times with similar results. (**, $p< 0.01$; *, $p< 0.05$; NS, not statistically significant; *t*-test).

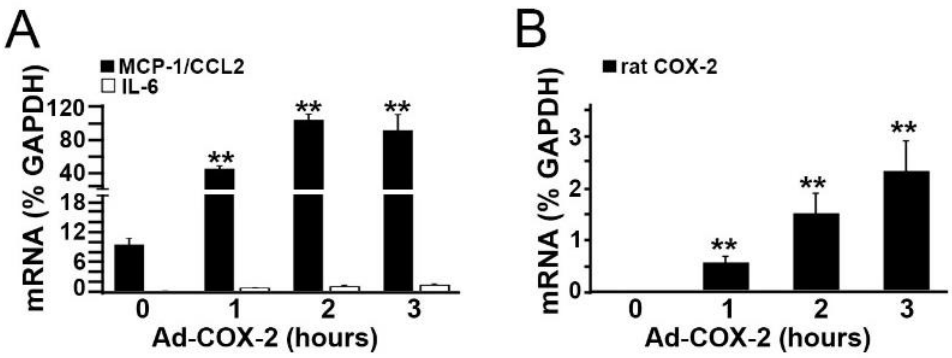


**Figure 14: COX-2 expression is sufficient in significantly inducing the expression of MCP-1/CCL2 in the absence of acute lipolysis.**

3T3-L1-CAR cells were transduced with a multiplicity of 200 adenoviral particles carrying rat COX-2 (Ad-COX-2) for the indicated times. Expression levels of MCP-1/CCL2, IL-6 (A), and rat COX-2 (B) were measured by qPCR analysis. Note that ectopic expression of COX-2 significantly increased MCP-1/CCL2 expression. Data are mean ± S.D. of triplicate determinations (**, $p < 0.01$; *t*-test).

To further investigate the role of lipolysis-triggered COX-2 activation *in vivo*, I *i.p.* injected C57BL/6 mice with and without celecoxib for one hour, followed by CL for three hours to stimulate acute ADRB3-mediated lipolysis. The EWAT pads were harvested after the mice were euthanized and analyzed for COX-1, COX-2, MCP-1/CCL2, and IL-6 expression. The COX-2 mRNA and protein expression were significantly up-regulated in response to the CL treatment, whereas the expression of COX-1 was not affected. Additionally, the CL treatment significantly up-regulated the gene expression levels of MCP-1/CCL2 and IL-6. The celecoxib significantly diminished the ISO-induced MCP-1/CCL2 expression, but did not affect the IL-6 gene expression. These results support my *in vitro* observations and indicate that ADRB3-mediated lipolysis activates COX-2, which regulates the MCP-1/CCL2 expression in adipocytes and adipose tissue.

