STUDIES OF FATTY ACID BINDING PROTEINS AND INFLAMMATORY LIPIDS IN ADIPOSE BIOLOGY

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Dedication

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Abstract

There are a number of factors spurred by obesity that contribute to the development of insulin resistance, such as adipose tissue inflammation and elevated circulating fatty acid levels. Fatty Acid Binding Proteins (FABP) are soluble proteins that bind long chain fatty acids and other hydrophobic molecules and facilitate their intracellular transport. Mice with genetic disruption of Adipocyte FABP (AFABP) exhibit an insulin sensitizing and anti-inflammatory phenotype on a high-fat diet compared to wild type littermates, however the molecular mechanisms are not completely understood. The goal of the studies presented herein was to gain further insights into the role of AFABP in the development of obesity-related insulin resistance. We identified a small molecule inhibitor of AFABP, HTS01037, that upon treatment of cultured cells recapitulates the beneficial phenotypes observed in AFABP knock out mice. Structural studies were undertaken to characterize the effect of ligand binding to AFABP utilizing x-ray crystallography. More specifically the structures of AFABP bound to one inflammatory lipid, 4-HNE, and the pan-specific FABP inhibitor, HTS01037 were determined. In addition, we found that inflammatory lipids, particularly leukotriene C_4, are elevated in obese adipose tissue and are produced by macrophages in response to fatty acid treatment in a FABP-dependent manner.

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CHAPTER 1

INSULIN RESISTANCE, FATTY ACID BINDING PROTEINS AND INFLAMMATORY LIPIDS

Kristina Hellberg wrote this chapter.

I. Adipose Biology and Obesity-Linked Insulin Resistance

Obesity is a growing health problem in the world and it has been described as an epidemic, affecting half a billion people worldwide. In 2009-2010, 35.7% of adult Americans, corresponding to 78 million people, were obese as indicated by a body mass index (BMI) over 30 kg/m^2. In the same years, almost 17% of American children were obese (1). This leads to major costs for the U.S. health care system and in 2008, it was estimated that overweight and obesity were responsible for almost 10% of the US medical expenditure (2). Obesity predisposes affected individuals to a plethora of diseases such as insulin resistance (the fore-runner of type 2 diabetes or non-insulin dependent diabetes mellitus), hypertension, and cardiovascular disease (3,4). These features associated with obesity are commonly referred to as the metabolic syndrome or syndrome X (5). Obesity and diabetes dramatically shorten the lifespan of individuals by promoting, for example, vascular disease, several different cancers, renal and liver disease, infectious disorders, mental disorders, and chronic obstructive pulmonary disease leading to premature death (6). In light of this, research is focusing on dissecting the molecular mechanism whereby obesity promotes insulin resistance.

The food that is consumed is used to generate energy for biochemical reactions and movement. When the energy intake is greater than the energy expenditure, excess energy is mainly stored as fat in adipocytes. A smaller amount of energy

is stored in the form of glycogen in liver and muscle and as protein in muscle. In a healthy individual these fuel stores are essential to provide energy in between meals, however, prolonged overnutrition without increased energy expenditure inevitably leads to overweight and ultimately obesity. In addition, some individuals are genetically predisposed to obesity. Furthermore, proper regulation of feeding behavior by the brain is important. For instance, activation of Agouti Related Protein (AgRP) neurons leads to hyperphagia and decreased energy expenditure, resulting in obesity (7,8). Obesity affects many organs in the body but according to the adipocentric view, the expansion of adipose tissue is the initiating event that ultimately generates systemic insulin resistance (9).

There are two types of adipose tissue, white and brown adipose tissue. White adipocytes are specialized in lipid storage. Lipids are stored in a large unilocular lipid droplet occupying most of the cell's cytoplasm. However, white adipose tissue (WAT) is also an endocrine organ releasing adipocyte-derived factors, termed adipokines, as well as more traditional cytokines (10). Brown adipocytes, with multilocular lipid droplets and numerous mitochondria, are morphologically very different from white adipocytes. The main purpose of brown adipose tissue (BAT) is to carry out non-shivering thermogenesis. The precursor of all adipocytes is mesenchymal stem cells (MSC) (11). To differentiate into adipocytes, the MSC first undergo commitment to preadipocytes, which can further differentiate into mature adipocytes upon adipogenic stimuli. Many known

and probably still unidentified factors play important roles in changing the transcriptional program to allow commitment and differentiation (12). The main regulators of terminal differentiation of white adipocytes are the transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT-enhancer binding protein (C/EBP) α, β and δ (13).

In a healthy individual, the increase in glucose and lipid levels in the blood in the postprandial state triggers release of insulin from pancreatic β-cells. Insulin binds to insulin receptors (IR) on insulin responsive cells such as adipocytes and initiates an intricate network of intracellular signaling events (Figure 1). The IR is a tyrosine receptor kinase that, when ligated, autophosphorylates itself followed by phosphorylation of target proteins, such as insulin receptor substrates (IRS) (14). Phosphatidylinositol 3-kinase (PI3K) is recruited to phosphorylated IRS by its src homology 2 (SH2) domain. PI3K phosphorylates the 3-position of phosphoinositides generating phosphatidylinositol-3,4,5-trisphosphate (PIP_3), among other phosphoinositides, that in turn acts as docking sites for proteins with pleckstrin homology (PH) domains such as phosphoinositide-dependent protein kinase 1 (PDK1) and Akt (or protein kinase B (PKB)), bringing them into close proximity resulting in PDK1-dependent phosphorylation and thereby activation of Akt. Akt is a serine/threonine kinase that, when activated, phosphorylates a plethora of downstream targets (15). For instance, phosphorylation of Akt substrate of 160kDa (AS160) allows relocalization of

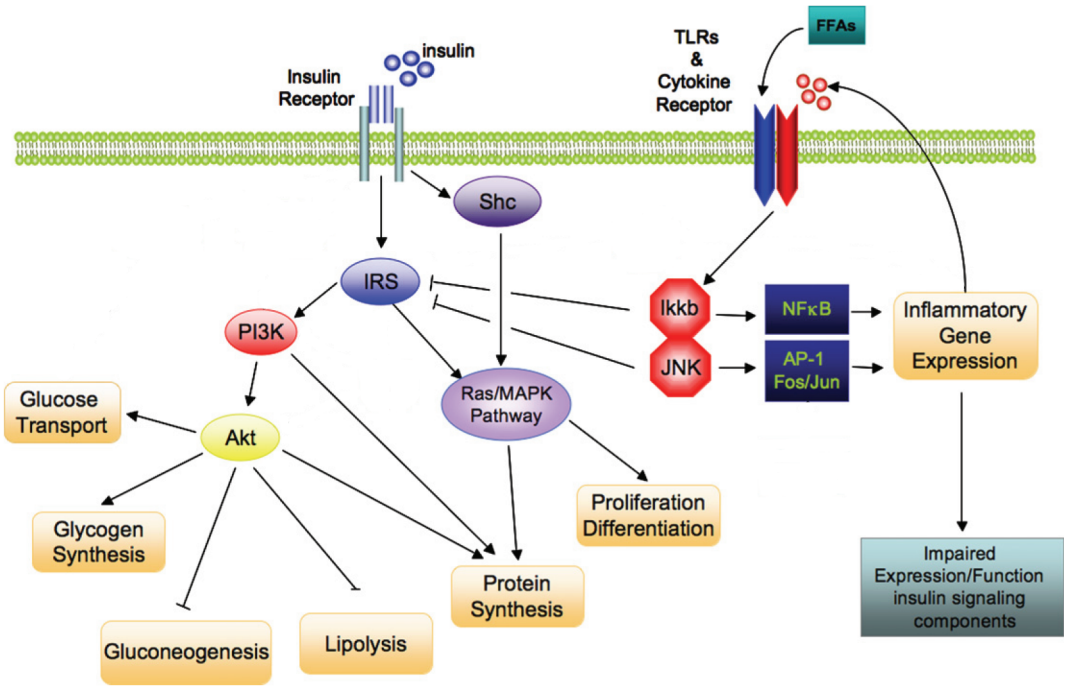

Figure 1. Insulin signaling and effects of inflammatory pathways. Insulin induce signaling through two main pathways, the PI3K/Akt pathway and the MAPK pathway, to regulate nutrient metabolism and cell growth. FA or inflammatory mediators results in the activation of the serine kinases, IKKβ and JNK, which activate transcription factors and reduce IRS signaling ability.

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glucose transporter 4 (GLUT4)-containing vesicles from intracellular stores to the plasma membrane, positioning GLUT4 to allow uptake of glucose from the circulation (16). Another Akt target is phosphodiesterase 3B (PDE3B), an enzyme that hydrolyzes cAMP (18,19). cAMP, generated by adenylyl cyclase in response to fasting or increased energy demand, stimulates cAMP-dependent protein kinase (PKA), which in turn activates hormone sensitive lipase (HSL). Previously HSL was considered the rate-limiting enzyme in lipolysis, the process whereby triacylglycerides are hydrolyzed into fatty acids and glycerol, however studies of HSL knock out mice have revealed that it mainly catalyzes the conversion of diacylglycerides into monoacylglycerides (20). The liberated fatty acids are released from adipocytes and transported to cells in need. Insulin activates PDE3B, reduces cAMP levels, and hence exhibits antilipolytic effects. Moreover, Akt stimulates lipogenesis at least in part via phosphorylating and inactivating glycogen synthase kinase 3 (GSK3). In addition, insulin activates the transcription factor sterol regulatory element-binding protein (SREBP)-1c, which turn on a number of genes encoding enzymes necessary for lipogenesis (21). Akt also promotes protein synthesis and cell growth by phosphorylating and inhibiting tuberous sclerosis 1 and 2 (TSC1/2), rendering mammalian target of rapamycin (mTOR) active to phosphorylate S6 kinase (S6K) and eIF-4E binding protein (4E-BP1) (22-24). In addition to the PI3K/Akt pathway, insulin promotes cellular growth via the mitogen activated protein kinase (MAPK) pathway (21). In liver and muscle, Akt-dependent phosphorylation and inactivation of GSK3 result

in glycogen synthesis through glycogen synthase (25). Additionally, insulin reduces glycogenolysis through negative regulation of glycogen phosphorylase. The collective efforts of glycogen synthase and glycogen phosphorylase result in replenished glycogen stores following a meal. Furthermore, Akt in the liver phosphorylates members of the Forkhead transcription factor family leading to nuclear exclusion and inactive cytoplasmic location of this transcription factor. Thus, expression of gluconeogenic genes such as phosphoenolpyruvate carboxykinase (Pepck) and glucose 6-phosphatase (G6Pase) are reduced resulting in suppression of hepatic glucose output (26-28). Taken together, insulin acts on several metabolic tissues to orchestrate a number of events to promote storage of the available nutrients and limit the use of the fuel reserves.

In the insulin resistant state, cells do not respond properly to the hormone insulin. To compensate for the elevated plasma glucose levels, pancreatic β-cells increase their insulin production and this is also accompanied by an increase in β-cell number (29). In some individuals, the β-cells ultimately cannot keep up with this increased demand of insulin and undergo β-cell failure resulting in hyperglycemia and hypoinsulinemia, a condition defined as type 2 diabetes.

There are a number of factors spurred by obesity that contribute to the development of insulin resistance. Such factors include, but are certainly not limited to, fatty acids, oxidative stress, adipokines, and inflammation.

Fatty acids. When the ability of adipocytes to store fat is impaired or if there is too much fat to store, levels of free fatty acids (FA) in the plasma are elevated. Hence, obese individuals experience increased circulating FA levels. Although FA released by adipocytes through lipolysis during a fast provide energy for peripheral tissues, prolonged exposure of elevated levels of FA, especially saturated long chain FA, has detrimental effects on cells. Indeed, FA treatment of cells causes activation of protein kinase C θ (PKCθ), which in turn activates c-jun N-terminal kinase (JNK) and inhibitor of nuclear factor κB (NF-κB) kinase β (IKKβ). These serine/threonine kinases are capable of phosphorylating IRS on inhibitory serine residues, preventing insulin-stimulated IRS tyrosine phosphorylation. As a result, glucose uptake is compromised generating an insulin resistant state (30). In addition to phosphorylating IRS, active IKKβ results in phosphorylation and degradation of inhibitor of NF-κB (IκB), and subsequent translocation of the transcription factor NF-κB to the nucleus inducing expression of pro-inflammatory mediators, including cytokines, that interfere with insulin signaling (see inflammation below) (31). Moreover, under conditions where circulating FA are elevated, lipid may accumulate ectopically in muscle, liver, and β-cells that are not capable of proper lipid storage thus causing lipotoxicity. Ectopic fat deposition can activate PKCθ resulting in impaired insulin signaling (32). Furthermore, lipid accumulation in β-cells can prevent insulin

secretion (33). In sum, excess FA signaling and ectopic lipid accumulation are implicated in insulin resistance.