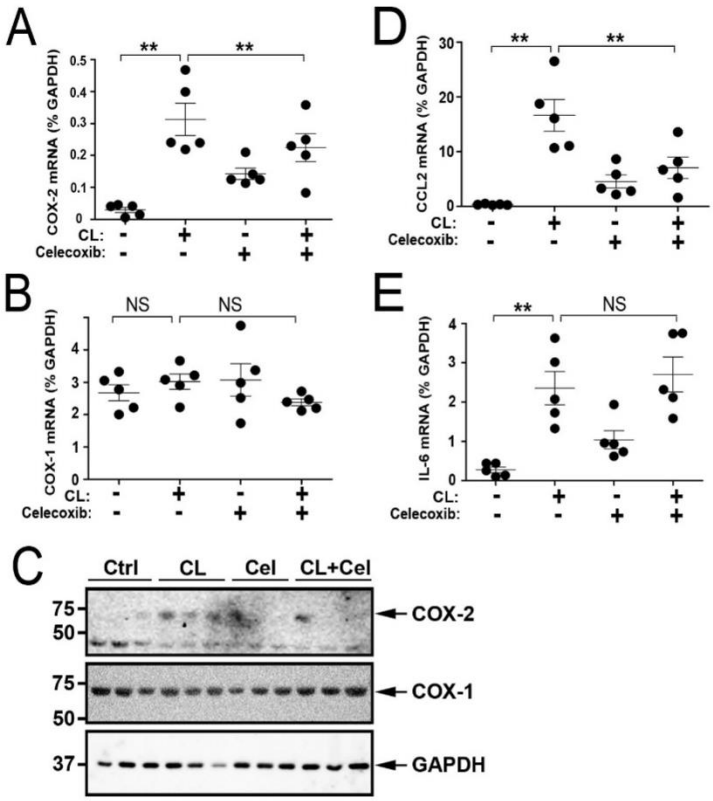


**Figure 15: COX-2 activity is required for the ADRB3-stimulated MCP-1/CCL2 up-regulation in mouse epididymal white adipose tissue.**

C57BL/6 mice (male, 8 weeks old) were *i.p.* injected with celecoxib (100 mg/kg) or control vehicle for 1 hour. Subsequently, mice were *i.p.* injected with or without CL 316-243 (10 nmol). Three hours later, epididymal white adipose tissues (EWAT) were collected, and measured for levels of COX-2 (A), COX-1 (B), MCP-1/CCL2 (D), and IL-6 (E) by qPCR analysis. (**, $p < 0.01$; NS, not statistically significant (n=5, Two-way ANOVA)). (C) Protein levels of COX-2 and COX-1 were measured by Western-blotting analysis.

Furthermore, I examined whether ADRB3-activated lipolysis stimulated the recruitment and infiltration of monocytes/macrophages to the adipose tissue and whether it was mediated by COX-2 activity. I analyzed the presence of monocytes/macrophages in the EWAT of the mice that were pretreated with and without celecoxib for one hour in the presence and absence of CL treatment for three hours, through immunohistochemistry. According to the F4/80 staining of the EWAT, the amount of macrophages that infiltrated the EWAT in the mice that were injected with CL was significantly elevated compared to the control. This result corresponds to the CL-induced MCP-1/CCL2 mRNA expression in the EWAT of mice. The celecoxib pretreatment inhibited the CL-increased F4/80 staining in the EWAT. The number of macrophages from multiple sections of EWAT was counted and is represented in Figure 16. The gene expression of macrophage marker, Mac-2, in the EWAT was also shown to be significantly up-regulated in response to the CL treatment. The celecoxib, in turn, abrogated the ISO-increased Mac-2 expression in the EWAT. Therefore, these results suggest that the activation of COX-2 by ADRB3-mediated lipolysis contributes to inflammation by regulating the recruitment of monocytes/macrophages to the adipose tissue.

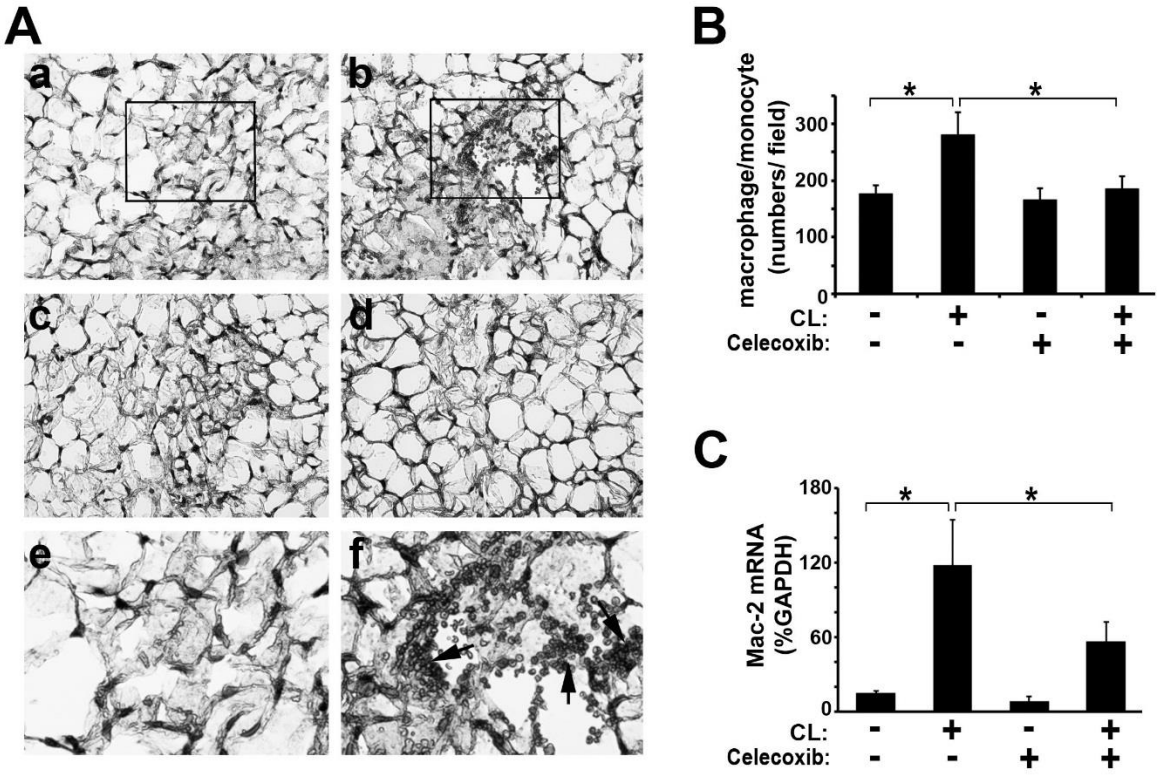


**Figure 16: COX-2 activity is necessary for the ADRB3-stimulated monocyte/macrophage infiltration in adipose tissue.**

C57BL/6 mice (male, 8 weeks old) were *i.p.* injected with celecoxib (100 mg/kg) or control vehicle for 1 hour. Subsequently, mice were *i.p.* injected with or without CL 316-243 (10 nmol). Three hours later, epididymal white adipose tissues (EWAT) were collected. (A) Paraffin sections (5 µm) of EWAT were immunohistochemically stained with anti-F4/80. a, vehicle; b, CL 316-243; c, celecoxib alone; d, celecoxib + CL 316-243; e and f, enlarged image of box area in a and b, respectively. Arrows, infiltrated macrophages/ monocytes. (B) Macrophages/ monocytes present in each treatment (4-5 microscopic fields) were scored. *, $p< 0.05$, *t*-test. (C) Levels of Mac-2, a macrophage marker, in the EWAT were quantitated by qPCR. (*, $p< 0.05$, *t*-test).

In summary, the results obtained from this study collectively suggest that the fatty acids generated from HSL-mediated lipolysis activate the JNK/NFκB/COX-2 signaling pathway in adipocytes. The activation of COX-2 results in the production and release of prostaglandins, prostacyclins, and thromboxanes. COX-2 activity also regulates MCP-1/CCL2 expression, which leads to the recruitment and infiltration of monocytes/macrophages to the adipose tissue. The presence and activation of macrophages may promote inflammation in the adipose tissue. Alternatively, the recruited macrophages may buffer local fatty acid concentrations by up-taking fatty acids that are released during adipocyte lipolysis. Therefore, my findings in this study characterize the lipid mediators derived from lipolysis in adipocytes and describe the discovery of a novel lipolysis-triggered pro-inflammatory cyclooxygenase pathway, involving COX-2, in the recruitment of monocytes to mouse adipocytes and adipose tissue.