Oxidative Stress. Physiological processes such as cellular respiration or certain enzymes, for example, NADPH oxidase, generate reactive oxygen species (ROS). Although ROS have signaling functions, excessive ROS production and/or dysfunction of antioxidant pathways responsible for detoxifying ROS result in accumulation of ROS and oxidative stress, a pathophysiological state that is harmful for the cell (34). Obesity is accompanied by increased oxidative stress in adipose tissue of mice and humans (35). Mechanistic studies utilizing cultured adipocytes indicated that FA, which are plentiful in obese adipose tissue, drive ROS production. Complementing these results, Tinahones *et al.* demonstrated a positive correlation between the degree of insulin resistance and oxidative stress in obese humans in the postprandial state (36). Polyunsaturated FA (PUFA) or polyunsaturated acyl chains of phospholipids are susceptible to attack from ROS, generating lipid hydroperoxide intermediates. Lipid hydroperoxide intermediates are unstable and undergo non-enzymatic Hock cleavage to form a variety of α,β-unsaturated reactive aldehydes, such as 4-hydroxy-2-nonenal (4-HNE), that can modify DNA or proteins if not detoxified by the cell. Proteins are modified on His, Lys or Cys residues in a process termed protein carbonylation (Figure 2) (37). Parallel to oxidative stress, protein carbonylation positively correlates with obesity in adipose tissue of mice and humans (38,39). Irreversible carbonylation

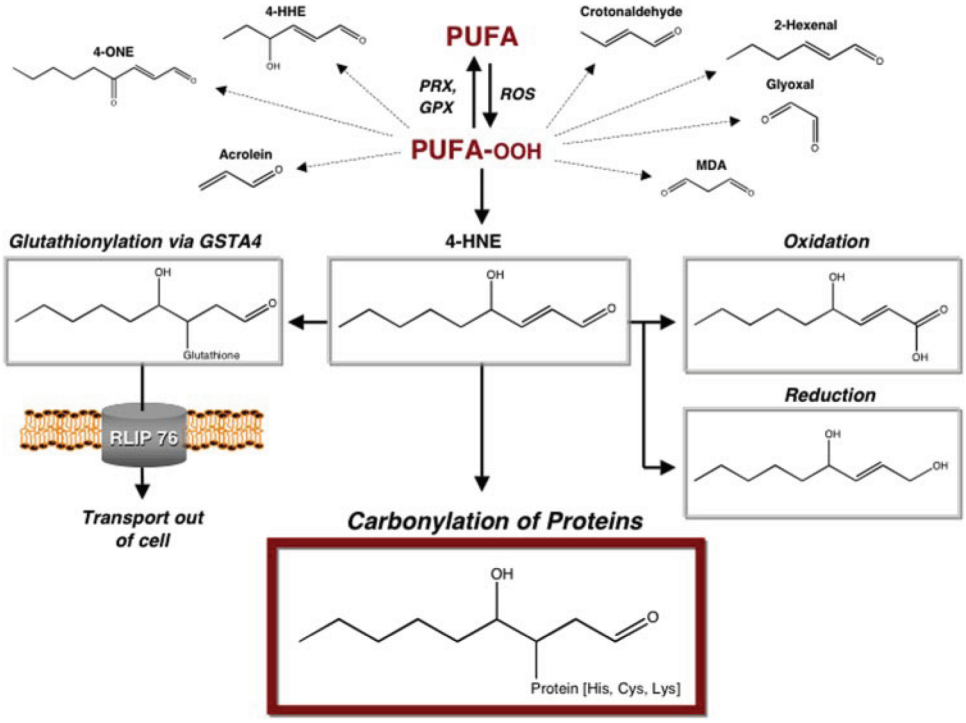

Figure 2. ROS-induced generation of 4-HNE. ROS stimulate peroxidation of polyunsaturated fatty acids (PUFA), generating unstable lipid hydroperoxides (PUFA-OOH). Non-enzymatic cleavage of PUFA-OOH results in a family of α,β-unsaturated aldehydes including 4-HNE. 4-HNE can be detoxified by oxidation or reduction or be conjugated to glutathione via GSTA4 and exported out of the cell. If 4-HNE fails to be metabolized it can covalently modify proteins on His, Cys, and Lys residues. PRX – peroxiredoxin and GPX – glutathione peroxidase.

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occurs on a wide spectrum of proteins affecting various cellular processes. For example, several ion and glucose membrane transporters, antioxidants, and stress-induced enzymes are inactivated by carbonylation furthering the oxidative imbalance (37). In addition, a battery of mitochondrial proteins involved in the electron transport chain and phosphate transport are subject to carbonylation, coinciding with mitochondrial dysfunction (40,41). Reduced antioxidant capacity, induced stress signaling pathways, and mitochondrial dysfunction following protein carbonylation can all contribute to obesity-linked insulin resistance.

Adipokines. In 1995 it was appreciated that adipose tissue is not simply an organ for triglyceride storage but also functions as an endocrine organ upon the finding that adipocytes secrete the hormone leptin (42). The amount of leptin released from adipocytes positively correlates with adipocyte size. Leptin signals to the central nervous system to reduce appetite and promote energy expenditure (43). It induces FA oxidation and therefore prevents ectopic lipid accumulation. Obese subjects develop leptin resistance, hence leptin delivery, as a therapeutic, is not feasible. Since the discovery of leptin, adipocytes have been found to secrete a wide variety of factors that influence whole-body metabolism, collectively referred to as adipokines (10). Consistent with the notion that ectopic lipid accumulation is detrimental for insulin sensitivity, overexpression of adiponectin, an insulin-sensitizing adipokine, promotes

expansion and fat storage in adipocytes (44). Adiponectin levels are reduced with obesity, which could explain the spill over of fat to other tissues.

Inflammation. Adipose tissue is composed of different cell types such as adipocytes, preadipocytes, endothelial cells, and a variety of immune cells such as macrophages (45,46), mast cells (47), neutrophils (48), T cells (49,50), B cells (51), and eosinophils (52).

In 2003 it was realized that obesity leads to increased levels of macrophages in adipose tissue preceding insulin resistance (45,46). The macrophage content was positively correlated with adipocyte size and body mass in humans and mice. In lean mice about 10% of cells in adipose tissue are macrophages and this percentage increase to 40% and 50% in extremely obese, leptin-deficient *ob/ob* mice and obese humans, respectively. Macrophages are generally described as classically activated macrophages (M1) or alternatively activated macrophages (M2) (53). These types of macrophages differ in expression of certain intracellular and cell surface markers as well as their functions. While M1 macrophages are considered pro-inflammatory and display bactericidal and phagocytic potential, M2 macrophages are involved in tissue repair and remodeling and are generally considered anti-inflammatory (54). In tissue, however, macrophages are a heterogeneous population and adopt intermediate phenotypes anywhere in between the two extremes (M1 and M2). Interestingly, macrophages residing in adipose tissue of lean mice are M2-like while adipose

tissue from obese mice contain predominantly M1 macrophages (55). Due to their pro-inflammatory effects, M1 macrophages have been linked to insulin resistance. Indeed, overexpression of monocyte chemoattractant protein-1 (MCP-1) in adipose tissue of mice results in enhanced infiltration of inflammatory macrophages and augmented insulin resistance (56,57). Conversely, knock out of MCP-1 prevents inflammatory macrophage infiltration and ameliorates insulin resistance. Along the same lines, disruption of PPARγ, the key regulator of alternative activation in macrophages, in myeloid cells renders mice insulin resistant (58). Again, pointing to the importance of immune cells in metabolic dysfunction. The role of inflammation in development of insulin resistance was further confirmed by the finding that mice with whole-body genetic disruption of the inflammatory kinase JNK are protected from obesity and insulin resistance (59). Moreover, mice with myeloid-specific JNK or IKKβ deletion have reduced inflammation and insulin resistance is alleviated (60,61). JNK and IKKβ result in activation of the transcription factors activator protein (AP) 1 and NF-κB, respectively, both of which have pro-inflammatory target genes, resulting in elevated levels of cytokines and other inflammatory factors. Cytokines promote lipolysis in adipocytes leading to a rise in circulating FA levels. The growing number of publications in this area clearly link obesity-induced inflammation to metabolic disease (62,63). Although macrophages have received much interest, other cell types also positively or negatively regulate the inflammatory state in adipose tissue (64).

Obesity affects many of the features discussed above but interestingly, it does not always promote insulin resistance. This reveals a couple of paramount questions: Why are some people protected from obesity-induced insulin resistance? What is the initiating factor(s), is there a certain threshold or combination of factors that drives insulin resistance in response to obesity? Further complicating matters, obesity is a multifactorial condition affecting adipose tissue as well as other organs. Although many genes have been knocked out or overexpressed affecting glucose homeostasis and insulin sensitivity in mice, the key event initiating insulin resistance in response to obesity remains unclear. Research aiming to identify this event will without a doubt be conducted in the future.

II. Adipocyte Fatty Acid Binding Protein

Fatty Acid Binding Proteins (FABPs) were discovered by Ockner *et al*. in 1972 (65). The 9 known FABP isoforms received their name after the tissue or cell type where they were first identified (66). All cells involved in active lipid metabolism express at least one FABP isoform. These proteins are highly expressed and usually constitute 1-5% of cytosolic protein (67). The adipocyte isoform of FABP, AFABP, was discovered by two laboratories almost simultaneously in the 1980s as a protein that is highly upregulated in differentiated adipocytes compared to preadipocytes (68,69). AFABP is expressed in adipocytes and in macrophages, although the levels are far greater in adipocytes. AFABP expression is upregulated in obesity. Another FABP isoform, epithelial FABP (EFABP) is expressed in a variety of cell types including adipocytes and macrophages. EFABP is more abundant than AFABP in macrophages, however in adipocytes EFABP levels are estimated to be 100-fold lower than that of AFABP (70). Furthermore, heart FABP (HFABP) is also expressed at low levels in macrophages.

High resolution structures of FABPs have been solved. Even though the sequence identity is not highly conserved between different isoforms, the x-ray crystal structure of AFABP is virtually superimposable with other FABPs. FABPs fold into 10 antiparallel β-strands, arranged into two orthogonal five-stranded β-sheets forming a β-barrel (71,72). Ligands bind inside the β-barrel and one end

of the barrel is closed off from the surroundings by side chains while the other end, where ligands enter the ligand-binding cavity, are partially restricted by a helix-turn-helix motif (73). It has proven difficult to unambiguously determine the role of this class of proteins. The generally accepted function of FABPs is to bind and sequester long chain FA and other hydrophobic molecules and facilitate their intracellular transport (74). Apart from affecting FA trafficking in the cell, AFABP interacts with different proteins such as PPARγ (75,76), HSL (77), Janus kinase 2 (Jak2) (78), and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (79) to alter their activity. Interestingly, in the cases where the interaction domain of proteins associating with AFABP has been mapped, a consensus sequence of ExYK, important for interaction has emerged. In proteins where residues necessary for interaction have not been identified, an ExYK motif is present in their sequence. In addition, FABP has to have a FA bound in the ligand-binding cavity for these interactions to occur, indicating that FABP functions as an intracellular FA sensor. AFABP may promote insulin resistance by, for example, interacting with HSL to induce lipolysis, resulting in elevated circulating FA. In addition, PPARγ activity is increased in AFABP KO mice, indicating that AFABP is capable of inhibiting PPARγ. PPARγ agonists, such as thiazolidinediones (TZD), improve insulin sensitivity in animal models and humans. Adida *et al.* found that AFABP can bind to helix 12 of PPARγ, the region required for recruitment of co-regulators, suggesting that AFABP can block co-activator binding.

EFABP, both *in vitro* and *in vivo*, is modified on Cys120 by 4-HNE, linking FABP biology to oxidative stress (80). In tissue extract from mice lacking EFABP other proteins experienced increased modification. Therefore, it was hypothesized that EFABP could serve as a scavenger of lipid species to protect other biomolecules from modification. In this sense, EFABP would act as an antioxidant. Grimsrud *et al*. later reported that AFABP is also modified by 4-HNE on Cys117 (39). It was estimated that 6-8% of AFABP is modified by 4-HNE in adipose tissue from obese mice. Although carbonylation of AFABP causes a 10-fold reduction in its affinity for FA, the exact outcome of modification remains unclear.