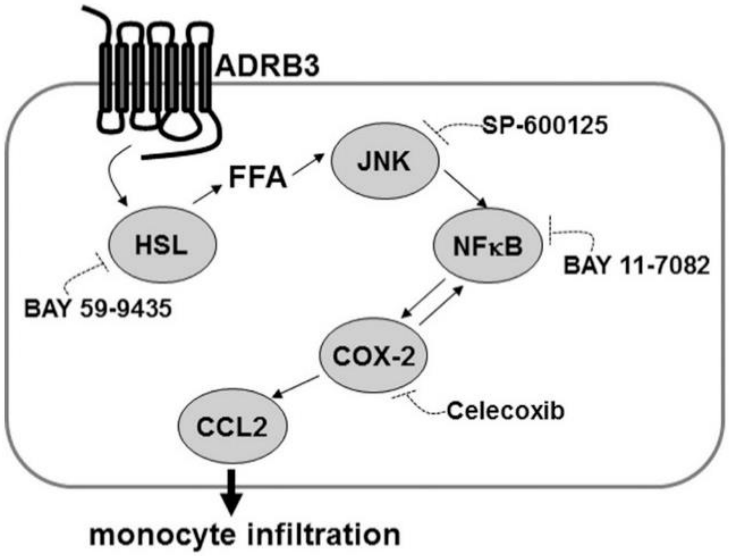


**Figure 17: Proposed mechanism of a pro-inflammatory lipolysis-stimulated cyclooxygenase pathway.**

The model of my findings reveal a COX-2-mediated mechanism through which HSL-driven lipolysis stimulates the infiltration of monocytes/macrophages in the adipose tissue. Various ways to inhibit this signaling pathway may reduce adipose inflammation triggered by acute lipolysis.

## CHAPTER 4: DISCUSSION

Excessive adipose lipolysis triggers adipose tissue inflammation and immune cell infiltration (84,112,128). However, the pro-inflammatory lipid mediators produced from adipose lipolysis remain elusive. Therefore, my doctoral study took on a lipidomic approach and utilized LC-MS/MS technology to successfully identify the eicosanoid lipid mediators generated during ADRB3/HSL-mediated adipose lipolysis for the first time. The acquired lipidomic profiles included lipids derived from cyclooxygenase, lipoxygenase, and epoxygenase enzymes and others from linoleic acid, EPA, and DHA. From my analysis, I was able to determine that in response to beta adrenergic stimulation, the production and secretion of approximately 63 eicosanoids were significantly increased in 3T3-L1 adipocytes. Furthermore, I discovered that the generation of 43 eicosanoids were dependent on the activation of ADRB3 and HSL in adipocyte lipolysis. These lipid mediator profiles serve as novel additions to the lipidomic and adipose biology fields.

In addition, the data obtained in this study strongly suggest that COX-2 is responsible for producing the elevated levels of prostaglandins, prostacyclins, and thromboxanes from arachidonic acid in response to beta-adrenergic activation of lipolysis in adipocytes. COX-2 has been shown to be a critical inflammatory molecule that is induced in various tissues and in obese individuals (129-131). Therefore, in the present study, I decided to focus on characterizing the involvement of COX-2 in lipolysis-triggered adipose inflammation. The experimental results show that COX-2 expression is significantly induced and activated in cultured adipocytes and adipose tissue in response to ADRB3 activation. The up-regulation of COX-2 was inhibited by selective pharmacological inhibition of HSL, indicating that the lipolysis-increased COX-2 expression is dependent on HSL activity. Furthermore, this study suggests that COX-2 up-regulates MCP-1/CCL2 production, which may play an important role in the recruitment and infiltration of immune cells in adipose tissue.