Even though AFABP and EFABP adopt very similar conformation and both bind hydrophobic molecules, evidence for isoform-specific functions exists. Tan *et al.* reported that AFABP interacts with PPARγ while EFABP interacts with PPARδ, indicating that AFABP and EFABP have unique functions in the cell (75). Subtle changes in electrostatic and hydrophobic topology of AFABP and EFABP may explain this preference discrepancy. Surrounding the helix-turn-helix motif, AFABP has a ridge of positive electrostatic surface potential that is slightly altered in intestinal, liver, and muscle FABP (81). In addition, surface exposed hydrophobic residues are distributed differently in these isoforms. Presumably, AFABP and EFABP also differ in these aspects, causing the proteins to interact with different partners.

Another clue for isoform-specific functions come from knock out mice. Spiegelman and colleagues discovered in 1996 that whole-body genetic disruption of AFABP in mice led to protection from development of insulin resistance on a high-fat diet, even though the AFABP knock out mice gained even more weight than wild type mice (82). Similar results were obtained in genetically obese leptin-deficient *ob/ob* mice (83). Apart from being more insulin sensitive, obese AFABP knock out (AKO) mice display a number of interesting features. Adipose tissue expression of the pro-inflammatory cytokine tumor necrosis factor α (TNFα) is lowered (82), the rate of lipolysis is reduced in adipocytes isolated from AKO mice (70,83,84), and the mice, as well as isolated adipocytes, have impaired non-esterified FA (NEFA) efflux from adipocytes resulting in a 3-fold increase in intracellular NEFA (70,85). Disruption of AFABP in mice results in a compensatory upregulation of EFABP in adipocytes, however, the total FABP levels are still 10-fold reduced compared to control mice (70). Moreover, AKO mice on a normal chow diet, a condition where improvements in insulin sensitivity and metabolic status are minor, are protected from atherosclerosis when crossed with atherosclerosis-prone apolipoprotein E (ApoE)-deficient mice (86). Atherosclerosis is an inflammatory disease, where the arterial wall thickens as a result of lipid deposition. Since macrophages, by transforming into foam cells, are key players in atherosclerotic lesions, the effect of AFABP in macrophages was further studied. AFABP-deficient macrophages

show an anti-inflammatory phenotype with diminished activation of inflammatory factors such as IKKβ, NF-κB, COX-2, and iNOS followed by attenuated secretion of pro-inflammatory cytokines (TNFα, IL-1β, IL-6, and MCP-1) (86,87). Additionally, PPARγ activity is enhanced in AKO macrophages impairing cholesterol ester accumulation, a parameter influencing formation of foam cells. Importantly, bone-marrow transplantation studies indicated that these results are due to the functions of AFABP exclusively in macrophages and appear unrelated to AFABPs role in lipid and glucose metabolism (86). In an attempt to tease apart the function of FABP in adipocytes and macrophages, Furuhashi *et al.* conducted a series of experiments in co-culture settings and bone-marrow chimeras (88). Absence of FABP in macrophages improved insulin signaling in adipocytes while inflammation in macrophages was reduced when FABPs were deleted in adipocytes. These experiments demonstrated that actions of FABP in both adipocytes and macrophages are responsible for the progression of insulin resistance. Apart from regulating inflammation in adipose tissue macrophages, AFABP actions are required for allergic airway inflammation. AFABP expression is strongly induced in bronchial epithelial cells by asthma-promoting Th2 cytokines. Intriguingly, AKO mice had reduced recruitment of leukocytes to the airways and diminished levels of cytokines in response to allergen stimulation (89). Immunohistochemistry and bone-marrow transplantation experiments suggest that AFABP in airway epithelial cells are responsible for the enhanced inflammation caused by allergen.

EFABP knock out (EKO) mice mimic the AKO mice in most aspects. EKO mice have improved glucose and insulin tolerance, however, opposite to AKO mice, EKO mice have slightly decreased body weight (90). Hence, it appears as if both AFABP and EFABP are involved in metabolic regulation. Fascinatingly, AFABP and EFABP double knock out (DKO) mice show an even more pronounced phenotype than single knock out mice (91). With no change in food consumption, body weight and fat mass are lower in DKO mice compared to wild type littermates. Glucose and insulin tolerance are improved in DKO mice, as well as insulin signaling in adipose tissue and muscle. Apart from being protected from insulin resistance and atherosclerosis, DKO mice do not develop fatty liver disease.

Even if EKO mice mimic the phenotypes seen in AKO mice, the effects are generally more modest, which implicates that AFABP and EFABP have different functions or that they share the same functions but the relative abundance of FABP is a major determinant. Arguing against the latter is the fact that the phenotypes observed in macrophages seem to be attributable to AFABP in spite of it being less abundant than EFABP in this cell type (86).

Since mice with genetic deletion of AFABP have several beneficial effects, different academic laboratories and pharmaceutical companies have aimed to

develop small molecule inhibitors of AFABP in the hope to recapitulate the results observed in knock out mice (92,93). One such inhibitor, BMS309403, reduced MCP-1 release from cultured macrophages (94). When administered orally to *Apoe*$^{-/-}$ mice fed a western diet, animals developed fewer atherosclerotic lesions accompanied by a reduction in foam cells formation and less accumulation of cholesterol esters. Adipose tissue from inhibitor-treated genetically obese mice contained less macrophages, had lower cytokine expression, and improved insulin signaling than the control group. Hence, obese BMS309403 treated mice experienced alleviated systemic insulin resistance. On the other hand, AFABP/EFABP dual inhibitors failed to protect diet-induced obese mice from insulin resistance even though they inhibited lipolysis in cultured adipocytes and prevented cytokine release from cultured macrophages (95). Even though some promising data exist in mice suggesting that AFABP antagonism is a promising strategy for therapeutic intervention, there are currently no pharmacologic inhibitors targeting AFABP on the market.

Mutation of the AFABP promoter (T-87C) in humans results in impairment of C/EBPα binding and lower AFABP expression, hence protection from the metabolic syndrome (96). This suggests that FABP function is conserved between man and mouse and emphasizes the importance of FABP biology in humans.

III. Leukotriene Biology in Obesity

Eicosanoids are a class of inflammatory lipid molecules generated from arachidonic acid (20:4 $\Delta^{5,8,11,14}$). Arachidonic acid is an essential polyunsaturated ω–6 FA that is commonly found in the sn-2 position of phospholipids in nuclear membranes. Upon elevation of intracellular calcium levels, cytosolic phospholipase A_2 ($cPLA_2$) translocates from the cytosol to the perinuclear membrane and interacts with the phospholipids by virtue of its C2 domain. $cPLA_2$ catalyzes a hydrolysis reaction to liberate arachidonic acid from phospholipids (97). The released arachidonic acid has several different fates. For example, cyclooxygenases (COX) can convert arachidonic acid into prostaglandins or thromboxanes. In addition, arachidonic acid can be channeled to 5-lipoxygenase (5LO) via 5LO activating protein (FLAP). 5LO can catalyze two reactions, the first is the oxygenation of arachidonic acid generating 5-hydroperoxyeicosatetraenoic acid (5-HpETE). At this point, 5-HpETE can either be reduced to 5S-hydroxyeicosatetraenoic acid (5S-HETE) or dehydrated by 5LO resulting in formation of leukotriene A_4 (LTA_4, 5(S)5,6-ozido-7,9,11,14-eicosatetraenoic acid). Moreover, arachidonic acid can be acted upon by 12LO and 15LO that converts it into 12- and 15-HETE, respectively. Furthermore, cytochrome P450 epoxygenases can act on arachidonic acid generating different epoxylipids. The majority of arachidonic acid-derived lipid mediators have pro-inflammatory effects that are important for normal functions, such as host defense, but become harmful when production is excessive.

The amount of arachidonic acid is 0.5% of total FA in adipose tissue. However, studies in humans suggest that the level of arachidonic acid is increased in adipose tissue of obese subjects and that there is a positive correlation between arachidonic acid levels and the metabolic syndrome (98,99).

LTA_4, generated from the cooperative actions of $cPLA_2$, 5LO and FLAP, can subsequently be converted into LTB_4 (5(S),12(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid) by LTA_4 hydrolase or conjugated to a glutathione moiety by LTC_4 synthase (LTC_4S) generating LTC_4 (5(S)-hydroxy-6(R)-S-glutathyionyl-7,9,11,14-eicosatetraenoic acid) (100). LTC_4 can be exported out of the cell and undergo further processing. Extracellularly, the glutamyl can be cleaved from the glutathione moiety of LTC_4 by γ-glutamyl transferase producing LTD_4. The glycine residue of LTD_4 can be removed by dipeptidase resulting in LTE_4 (Figure 3). LTC_4, LTD_4 and LTE_4 are collectively referred to as cysteinyl leukotrienes (CysLT, also known as slow-reacting substance of anaphylaxis). Enzymes that make up the leukotriene machinery are primarily expressed in inflammatory cells and the leukotrienes are involved in diseases with an inflammatory component. Leukotrienes signal through G-protein coupled receptors on target cells. There are two LTB_4 receptors termed BLT1 and 2. CysLT bind two receptors, CysLTR1 and 2. However, several investigations suggest that one or more receptors for CysLT remain to be identified (101). This is based on the fact that using a dual

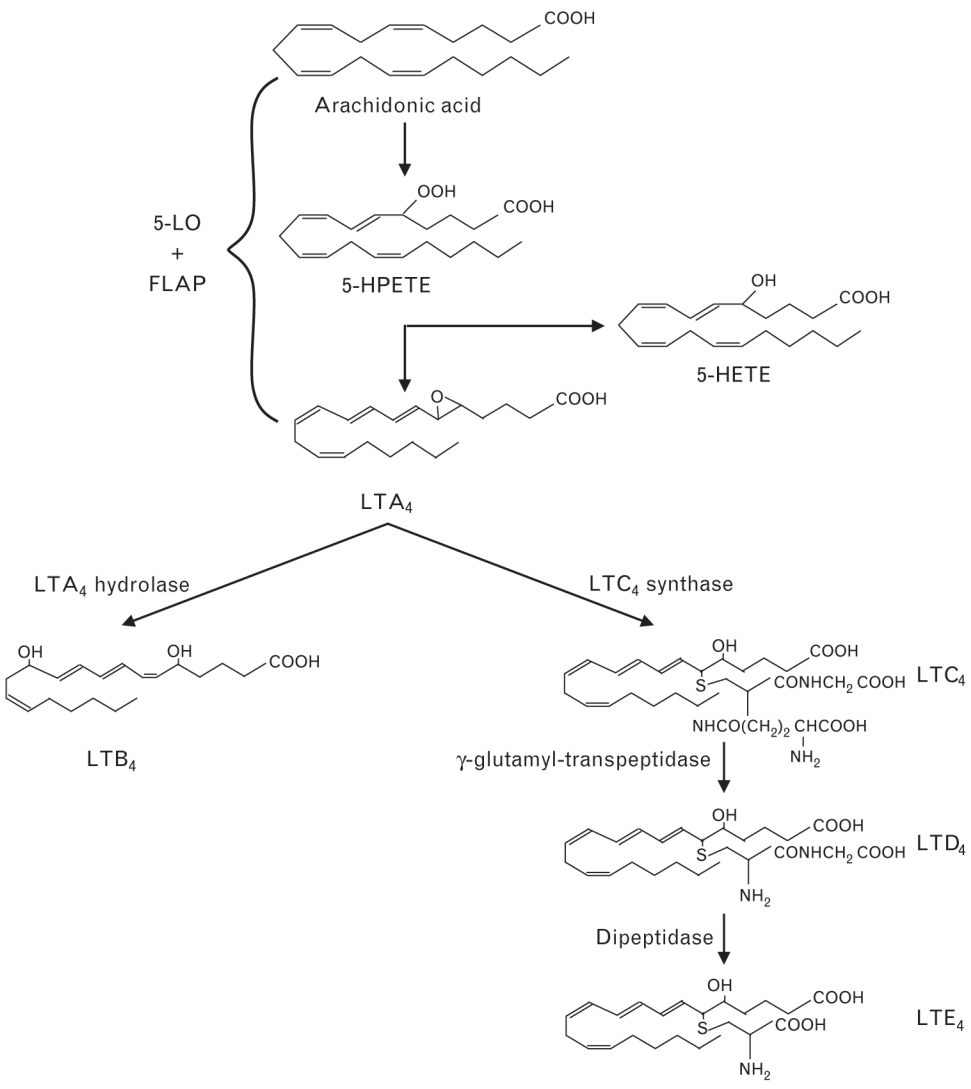

Figure 3. Schematic of leukotriene synthesis.