As previously reported, adipose lipolysis activates JNK and p38 stress kinases, which play important roles in lipolysis-stimulated production of pro-inflammatory cytokines/chemokines

(84,112). In the present study, I observed that beta adrenergic activation induced nuclear translocation of NFκB, a known downstream molecular target of JNK and transcription factor that plays a key role in regulating inflammation. Moreover, the lipolysis-induced COX-2 up-regulation was suppressed by pharmacological inhibitions of JNK or NFκB. Lipolysis is known to generate free fatty acids through the hydrolysis of triglycerides and the direct treatment of adipocytes with palmitic acid, a free fatty acid, activated the JNK/NFκB/COX-2 signaling pathway. Palmitic acid is a saturated fatty acid that is abundant in the Western diet and can contribute to adipose inflammation that is seen in obese individuals (132). Although palmitic acid was the only fatty acid used to treat the 3T3-L1 mouse adipocytes in this study, it can be speculated that other saturated fatty acids and omega-6 fatty acids could also activate this pro-inflammatory signaling pathway. Derivatives of DHA and EPA and other omega-3 fatty acids exert anti-inflammatory effects and therefore, I do not predict they would trigger this proposed cyclooxygenase pathway in the adipose tissue. These results together suggest that free fatty acids produced by adipose lipolysis activate the JNK/NFκB pathway, leading to COX-2 up-regulation.

In addition, it was previously reported that the lipolysis-induced expression of IL-6 is mediated by JNK activation (84). However, unlike MCP-1/CCL2, pharmacological inhibition of NFκB or COX-2 had no significant effect on the lipolysis-stimulated IL-6 expression. In this regard, my collaborators and I recently reported that the lipolysis-induced up-regulation of IL-6 is mediated by the production of sphingosine-1-phosphate, and this pathway requires the up-regulation of sphingosine kinase 1 (SphK1) via the JNK/AP-1 pathway (112). Collectively, these results indicate that the regulation of MCP-1/CCL2 and IL-6 both involve the generation of lipid mediators, but the specific pathways (COX-2 and SphK1) diverge following JNK activation.

In the present study, I found that ADRB3-activated lipolysis triggered an acute infiltration of monocytes and macrophages in the epididymal white adipose tissues of mice. The physiological or patho-physiological significance of the lipolysis-driven monocyte/macrophage infiltration warrants future investigation. Our previous studies suggest that the lipolysis-driven

infiltration of monocytes/macrophages regulates inflammation, apoptosis, and remodeling of adipose tissues (83,84,112,133). In addition, it has been suggested that adipose macrophages can buffer local fatty acid concentrations through the uptake of fatty acids and suppression of adipocyte lipolysis (134). Pretreatment of the selective COX-2 inhibitor, celecoxib, prevented the recruitment and infiltration of the majority of the macrophages in the adipose tissue. Therefore, for the first time, it has been discovered that ADRB3/HSL-mediated lipolysis initiates a cyclooxygenase pathway, via COX-2, that contributes to inflammation in the adipose tissue.

The mechanism of how COX-2 could potentially regulate the expression of MCP-1/CCL2 in adipocytes is not yet known. The lipidomic analysis showed that prostaglandin E2 (PGE2), a pro-inflammatory prostaglandin involved in numerous inflammatory processes (135-138), is one of the most abundant lipidomic metabolites generated from the COX-2 enzyme during lipolysis. PGE2 is able to exert its signaling effects by binding to its own family of G protein coupled receptors, EP-1, EP-2, EP-3, and EP-4. In addition, it has been reported that PGE2 treatment up-regulates MCP-1/CCL2 expression in mesangial cells (139). However, I was unable to demonstrate that the exposure of 3T3-L1 adipocytes to PGE2 alone (up to 50 μM for up to 24 hours) could up-regulate MCP-1/CCL2 (not shown). Furthermore, experimental attempts using various combinations of other prostaglandins (*e.g.* PGD2, PGJ2, d12-PGJ2, 0-25 μM) to treat 3T3-L1 adipocytes were also ineffective. It is possible that exogenous PGE2 suppresses COX-2-dependent pro-inflammatory signaling by activating the EP-4 receptor (140,141). Thus, it can be speculated that either a combination of prostaglandins and/or other lipid mediator(s) could be responsible for the COX-2-mediated up-regulation of MCP-1/CCL2. Alternatively, it is possible that the up-regulation of MCP-1/CCL2 is mediated by intracellular effects of the eicosanoids generated by the COX-2 pathway. Future studies are needed to expose the molecular link between the specific lipolysis-stimulated COX-2 products and the MCP-1/CCL2 expression in adipocytes.