Image reprinted from (103) with permission from Wolters Kluwer Health.

antagonist of CysLTR1 and 2 does not block all effects of leukotriene treatment (102). Furthermore, both CysLTR1 and 2 have very weak, if any, affinity for LTE_4, supporting that it signals through other functional receptors.

Ligation of the receptors activates G-proteins and initiates signaling cascades generally resulting in increased intracellular calcium, activation of MAPK as well as other kinases, and NF-κB giving rise to a multitude of biological actions. Predominantly, leukotrienes have been studied in inflammatory airway disease where increased levels are associated with asthma and allergic rhinitis (100). The main functions of LTB_4 are to act as a potent chemoattractant, promote degranulation and ROS production causing recruitment, adherence, and activation of leukocytes. CysLT are highly potent smooth muscle contraction agents leading to bronchoconstriction of the airways. They also enhance mucus secretion and vascular permeability, thereby supporting inflammatory cell recruitment. In a combined effort, leukotrienes coordinate movement of leukocytes to the site of inflammation to promote allergic responses and host defense.

Apart from being involved in asthma, leukotrienes have been proposed to be important players in other inflammatory diseases such as atherogenesis. Indeed, levels of 5LO and FLAP, and their products, are increased in atherosclerotic plaque (104,105). Genetic knock out or pharmacologic inhibition of enzymes

producing leukotrienes or interfering with downstream signaling of leukotrienes ameliorate atherosclerosis in mice (105-107). It has been suggested that leukotrienes play a role in early stages of atherogenesis so that progression of atherosclerosis in the absence of leukotrienes is slowed down (108). Presumably, once the plaque has formed there is no difference between animals with interrupted leukotriene synthesis and wild type animals. However, more work is needed to elucidate the role of leukotrienes in atherosclerosis.

More recently, there has been an increasing interest in the involvement of various lipid mediators, such as HETEs and leukotrienes, in obesity and insulin resistance (103,109).

HETEs. mRNA levels of 12LO and leukocyte 12/15LO are increased in isolated adipocytes and adipose tissue of obese mice and rats compared to lean counterparts (110,111). This was accompanied by increased HETE production. In addition, 12/15LO mRNA increases during adipogenesis in cultured cells and FA stimulate the levels further (110). The products of the 12/15LO pathway, 12-HpETE and 12S-HETE, induce secretion of pro-inflammatory cytokines and activate JNK resulting in impaired insulin signaling, evident by increased phosphorylation of serine residues of IRS, in differentiated 3T3-L1 adipocytes (110).

Leukotrienes. Information about leukotrienes in obesity and insulin resistance has been more limited. But in 2010 it was established that the leukotriene synthesizing machinery and the downstream leukotriene receptors are present in adipose tissue, in isolated primary adipocytes as well as in the stromal vascular fraction (112). Interestingly, the expression of FLAP and 5LO are increased in adipose tissue with obesity and inflammation (105,111). Consistently, LTB_4 and CysLT levels are elevated in adipose tissue from obese high-fat fed mice (113). Cells in the stromal vascular fraction are the main producers of leukotrienes even if adipocytes are capable of producing smaller amounts. Treatment of adipose tissue explants with LTB_4 and LTD_4 increased the activity of the pro-inflammatory transcription factor NF-κB (112). Importantly, 5LO knock out mice or wt mice treated with a 5LO inhibitor, zileuton, have reduced macrophage infiltration into adipose tissue and improved insulin sensitivity when placed on a high-fat diet compared to control mice (113). Similarly, treatment of mice fed a high-fat diet with a FLAP inhibitor, Bay-X-1005, reduced 5LO products in adipose tissue, improved insulin sensitivity, diminished inflammatory cell infiltration into adipose tissue and reduced lipid accumulation in liver (112). Along the same lines, disruption of the LTB_4 receptor BLT1 in mice fed a high-fat diet improved insulin signaling in skeletal muscle, decreased inflammation in adipose tissue and alleviated systemic insulin resistance (114). Taken together, these studies show that leukotrienes are in fact made in adipose tissue, and play a role in development of obesity-induced insulin resistance.

Several other mouse models engineered to lack proteins in the leukotriene pathway have been generated and all lead to developmentally normal offspring (115-118). Unfortunately, unlike the 5LO and BLT1 knock out mice mentioned above, these studies were performed before the involvement of leukotrienes in obesity-induced insulin resistance was realized and therefore mice were not challenged with a high-fat diet.

Interestingly, mice deficient in the leukotriene machinery have increased adiposity without inflammation and protection from insulin resistance on a high-fat diet (119,120). This is the same constellation of phenotypes also observed in AKO mice. Therefore, a connection between leukotrienes and AFABP is plausible. Indeed, FABPs can stabilize LTA_4. LTA_4 contains an epoxide moiety that is highly susceptible to spontaneous water hydrolysis generating molecules that do not serve as precursors for the downstream leukotrienes. Thus, LTA_4 has a short half-life in aqueous buffer. In the presence of FABPs, the half-life is increased from 30 seconds to around 15 minutes (121), implicating that FABPs may mediate downstream leukotriene production by increasing steady-state levels of the precursor LTA_4. Furthermore, obese asthmatics secrete higher amount of LTE_4 in the urine than lean asthmatics (122), indicating that leukotriene synthesizing capacity is increased in conditions where AFABP expression is elevated.

Collectively, these results led to the hypothesis that leukotrienes are produced in a FABP-dependent manner and may be implicated in propagation of insulin resistance and its sequelae. Targeting leukotriene production or their downstream signaling capabilities in adipose tissue could be a new therapeutic strategy to prevent obesity-induced complications.

IV. Current Objectives

The association between obesity and inflammation is well established. Mice with genetic disruption of AFABP exhibit an insulin sensitizing and anti-inflammatory phenotype on a high-fat diet compared to wild type littermates, however the molecular mechanisms are not completely understood.

The goal of the studies presented herein was to gain further insights into the role of AFABP in the development of obesity-related insulin resistance. The effect of ligand binding on AFABP was characterized by x-ray crystallography. More specifically by solving structures of AFABP bound to one inflammatory lipid, 4-HNE, and one pan-specific FABP inhibitor, HTS01037. In addition, leukotriene production in adipose tissue and macrophages was evaluated as well as its potential link to AFABP.

V. References

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CHAPTER 2

X-RAY CRYSTALLOGRAPHIC ANALYSIS OF ADIPOCYTE FATTY ACID BINDING PROTEIN (aP2) MODIFIED WITH 4-HYDROXY-2-NONENAL

This chapter is a reprint of an original publication with minor alterations:

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Kristina Hellberg performed experiments and wrote the paper.

Fatty acid binding proteins (FABP) have been characterized as facilitating the intracellular solubilization and transport of long-chain fatty acyl carboxylates via noncovalent interactions. More recent work has shown that the adipocyte FABP is also covalently modified *in vivo* on Cys117 with 4-hydroxy-2-nonenal (4-HNE), a bioactive aldehyde linked to oxidative stress and inflammation. To evaluate 4-HNE binding and modification, the crystal structures of adipocyte FABP covalently and noncovalently bound to 4-HNE have been solved to 1.9 Å and 2.3 Å resolution, respectively. While the 4-HNE in the noncovalently modified protein is coordinated similarly to a carboxylate of a fatty acid, the covalent form show a novel coordination through a water molecule at the polar end of the lipid. Other defining features between the two structures with 4-HNE and previously solved structures of the protein include a peptide flip between residues Ala36 and Lys37 and the rotation of the side chain of Phe57 into its closed conformation. Representing the first structure of an endogenous target protein covalently modified by 4-HNE, these results define a new class of *in vivo* ligands for FABPs and extend their physiological substrates to include bioactive aldehydes.

Introduction

Obesity-linked insulin resistance and a variety of metabolic comorbidities are associated with inflammation and have been causally linked to an increase in reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and hydroxyl radicals (1–4). Of the various deleterious effects of ROS, particularly hydroxyl radicals, oxidation of polyunsaturated fatty acids (PUFA) and phospholipids in membranes followed by Hock cleavage generates a family of lipid-derived reactive aldehydes with the most common being malondialdehyde and 4-hydroxy-2-nonenal (also known as (2E,4R)-4-hydroxynon-2-enal or 4-HNE) (5–7). 4-HNE is an α,β-unsaturated 9 carbon aldehyde that undergoes Michael addition reactions with the side chains of cysteine, histidine and lysine residues. As such residues are frequently used in catalysis, alkylation reactions typically result in enzyme inactivation followed by targeted degradation leading to overall net loss of function (8). Grimsrud *et al*. recently discovered in murine adipose tissue that the adipocyte fatty acid binding protein (AFABP also known as aP2) is the soluble protein most highly modified by 4-HNE (9). Approximately 6–8% of the AFABP in adipose tissue of obese mice is covalently modified on Cys117 by 4-HNE and results in decreased affinity of the protein for fatty acids (9). AFABP facilitates the diffusion of fatty acids in the adipocyte and mediates transport of fatty acids derived from triacylglycerol breakdown (10–12). Molecular disruption of AFABP in mice leads to reduced lipolysis and mild obesity as well as the surprising finding of an anti-diabetic and anti-atherogenic phenotype (12–

17). The latter result has spurred on the development of a variety of AFABP targeted small molecule drugs that may be efficacious in unlinking obesity from diabetes and atherogenesis (18,19). Like other intracellular lipid-binding proteins, the structures of AFABP in both its apo and several different holo forms consist of a β-barrel formed by 10 antiparallel β-strands (referred to as A-J) defining an interior water filled binding cavity that serves as the ligand-binding site (20–23). One end of the b-barrel is closed by the packing of protein side chains while the other end is partially restricted by a helix-turn-helix motif that serves as the portal for ligand entry/exit. The ligand-binding cavity contains several structurally conserved water molecules that are present in both apo- and holo-protein. The helix-turn-helix motif of AFABP and other FABP isoforms are important for fatty acid transfer from FABPs to membranes (24) and four surface-exposed charged residues in this motif represent a protein-protein interaction domain (25,26). Physiological ligands for AFABP are long-chain fatty acids and carboxylate derivatives that bind to the protein noncovalently through acyl chain hydrophobic and entropic interactions and salt bridges between the carboxylate and Arg126 and Tyr128. AFABP binds avidly to a variety of long-chain fatty acids (Kd 5-50 nM) with the affinity declining substantially when the fatty acid is less than 12 carbons (27,28). The discovery that AFABP is modified by 4-HNE *in vivo* represents a major shift in our understanding of AFABP biology. Firstly, 4-HNE has only 9 carbons and is shorter than any ligands previously believed to interact with the adipocyte fatty acid binding protein *in vivo*. Secondly, 4-HNE does not

possess a carboxyl group but is an aldehyde. Thirdly, AFABP and 4-HNE interact through a covalent linkage. Taken together, the above observations suggest that the cellular functions and specificity of AFABP is much broader than previously anticipated and provides new interpretation of the anti-diabetes function of AFABP inhibitors. Herein we report the crystal structure and analysis of AFABP with noncovalently bound 4-HNE and covalently modified with 4-HNE. To the best of our knowledge this is the first conformation of an endogenously modified AFABP as well as the first structure of 4-HNE covalently modifying an endogenous target protein.

Results

Enrichment of 4-HNE Modified AFABP. Bacterially derived apo mouse AFABP was modified with 4-HNE for 30-70 minutes and the reactions quenched by addition of β-mercaptoethanol. The modified protein was applied to a long-chain acyl carboxylate affinity column and eluted with 1M NaCl (29). Protein in the bound and unbound fractions was analyzed by mass spectrometry (Figure 1) where covalent 4-HNE modification was accompanied by a 156 Da increase in the detected molecular mass of the protein. The bound fraction contained approximately 90% 4-HNE modified protein while a sample not subject to affinity chromatography represented a 70:30 mixture of modified and unmodified AFABP, respectively. Crystals were obtained from protein prepared without or with affinity enrichment and represented two forms; one with partial occupancy, noncovalently bound 4-HNE and one with covalently bound 4-HNE, respectively.