In this study, the celecoxib treatment alone was observed to slightly, but significantly, increase levels of COX-2, MCP-1/CCL2, and IL-6 in the epididymal white adipose tissues of mice (see Figure 15). The mechanism for elevated expression of those adipose inflammatory markers by COX-2 inhibition alone is currently unknown. I found that a noticeable quantity of PGE2 is secreted by cultured adipocytes, and this level of PGE2 was significantly reduced by celecoxib treatment (Figure 9 and Table 3). As discussed earlier, PGE2 was shown to suppress lipolysis (140-143). Thus, it is possible that the basal level of PGE2 secreted by adipocytes functions to suppress adipose lipolysis and inflammation, and this process would be reversed somewhat by COX-2 inhibition.

Another aspect of the study that is currently unknown is the source of the arachidonic acid that served as the substrate for the production of the majority of the eicosanoids. In most cells, a major source of arachidonic acid is stored in its esterified form within the cell membrane. In response to external stimuli, phospholipase A2 (PLA2), cleaves the phospholipids in the cell membrane which releases arachidonic acid into the cytosol where it can be metabolized to form different eicosanoids. Arachidonic acid can also be incorporated in triglycerides that are stored in the lipid droplets of adipocytes. When lipolysis is initiated, triglycerides are hydrolyzed resulting in the release of free fatty acids, including arachidonic acid. The free form of arachidonic acid can be used as a substrate for the biosynthesis of pro-inflammatory lipid mediators by the COX, LOX, and cytochrome P450 enzymes. One strategy that tests the hypothesis that the source of the arachidonic acid used as the substrate for COX-2 could originate from triglycerides, involves using stable isotopic labeling of arachidonic acid to treat 3T3-L1 adipocytes (144). The incorporation of the labeled arachidonic acid into the triglycerides stored in the lipid droplets and its fate in response to cellular manipulation can be tracked. If the elevated levels of COX metabolites generated from ADRB3-stimulated lipolysis possess the isotopic label from the arachidonic acid that was used to treat the 3T3-L1 cells, then it can be concluded that the

substrate for COX-2 originated from arachidonic acid in the triglycerides that was released during the lipolysis process.

Although the ADRB3/HSL signaling pathway is a well-established model to study lipolysis in mice, the minute amounts of ADRB3 detected in humans is not effective in regulating lipolysis. Therefore, the ADRB3 itself cannot be an option for a therapeutic target in humans. The results from this study still have merit, however, as the lipolysis process in mice and humans involves the activation of the beta-adrenergic or glucagon receptors and the subsequent activation of PKA. The lipidomic profiles and the lipolysis-initiated pro-inflammatory cyclooxygenase pathway in this study were obtained from mice and it is imperative to confirm the results in humans before moving forward to drug discovery and therapeutic intervention. A variety of non-steroidal anti-inflammatory drugs (NSAIDs) are currently available on the market and prescribed to individuals suffering from hypertension and inflammatory diseases, such as rheumatoid arthritis. However, to my knowledge, there are no studies that link the effect of NSAIDs to lipolysis-induced adipose inflammation in obesity. The novel results from this study can be used as a platform to propel future research by providing avenues for targeting lipolytic-specific COX-2 products that are up-regulated in obesity.

My doctoral research project significantly impacts the lipidomic field, especially as it relates to adipose tissue inflammation. The bioactive lipid profiles revealing the eicosanoids generated and released upon stimulation of lipolysis add a new dimension to our overall understanding of lipidomics and adipose tissue biology. Scientific researchers that have an interest in a specific lipid or class of lipids are now able to reference the lipidomic profiles acquired in this study and use this information to guide their future research projects. The discovery of a new lipolysis-stimulated pro-inflammatory cyclooxygenase mechanism, involving COX-2, advances our understanding of the molecular players involved in the lipolytic process and their patho-physiological roles in adipose inflammation.