Overall Structure and 4-HNE Binding. Crystal structures were analyzed for AFABP containing noncovalently and covalently bound 4-HNE. Statistics for the diffraction data and subsequent model refinement are shown in Table 1. The overall folds for both forms are very similar to previously solved structures of AFABP with fatty acid ligands, but present unique conformational changes in a number of regions (Figure 2). The overall fold consists of 10 anti-parallel β-strands arranged into a β-barrel and two α helices forming a lid to the ligand-binding cavity where 4-HNE is located. The ligand-binding cavity also contains

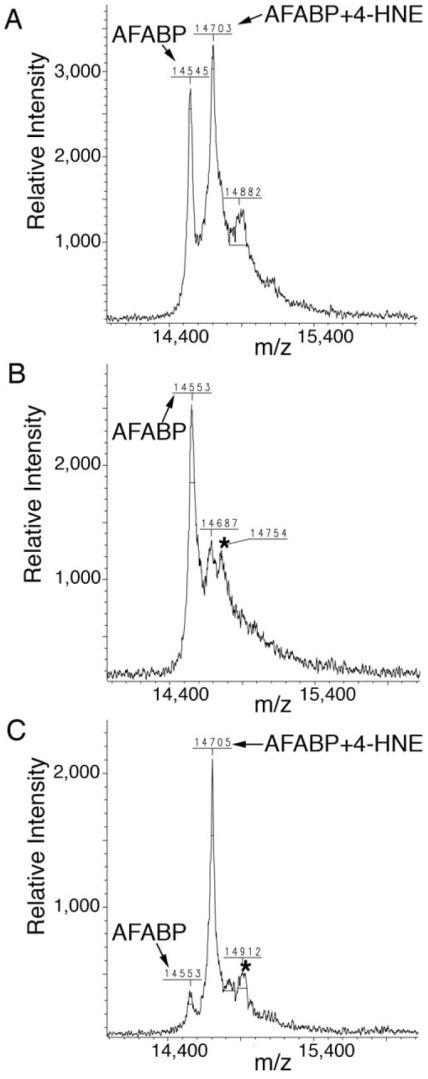


Figure 1. Enrichment of covalently 4-HNE modified AFABP. Shown are mass spectrometric analysis spectra of (A) sample after modification of AFABP with 4-HNE, (B) flow through fraction from the long-chain fatty acid affinity column, (C) eluted fraction from the long-chain fatty acid affinity column. Unmodified AFABP as well as AFABP modified with 4-HNE is indicated with arrows. * indicates a matrix adduct. Errors in the m/z values are within the mass accuracy of the instrument used.

Table 1. X-ray Data Collection and Refinement Statistics

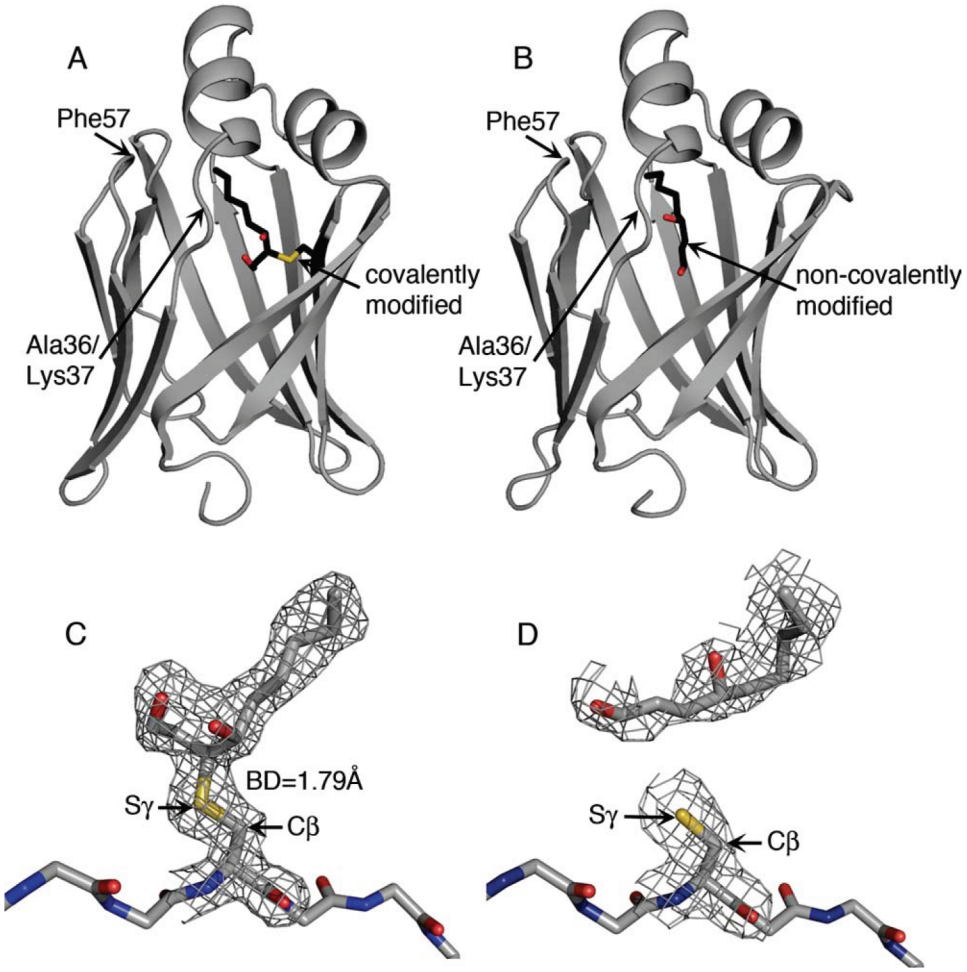
	Covalent 4-HNE[a]	Non-covalent 4-HNE[a]
Data collection		
Space group	$P2_12_12_1$	$C222_1$
Cell dimensions		
a, *b*, *c* (Å)	50.3, 81.3, 92.7	77.6, 93.2, 49.1
Resolution (Å)	20.0-1.8 (1.94-1.90) *	50.0-2.3 (2.34-2.30)*
R_{sym} (%)	6.8 (45.6)*	6.5 (33.2)*
$I / \sigma I$	20.3 (3.2)*	16.6 (2.9)*
Completeness (%)	97.4 (100.0)*	93.7 (73.5)*
Redundancy	4.4 (4.4)*	3.6 (3.2)*
Refinement		
Resolution (Å)	19.86-1.81	19.97-2.30
No. reflections	33276	7381
R_{work} / R_{free} (%)	19.7/22.8	21.4/26.3
No. atoms		
Protein	2039	1022
Ligand/ion	22/5	11/21
Water	365	91
B-factors (Å^2)		
Protein	23.8	32.7
Ligand/ion	31.0/16.3	64.7/68.3
Water	37.1	45.3
R.m.s. deviations		
Bond lengths (Å)	0.010	0.016
Bond angles (°)	1.323	1.160

[a]Data were collected from one crystal. *Values in parentheses are at resolution limit.

ordered water molecules, some of which are conserved with respect to the family of known structures.

Dimerization. For the covalent 4-HNE structure there are two molecules in the asymmetric unit cell and they are related by a local two fold symmetry axis. The root mean square deviation (RMSD) between the Cα atoms in the A and B chain is 0.182 Å. The total solvent accessible surface areas for chain A and chain B are 7145 and 7294 $Å^2$, respectively, as calculated using Surface Racer 5.0 (30) with a probe radius of 1.4 Å. The total solvent accessible surface area for the dimer in the asymmetric unit cell is 12905 $Å^2$ resulting in a large contact area between the two monomers of 1535 $Å^2$. This suggests that the AFABP with 4-HNE covalently attached could potentially form a biological dimer since crystallographic contact areas are usually small. Residues 44-73, corresponding to β-strands C, D, and E, are located in the likely dimerization interface. Three holo-AFABP structures have been proposed to lead to dimerization formation previously based on crystal packing (31,32). These ligands are linoleic acid, troglitazone and 1-anilinonaphthalene-8-sulfonate (ANS). The noncovalent form crystallizes with one molecule in the asymmetric unit cell. This monomer has a total accessible surface area of 6587 $Å^2$. However, a plausible dimer can be found in the crystal packing that closely resembles the dimer found in the covalent form of the protein with a RMSD of the Cα atoms in the covalent and noncovalent 4-HNE dimers of 0.507 Å. The monomers in the noncovalent dimer are related by a

Figure 2. The structure of AFABP with covalently and noncovalently bound 4-HNE. The cartoons illustrate the overall structure of AFABP (A) covalently or (B) noncovalently modified with 4-HNE. AFABP is displayed in cartoon representation with the 4-HNE molecule located in the ligand-binding cavity represented in sticks. The position of the Ala36/Lys37 region and Phe57 is indicated. Electron density surrounding the 4-HNE molecule and Cys117 of AFABP in the (C) covalent and (D) noncovalent forms are compared. 4-HNE, Cys117, and the AFABP backbone around Cys117 are shown in stick representation. The $2|F_O|-|F_C|$ maps are contoured at 1.0 σ. The Cβ and Sγ atoms of Cys117 are indicated in the figure. The bond distance (BD) between C3 of the covalently bound 4-HNE and Sγ of Cys117 is given. Carbons are colored grey or black, oxygens red, nitrogens blue and sulfurs yellow.


A
Phe57
covalently
modified
Ala36/
Lys37
B
Phe57
non-covalently
modified
Ala36/
Lys37
C
BD=1.79Å
Sγ
Cβ
D
Sγ
Cβ

crystallographic two-fold symmetry axis. The total solvent accessible surface area of this dimer is 11611 $Å^2$ leading to that 1563 $Å^2$ of the surface area is lost in the dimer compared to two individual monomers, a number very similar to the area lost in the covalent form. Despite the prediction that AFABP may form a dimer, size exclusion chromatography revealed that apo-AFABP and HNE-modified AFABP chromatograph as monomeric proteins and not a dimeric assembly (results not shown). This point is further evaluated in the Discussion.

4-HNE Modification. Analysis of the covalently modified AFABP revealed that the chemical modification from the α,β-unsaturated aldehyde results in a covalent bond to the sulfur atom of side chain of Cys117. The result is a protein-thioether bond (Figure 2C). Consistent with the mass spectrometry results (Figure 1) the electron density for the ligand is as strong as the electron density for most of the residues in the protein suggesting that essentially all of the protein was covalently modified. In the structure, the sulfur atom of Cys117 is covalently attached to 4-HNE in an S-configuration at carbon 3 (C3) leading to an R-configuration at C4. To ensure that the chirality of C3 was evaluated correctly, C3 was modeled in an R-configuration and one additional round of refinement performed. This generated more negative electron density in the F_o-F_c omit map, especially in the A chain, consistent with the conclusion that C3 adopts an S-configuration. In contrast to the covalent 4-HNE structure, 4-HNE in the noncovalent form is too far away from the side chain sulfur of Cys117 to be

covalently attached (Figure 2D). In addition, the electron density of the ligand in this noncovalent form is much weaker compared to the density for side chains in the protein suggesting that substitution was not stoichiometric.

Ligand Coordination. Since 4-HNE is attached covalently to Cys117 the ligand does not reach as far into the binding cavity as does a fatty acid. In the covalent 4-HNE structure the ligand aldehyde is positioned to far away (4.69 Å) to allow direct interaction with Tyr128 that has been identified previously to coordinate fatty acid carboxyl groups together with Arg126 (21) (Figure 3A,C). The distance between the aldehyde of 4-HNE and Arg126 is 4.47 Å, while the distance to the fatty acid oleic acid (PDB ID 1LID) is 2.77 Å. Instead, there is an additional water molecule in the covalent 4-HNE structure located 2.69 Å from the aldehyde of the ligand that is not present in the structure with oleic acid. This water molecule forms hydrogen bonds with both the free aldehyde and with Arg126 thereby bridging the two (Figure 3A). In contrast to the covalent 4-HNE structure, the noncovalent 4-HNE bound form reveals that the ligand aldehyde extends as far into the ligand-binding cavity as does the carboxyl group of a fatty acid, and the aldehyde aligns well with the carboxyl oxygen of a fatty acid facing Arg126 and Tyr128. This permits the aldehyde to interact directly with both Arg126 and Tyr128 (Figure 3B) without the need for any bridging water molecules. In addition to Arg126 and Tyr128, Arg106 has also been implicated in the coordination of oleic acid by indirectly interacting with the fatty acid carboxylate via a water

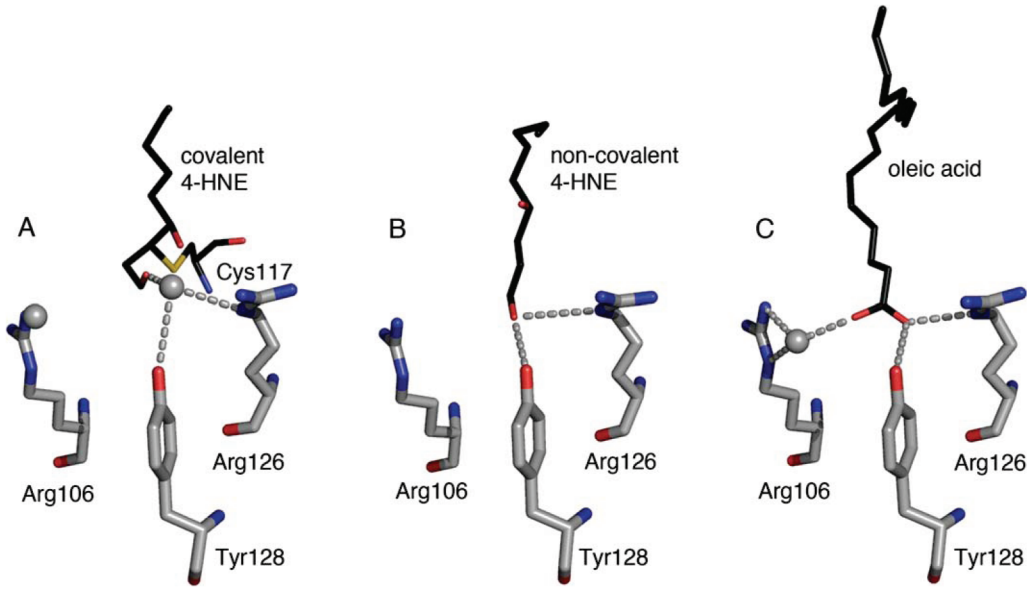


Figure 3. Coordination of the 4-HNE aldehyde function. The free aldehyde of covalently attached 4-HNE is located about 4.5 Å away from Arg126 and Tyr128, and instead of being directly coordinated to these residues a water bridges the aldehyde to Arg126 and Tyr128. Residues Arg106, Arg126 and Tyr128 are represented as sticks in grey and (A) covalent 4-HNE, (B) non-covalent 4-HNE and (C) oleic acid (PDB ID 1LID) are shown in black. Oxygen and nitrogen functional groups are colored in red and blue, respectively. Water molecules are depicted as grey spheres. Hydrogen bonds are indicated by dashed lines. The water molecule on the left in the covalent 4-HNE structure is hydrogen bonding with Arg106.

molecule (21) (Figure 3C). This water molecule is absent in the noncovalent structure. In the covalent structure it is present and forms a hydrogen bond to Arg106 but it is located approximately 5 Å away from the free aldehyde of 4-HNE preventing it from forming an interaction. The hydrophobic contacts between AFABP and the hydrocarbon chain of 4-HNE are similar in the covalent and noncovalent structure and also exhibit some similarity to those utilized for oleic acid binding (Table 2). Since oleic acid is longer than 4-HNE (18 C compared to 9 C), it protrudes farther from the head group coordination site than does 4-HNE. Consistent with this, the acyl chain of 4-HNE is positioned more than 4.5 Å away from Thr29 (helix 2), Val32 (helix 2), M40 (β-strand B), K58 (loop between βC-D) and A75 (loop between βE-F), while the acyl chain oleic acid is within 4.5 Å from these residues. In contrast, the hydrocarbon chain of covalently bound 4-HNE is in close proximity to E116, while the hydrocarbon chains of oleic acid and the noncovalent ligand are not. The covalent 4-HNE bound form is also contacting Ile104 as is oleic acid but the noncovalent 4-HNE is farther away from this residue. In addition to Tyr128, the noncovalent 4-HNE (but not the covalently bound form) is within 4.5 Å distance from Ala33. These results suggest in sum that even though mostly similar contacts are made between AFABP and the three ligands evaluated, ligand specific interactions do take place.

Peptide Flip. In some previously solved crystal structures of apo- and holo-AFABP a peptide flip can be observed centering on the amide bond between

Table 2. Residues Within 4.5 Å From the Different Ligands

Residue	Covalent 4-HNE	Non-covalent 4-HNE	Oleic acid
F16 (s.c)	X	X	X
Y19 (s.c)	X	X	X
M20 (s.c)	X	X	X
V25 (s.c)	X	X	X
T29 (s.c, m.c)			X
V32 (s.c)			X
A33 (s.c, m.c)		X	X
M40 (s.c)			X
F57 (s.c)	X	X	X
K58 (s.c)			X
A75 (s.c, m.c)			X
D76 (s.c)	X	X	X
R78 (s.c)	X	X	X
I104 (s.c)	X		X
V115 (s.c)	X	X	X
C117 (s.c, m.c)	X	X	X
R126 (s.c)	X	X	X
Y128 (s.c)		X	X

Structures were analyzed with PyMol (DeLano Scientific Co.). s.c and m.c refer to side chain and main chain atoms, respectively.

Ala36 and Lys37 (21). This peptide flip is accompanied by a perturbation of the adjacent residues leading to alterations in the orientation of both main chain and side chain atoms. As shown in Figure 4, the covalent 4-HNE and noncovalent 4-HNE structures reveal a similar flip in the peptide bond relative to the apo-AFABP structure and with the ligand linoleic acid. The flip of the amide bond between Ala36 and Lys37 leads to a rotation of approximately 180° in the backbone carbonyl group of Ala36; similar to that found in the AFABP structure with oleic acid (21). In the apo-AFABP structure the Ala36 carbonyl group cannot hydrogen bond to any residue while in the linoleic acid structure a water molecule is located within hydrogen bonding distance. In both the covalent and noncovalent 4-HNE structures, as well as the structure with oleic acid, the carbonyl group of Ala36 forms a hydrogen bond with a water molecule (2.48, 2.81 and 2.61 Å, respectively). Interestingly this water molecule is also within hydrogen bonding distance of the guanidinium group of Arg126, the residue that coordinates the carboxyl group of fatty acids. This water molecule is present in the apo structure and can form a hydrogen bond to the nitrogen backbone atom of Ala36 although the distance is slightly increased (3.15 Å) compared to when Ala36 undergo the peptide flip.

The Portal Amino Acid Phe57. An important and potentially regulatory feature in previous crystal structures of AFABP with different ligands concerns Phe57. Phe57 is positioned in a loop region between β-strand C and D, facing the helical

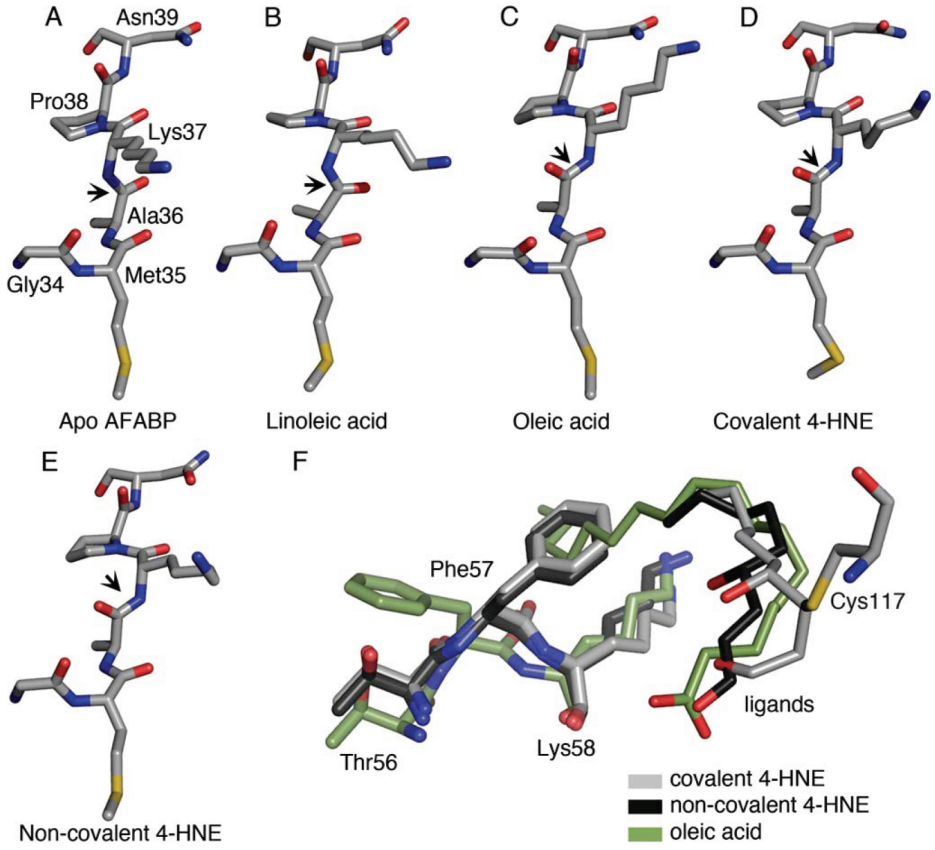


Figure 4. The peptide flip around Ala36 and Lys37. Residue Gly34 to Asn39 are shown in stick representation in (A) apo AFABP (PDB ID 1LIB), (B) AFABP with linoleic acid (PDB ID 2Q9S), (C) AFABP with oleic acid, (D) AFABP with covalently attached 4-HNE, and (E) noncovalent 4-HNE. The Ala36/Lys37 peptide bonds are indicated by arrows. Carbon atoms are colored grey, oxygen atoms red, nitrogen atoms blue and sulfur atoms are colored yellow. (F) The portal region of AFABP and the orientation of Phe57. The structures illustrated are AFABP with oleic acid shown in green, AFABP with covalent 4-HNE in grey and noncovalent 4-HNE in black. The side chains of Thr56, Phe57, and Lys58 are shown in stick representation and ligands in line representation.

domain, and its side chain can adopt two distinct conformations referred to as open or closed (21). This residue has been proposed to act as a gating function affecting access from the external environment to the ligand-binding cavity (33). In the apo structure the side chain of Phe57 is oriented inwardly in what is referred to as the closed position, but when bound with some long-chain fatty acids, the ω-terminus of the bound lipid forces rotation of Phe57 outwardly in what is referred to as the open conformation (21). In both the covalent and noncovalent 4-HNE structures, the side chain of Phe57 adopts the closed form (Figure 4F). Since 4-HNE is only nine carbons long, it does not protrude as far from the head group coordination towards the portal region as does a long-chain fatty acid allowing the rotation of Phe57 into the closed conformation.

Discussion

Herein we report the structure of AFABP with 4-HNE bound in the ligand-binding cavity. 4-HNE is produced endogenously in the cell as a product of lipid oxidation and this is, to the best of our knowledge, the first structure of AFABP covalently modified with an endogenous ligand and the first structure of 4-HNE covalently modifying an endogenous binding protein. However, this is not the first report of FABPs binding to ligands covalently. Previous studies have suggested that the liver isoform of FABP can bind metabolites of the genotoxic carcinogens 2-acetylaminofluorene and aminoazo dyes in a covalent manner (34,35). Based on MALDI-TOF and LC/MS-MS analysis, it was previously concluded that 4-HNE bound covalently to Cys117 of AFABP (9) and our structural results herein confirm that finding. All structures of AFABP, solved to date are more or less superimposable and this was also the case for the AFABP covalently or noncovalently modified with 4-HNE. The overall structures consist of a β-barrel and a helix-turn-helix motif that define an interior cavity that functions as the ligand-binding site. There is an extensive hydrogen-bonding network in the cavity involving both ordered water molecules and amino acid residues and in both structures solved herein, 4-HNE is positioned in the large ligand-binding cavity of AFABP with the free aldehyde group oriented farthest away from the cavity portal and the hydrocarbon tail oriented towards the opening. The modification fills most of the cavity's empty volume and makes further uptake of fatty acids unlikely. This is consistent with the previous observation that the affinity for fatty acids is

decreased in the modified protein (9).

The attachment of 4-HNE to Cys117 occurs through a Michael addition reaction whereby the sulfur atom of Cys117 acts as a nucleophile and attacks C3 of 4-HNE, thereby leading to a covalent interaction. (R)-4-HNE was used for the binding reactions and as such, the chirality at C4 is not affected by the modification. Upon reduction of the carbon-carbon double bond between C2 and C3 of 4-HNE, a new chiral center is generated in the modification process at C3 that adopts an S configuration. It has been demonstrated previously that 4-HNE attached to an amino acid residue in a protein can be stabilized by undergoing cyclization to form a hemiacetal (7). The electron density of the covalent structure is ambiguous in this matter and does not exclude this possible orientation. Since C3 adopts the S configuration, the distance between the hydroxyl group of 4-HNE and the carbonyl aldehyde is 2.08 Å. This distance would be increased if C3 were to adopt an R configuration thereby eliminating possible hemiacetal formation.