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## ABSTRACT

**IDENTIFICATION OF LIPOLYSIS-DERIVED LIPID MEDIATORS AND THE ACTIVATION OF A PRO-INFLAMMATORY CYCLOOXYGENASE PATHWAY, VIA CYCLOOXYGENASE-2, IN ADIPOSE TISSUE**

by

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**Advisor:** Dr. Menq-Jer Lee

**Major:** Pathology

**Degree:** Doctor of Philosophy

Adipose lipolysis triggers pro-inflammatory responses that play critical roles in insulin resistance and associated metabolic syndrome. However, pro-inflammatory mediators generated by adipose lipolysis, particularly in the context of lipid mediators, are poorly defined. In this study, the activation of the beta-3 adrenergic receptor (ADRB3)/hormone sensitive lipase (HSL) pathway, a well-employed model system, was utilized to characterize the pro-inflammatory lipid mediators generated by adipose lipolysis. Cultured adipocytes were treated with an ADRB3 agonist and the media was analyzed for eicosanoids using the LC-MS/MS lipidomic method. Among the characterized eicosanoids, I found that approximately 43 metabolites generated by cyclooxygenase (COX), lipoxygenase, and cytochrome P450 enzymes were significantly produced in response to ADRB3/HSL-stimulated lipolysis in adipocytes. Mechanistically, I observed that lipolysis induced cyclooxygenase 2 (COX-2), not COX-1, expression in an HSL-dependent manner in adipocytes and in the epididymal white adipose tissue (EWAT) of C57BL/6 mice that were injected with a specific ADRB3 agonist, CL-316243 (CL). Additionally, JNK and NFκB are activated by ADRB3-mediated lipolysis and regulate the increased COX-2 expression. Moreover, treatment with a pharmacological COX-2 inhibitor, celecoxib, decreased the COX metabolites in the media of ADRB3-stimulated adipocytes. Inflamed adipose tissue involves the increased presence and activation of macrophages that are recruited to the tissue by the pro-

inflammatory cytokine, MCP-1/CCL2. Interestingly, not only was MCP-1/CCL2 expression significantly increased in ADRB3/HSL-mediated lipolysis, but its expression was also dependent on JNK/NFκB/COX-2 activation. Furthermore, I observed that celecoxib pretreatment significantly blocked macrophage infiltration in the EWAT of mice treated with CL. In summary, I have shown for the first time that ADRB3/HSL signaling activates a pro-inflammatory cyclooxygenase pathway via COX-2, in adipose tissue.

## AUTOBIOGRAPHICAL STATEMENT

## ALLISON GARTUNG

Allison Gartung was born and raised in Lansing, Michigan and always had an interest in mathematics and science. She graduated Valedictorian from Grand Ledge High School in 2006. In 2010, she graduated Summa Cum Laude from Ferris State University, where she was in the Honors Program and made the Dean's List every semester. Aside from academia, she was involved in numerous student organizations on campus, including Big Brothers Big Sisters of Mecosta County, Student Alumni Gold Club, and the American Chemical Society – Ferris State Chapter. She was section leader in the Ferris State Pep Band and on the Fall 2009 Homecoming Court. In Fall 2010, she decided to continue her education in science by enrolling in the Pathology graduate program at Wayne State University School of Medicine. She began her dissertation study in Dr. Menq-Jer Lee's laboratory in the summer of 2011 and has since contributed to several publications including a first author manuscript in the Journal of Biological Chemistry (currently in press) and a co-author of one review and five other manuscripts. Following the defense of her dissertation on June 10, 2016, she will be joining a cancer research laboratory as a postdoctoral fellow, under the direction of Dipak Panigrahy (M.D.) and John Lawler (PhD), at the Beth Israel Deaconess Medical Center, a teaching affiliate of Harvard Medical School, in Boston, Massachusetts.