Fatty acid carboxylates in the ligand-binding cavity of AFABP are coordinated through hydrogen bonds with the guanidinium group of Arg126 and the hydroxyl group of Tyr128 (21). Structural analysis of AFABP covalently modified with 4-HNE demonstrates that due to the Cys117 4-HNE thioether bond, the aldehyde function is positioned too far away from Arg126 and Tyr128 for direct interaction.

To compensate for this distance, an extra water molecule is located at a similar position as the fatty acid carboxylate that bridges the free aldehyde oxygen to the guanidinium group of Arg126 thereby allowing an indirect interaction. In contrast, the noncovalent 4-HNE reaches farther into the ligand-binding cavity and is located at a position equivalent to a carboxylate in a fatty acid. This permits direct interactions between the free aldehyde oxygen and the side chains of both Arg126 and Tyr128 in a similar manner as a fatty acid carboxylate. In both structures, the hydrocarbon chain of 4-HNE adopts an orientation similar to the hydrocarbon chain of oleic acid and the close contacts between the ligands and the protein are highly conserved. The 4-HNE chains are straighter than oleic acid, resulting in C9 of the covalent 4-HNE ending at the same position as C10 of oleic acid and C9 in the noncovalent structure co-localizing with C11 of oleic acid. However, 4-HNE in the covalent form, 4-HNE in the noncovalent form and oleic acid all make a few unique interactions with the protein, as summarized in Table 2.

Previously all studies on AFABP have shown it to function as a monomer in solution. However, recently fluorescence anisotropy and small angle x-ray scattering have indicated that the protein in its apo form can exist as a homodimer (31) The crystal lattice packing of the apoprotein suggests that this potential homodimerization occur through interactions involving the helix-turn-helix motif. The same crystal lattice packing was obtained for AFABP complexed

with several different ligands such as oleic acid, palmitic acid and hexadecanoic acid. Gillilan *et al*. observed a shift in crystal lattice packing when certain ligands are used (troglitazone, linoleic acid and 1,8-ANS) to a dimer form characterized by monomers interacting through β-sheets instead of the helix-turn-helix motifs (31). Based on crystal lattice packing and calculation of contact interface AFABP bound either covalently or noncovalently to 4-HNE is predicted to adopt a β-sheet dimer form. However, sedimentation equilibrium analysis indicates that the apoprotein is a monomer and if any dimer exists, the K_d for monomer–monomer interaction is quite high, minimally millimolar (Hellberg, Xu, and Bernlohr, unpublished data). Moreover, size exclusion chromatography supports a monomeric form of the protein in both the apo form and 4-HNE modified form. As stated previously, the affinity of AFABP for fatty acids is decreased upon 4-HNE-modification and it is therefore unlikely that modified AFABP bind additional fatty acids (9). It remains undetermined if 4-HNE-modified AFABP facilitates heterotypic protein-protein association as does AFABP bound with fatty acid, which interacts with the hormone sensitive lipase (HSL) (25) and Janus kinase 2 (Jak2) (36) and regulates cellular metabolism and signalling.

A major conclusion reached from analysis of a variety of AFABP structures is that the side chain of Phe57 can be rotated inwardly or outwardly to adopt a closed or an open conformation, respectively, in response to different ligands. This residue is located in a loop region adjacent to the entrance of the ligand-binding cavity

and has been the subject of considerable debate as to its function as a gatekeeper for ligand entry/exit (23,33,37). The side chain shift from a closed orientation to an open orientation is seen with some ligands and not with others. One possible explanation is that the favored conformation is the closed position and that steric hindrance from a bound lipid forces Phe57 to rotate to the open conformation. Consistent with this, Phe57 is oriented inwardly in the apo structure, with bound linoleic acid and arachidonic acid, as well as in both the noncovalent and the covalent 4-HNE structures. Linoleic acid and arachidonic acid adopt a bent conformation when bound to AFABP and are accommodated in the ligand-binding cavity without nearing Phe57. As 4-HNE is only 9 carbons long it is also deep inside the ligand-binding cavity enabling Phe57 inward rotation. In contrast, bound oleic acid and palmitic acid protrude out of the ligand-binding cavity and consequently Phe57 is rotated into its open form. In this model, Phe57 does not serve as a molecular gatekeeper but the position of its side chain is defined somewhat by the type of ligand bound.

AFABP has been implicated in the development of insulin resistance since mice deficient of this protein exhibit improved insulin sensitivity along with decreased inflammation (13,17,38) The previous work of Grimsrud *et al*. and Bennaars-Eiden *et al*. have suggested that a major function for FABPs is as antioxidant proteins functioning to bind to and sequester reactive aldehydes from other proteins (9,39). In this capacity, AFABP fatty acid binding activity would be

potentially diminished. Since AFABP is perhaps the most abundant protein in adipocytes and as such, loss of a small fraction due to 4-HNE modification would not likely affect fatty acid trafficking significantly. In contrast, loss of AFABP in the null mice would eliminate a major antioxidant protein and likely lead to increased covalent modification of secondary targets in adipocytes. Similarly, high affinity small molecule pharmacologic agents that target AFABP may have the identical effect, to inhibit the anti-oxidant activity of the FABPs and in turn, lead to covalent modification of secondary targets. If modification of such targets were anti-inflammatory, metabolic improvement may be realized. While the identification of such secondary targets remains speculative, previous studies by Karin and co-workers have reported that IκB kinase is covalently inactivated by Michael addition adducts resulting in diminished inflammatory signaling (40). Future studies will be needed to assess this possibility.

Materials and Methods

Purification, 4-HNE Modification and Crystallization of AFABP. Mouse AFABP was expressed in E. coli BL21(DE3) pLysS cells and purified as described previously with minor modifications (41). Briefly, native protein was purified using a combination of acid and protamine sulfate fractionation followed by delipidation with hydroxyalkoxypropyl dextran resin and gel filtration chromatography using Superdex G-75. 4-HNE modification of AFABP was conducted as previously described (9). The extent of modification was analyzed using a Bruker Biflex III matrix-assisted laser desorption ionization (MALDI) with time of flight (TOF). AFABP with 4-HNE noncovalently bound was concentrated to 10 mg/mL and dialyzed in 12.5 mM HEPES pH 7.5, 1 mM DTT. The sitting drop vapor diffusion method with 1 μL protein solution and 1 μL well solution was utilized to obtain crystals. The best crystal was found in conditions containing 0.1 M HEPES, 1.6 M NaH_2PO_4/K_2HPO_4 pH 7.5. The crystals grew within 2 weeks in 18 °C.

To optimize the purification scheme, a long-chain fatty acid affinity column (29) was added in the purification procedure followed by 4-HNE modification in room temperature for 70 minutes. The sample was dialyzed and loaded onto the long-chain fatty acid affinity column to enrich for modified protein, which was eluted with 1 M NaCl. Samples from the modification process were analyzed with MALDI-TOF. The eluted fraction, containing covalently modified protein, was concentrated, dialyzed and crystallized as described for the noncovalently bound

4-HNE protein above. The best crystal of AFABP covalently modified with 4-HNE grew in 1.9 M NaH_2PO_4/K_2HPO_4 pH 7.5. Crystals were obtained after 4 days in 18 °C.

X-ray Data Collection and Processing. Crystals were frozen in the well solution supplemented with 20% glycerol as a cryoprotectant. Diffraction data were collected at Argonne Advanced Photon Source (APS) at beamline 23-ID-B for the noncovalently modified AFABP and in house using a Rigaku MicroMax 007HF generator with a Cu anode and a Rigaku Raxis IV ++ detector for the covalent form of AFABP. Collected data were integrated and scaled using the HKL2000 software (42). Table 1 shows statistics from the data collection.

Structure Determination and Refinement. Molecular replacement was used to solve the structures of both noncovalent and covalently modified AFABP. AFABP bound to palmitic acid (PDB ID 1LIE) with the ligand removed was used as a probe for the noncovalent structure while a modified version of the same structure was used for the covalently modified structure. Refinement was performed using Refmac5 in the CCP4 suite and manual model building was done in Coot (43–45). Analysis and figure preparation were performed using PyMol (46).

Coordinates. The coordinates and structure factors for the structures have been

deposited in the Protein Data Bank. The accession number for AFABP with noncovalently bound 4-HNE is 3JSQ, and for AFABP covalently modified with 4-HNE 3JS1.

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CHAPTER 3

IDENTIFICATION AND CHARACTERIZATION OF A SMALL MOLECULE INHIBITOR OF FATTY ACID BINDING PROTEINS

This chapter is a reprint of an original publication with minor alterations:

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Kristina Hellberg performed experiments from figure 2, 3 and table 2, performed computer modeling, wrote the corresponding text sections and helped edit the manuscript.

Molecular disruption of the lipid carrier AFABP/aP2 in mice results in improved insulin sensitivity and protection from atherosclerosis. Because small molecule inhibitors may be efficacious in defining the mechanism(s) of AFABP/aP2 action, a chemical library was screened and identified HTS01037 as a pharmacologic ligand capable of displacing the fluorophore 1-anilinonaphthalene 8-sulfonic acid from the lipid binding cavity. The X-ray crystal structure of HTS01037 bound to AFABP/aP2 revealed that the ligand binds at a structurally similar position to a long-chain fatty acid. Similar to AFABP/aP2 knockout mice, HTS01037 inhibits lipolysis in 3T3-L1 adipocytes and reduces LPS-stimulated inflammation in cultured macrophages. HTS01037 acts as an antagonist of the protein-protein interaction between AFABP/aP2 and hormone sensitive lipase but does not activate PPARγ in macrophage or CV-1 cells. These results identify HTS01037 as an inhibitor of fatty acid binding and a competitive antagonist of protein-protein interactions mediated by AFABP/aP2.

Introduction

Fatty acid binding proteins (FABPs) are small molecular weight proteins containing a central cavity that binds long chain fatty acids or other hydrophobic ligands (1). Each FABP gene exhibits a unique expression pattern and all cells that carry out extensive lipid metabolism express one or more FABP(s). The primary sequences of the nine family members vary significantly (as little as 20% amino acid identity), however their tertiary structures are virtually superimposable. FABPs are abundantly expressed and function to promote intracellular fatty acid solubilization, trafficking, and metabolism (1,2). The adipocyte member of the FABP family (AFABP, also known as aP2) is expressed in both adipocytes and macrophages, two cell types that play major roles in overall whole body metabolic homeostasis (3).

To delineate the specific physiological function(s) of AFABP/aP2 in adipose tissue, knockout mice have been generated, and although these animals develop obesity in response to a high-fat diet, they exhibited significant resistance to a cluster of pathologies including decreased insulin sensitivity, asthma, and atherogenesis (4-6). As shown by both *in situ* and *in vivo* technologies, adipocyte lipolysis was reduced under both basal and stimulated conditions (7-9). Furthermore, inflammatory cytokine production in response to LPS was reduced in macrophages from AFABP/aP2 knockout mice (10).

All members of the FABP family share a similar structure derived from two α-helices and 10 antiparallel β-strands folded into two β-sheets (11-13). The overall protein fold is typically called a β-barrel or β-clam and importantly for function forms an internal water-filled ligand-binding cavity. Although this cavity binds hydrophobic ligands, it contains a hydrogen bond network involving the side chains and main chain atoms linked through numerous disordered and ordered water molecules. The comparative ligand binding properties of many FABPs for various fatty acids has been defined by Kleinfeld and colleagues, and although there are subtle isoform-specific differences, the proteins as a class bind a variety of long chain fatty acids (14). Molecular disruption of AFABP/aP2 may increase the bioavailability of fatty acids for metabolism by alternate pathways: a process that may underlie the broad metabolic phenotype of the knockout mice.

Recent work has demonstrated that beyond fatty acid solubilization and trafficking, AFABP/aP2 physically associates with at least three different intracellular proteins in a fatty acid dependent manner, suggesting that the protein may serve a regulatory role as a lipid sensor. Work by Jenkins-Kruchten *et al*. (15) and subsequently by Smith *et al*. (16) have shown that AFABP/aP2 physically associates with the adipocyte hormone sensitive lipase (HSL) in a reaction that requires a fatty acid bound to the FABP. Such an interaction is likely to be regulatory and would feedback-inhibit lipolysis. More recently, Thompson *et al*. (17) have shown that AFABP/aP2 interacts with Jak2 in a fatty acid dependent

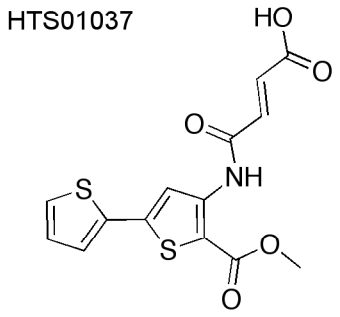
manner and affects IL-6 dependent signaling in macrophages. In addition, work from the Spener and Noy laboratories has shown that AFABP/aP2 interacts with the nuclear hormone receptor peroxisome proliferator activated receptor γ (18,19). The results of all protein-protein interaction studies suggest that AFABP/aP2 may act as a lipid sensor and affect adipocyte metabolism, signaling, and gene expression via a series of targeted interactions.

To evaluate the molecular mechanism of AFABP/aP2 function in adipocytes and macrophages, we reasoned that small molecule inhibitors of AFABP/aP2 may be efficacious tools. To that end, we report herein the identification and analysis of a small molecule inhibitor of AFABP/aP2 that not only blocks fatty acid binding but also antagonizes physical interaction with HSL. Such a molecule reproduces many of the phenotypes of the AFABP/aP2 null mouse with regard to lipid metabolism in fat cells and inflammation in macrophages and may be useful in delineating FABP function.

Results

Identification of a Small Molecule Ligand of AFABP/aP2. To evaluate the various models of AFABP/aP2 function, knockout mice have been generated and their phenotype characterized (4-6). AFABP/aP2 null mice exhibit decreased rates of basal and stimulated lipolysis, as well as reduced lipogenesis (7-9,20). Although still prone to diet-induced obesity, the knockout mice maintain an improved metabolic state, including increased insulin sensitivity, increased insulin secretion, and resistance to atherosclerosis and asthma (4-6).

To identify a small molecule that could antagonize the function of AFABP/aP2 and potentially mimic the AFABP/aP2 null phenotype, we took advantage of a fluorescent displacement assay (21) allowing high throughput screening of a small molecule library. Of the approximately 5000 compounds, seven positive hits were obtained in the first round, of which five were reproduced in a second round of screening. Four of these reproducibly displaced the fluorescent probe. One molecule was selected for further study, HTS01037, which functioned as a high affinity ligand of AFABP/aP2 (Figure 1) with an apparent Ki of 0.67 ± 0.18 μM (Table 1). To test whether HTS01037 is specific for AFABP/aP2 or would bind to other members of the FABP family, liver, intestinal, heart muscle, and epithelial FABPs were also tested for binding. The results show that HTS01037 binds to other FABPs, although with somewhat reduced affinities, indicating that the small molecule is somewhat selective for AFABP/aP2 but at higher



4-{[2-(methoxycarbonyl)-5-(2-thienyl)-3-thienyl]amino}-4-oxo-2-butenoic acid

Figure 1. Chemical structure of HTS01037.

Table 1. Binding of HTS01037 to FABPs[a]

Protein	Apparent Ki (μM)	P value
AFABP/aP2	0.67 +/- 0.18	
EFABP	3.40 +/- 0.60*	0.04
HFABP	9.07 +/- 1.71*	0.04
IFABP	6.57 +/- 1.55	0.06
LFABP	8.17 +/- 1.28*	0.03

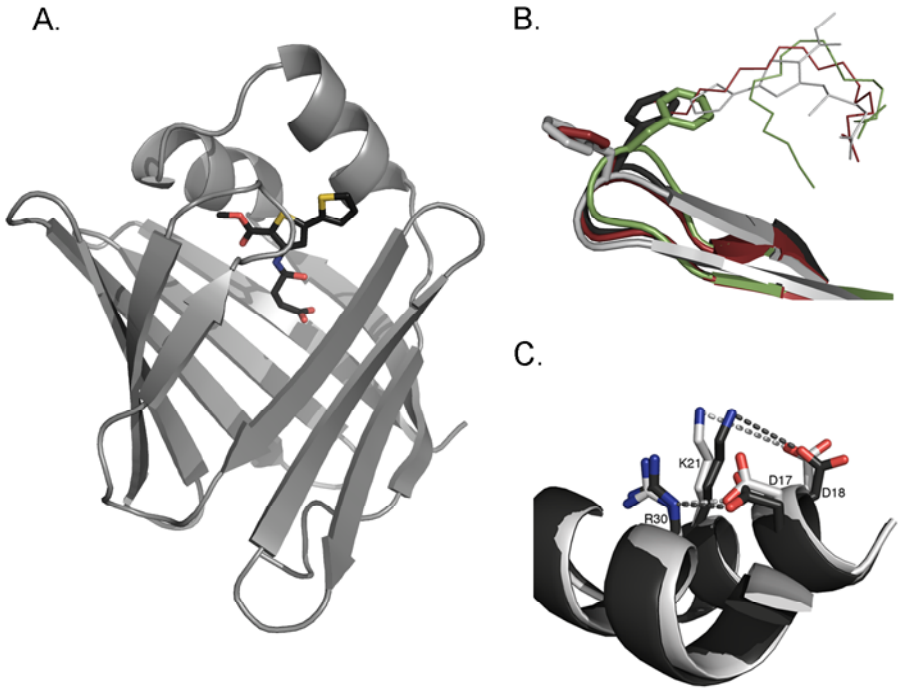
[a] The displacement of 1,8-ANS by HTS01037 reported as the mean and standard error of the mean (n=3). P value relative to AFABP/aP2.

concentrations is a pan-specific FABP inhibitor (Table 1).

Determination of the X-Ray Crystal Structure of HTS01037 *Complexed to AFABP/aP2.* Various X-ray structures of FABP family members have been solved that upon comparison reveal a conserved three-dimensional fold of a β-barrel or β-clam shape. For AFABP/aP2, both apo and holo structures have been determined (22-27). Using molecular replacement methods, we determined the structure of AFABP/aP2 with HTS01037 bound in the ligand cavity at 1.7 Å resolution (Figure 2A).

Not surprisingly, the crystal structure of AFABP/aP2 with HTS01037 shares the same conserved fold with the previously solved crystal structures. It consists of 10 antiparallel β-strands folded into two β-sheets, which form a β-barrel with an internal ligand-binding cavity. A helix-turn-helix motif serves to close the main opening to the β-barrel from the surroundings. The volume of the ligand-binding cavity is large (1099 $Å^3$ as estimated with CASTp with a probe radius of 1.4 Å (28)) and contains both ordered and disordered water molecules. Even though there is an increase in the number of water molecules in the structure of AFABP/aP2 with HTS01037 compared to the apo form (PDB ID 1LIB) and AFABP/aP2 with bound oleic acid (PDB ID 1LID), the positions of many ordered water molecules are conserved in the ligand-binding cavity between the structures.

Figure 2. Structure of AFABP/aP2 complexed with HTS01037. (A) Ribbon representation of the crystal structure of AFABP/aP2 with HTS01037 highlighted in black and shown in sticks in the ligand-binding cavity. (B) The side chain of Phe57 from different structures are aligned and represented as sticks. AFABP/aP2 with HTS01037 is shown in light gray, apo AFABP/aP2 (PDB ID 1LIB) in black, AFABP/aP2 with linoleic acid (PDB ID 2Q9S) in green, and AFABP/aP2 with oleic acid (PDB ID 1LID) in brown. Ligands are represented as lines and are color coded as mentioned above. (C) The helical domain is shown in cartoon representation and residues in the charge quartet are represented as sticks. AFABP/aP2 with HTS01037 is shown in light gray and apo AFABP/aP2 in black. Distances between the ion pairs are represented as dashed lines and color coded as the proteins. (D) The peptide flip between alanine 36 and lysine 37 are shown. AFABP/aP2 with HTS01037 is shown in light gray, apo AFABP/aP2 in black. Arrow points to the main chain carbonyl of alanine 36. Figure was prepared using the program PyMol (43).


A.
B.
C.
K21
D17
D18
R30

The presence and positioning of HTS01037 in the ligand-binding cavity is unambiguous due to the tight binding of the ligand and the high resolution of the data (Figure 3B). HTS01037 adopts a planar conformation and binds, as do fatty acids, in the ligand-binding cavity with its carboxyl group coordinated by hydrogen bonds to R126 and Y128 (Figure 3A). In the structure of AFABP/aP2 complexed with oleic acid, the carboxyl group is also coordinated through hydrogen bonds to R106 via a bridging water molecule. This is not the situation in the HTS01037 structure where the corresponding water molecule is shifted and positioned too far away from the carboxyl group to bridge it to R106.

Besides interactions between the carboxylate and the side chains of R126 and Y128, structural examination of HTS01037 reveals a number of additional interactions with residues of the cavity and water molecules. The carbonyl oxygen of the ester bond of HTS01037 interacts through a hydrogen bonding with a water molecule located 3.04 Å away. This water molecule in turn is involved in the large water network inside the ligand-binding cavity of the protein. In addition, the distance between the ester carbonyl group and the nitrogen atom of molecule HTS01037 is less than 3 Å, suggesting that an intramolecular hydrogen interaction may be formed between these two atoms. The amide nitrogen can also form a hydrogen bond (3.34 Å) to the same water molecule as does the ester carbonyl oxygen. Second, HTS01037 contains two thiofuran rings, referred to as proximal and distal relative to the carboxylate function. The

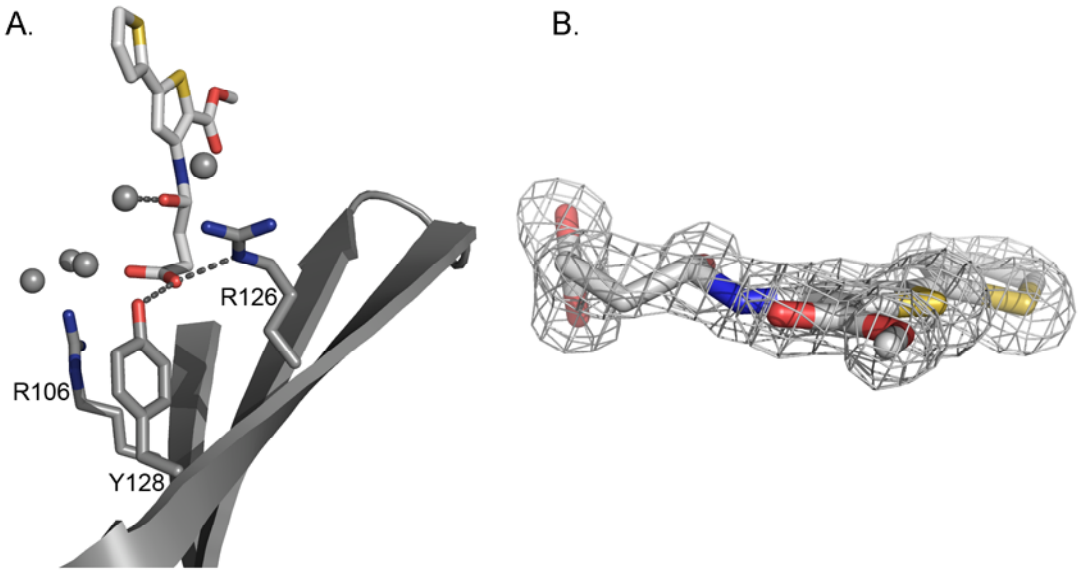


Figure 3. Structure of HTS01037 bound to AFABP/aP2. (A) Coordination of HTS01037 in AFABP/aP2. Residues previously implicated in coordination of fatty acids are shown in sticks. Waters are shown as gray balls, and hydrogen bonds to HTS01037 are represented as dashed lines. (B) Electron density of HTS01037 with the $2F_o$ - F_c map contoured at 1.5 σ. Figure was prepared using the program PyMol.[43]

proximal thiofuran ring of HTS01037 is positioned near the ether oxygen of the ester group potentially forming an H-bond. The sulfur atom of the distal thiofuran of HTS01037 is within H-bonding distance with the main chain carbonyl of A75. Despite the extensive interaction network between HTS01037 and cavity amino acids and water molecules, no major conformational shifts in the AFABP/aP2 structure are observed upon binding of the ligand, which is consistent with previously reported structures of AFABP/aP2 in complex with different ligands. However, three regions deserve further examination: the side chain of F57, the charge quartet, and the A36-K37 peptide flip.

F57 is a residue positioned in a loop region lining the opening to the ligand-binding cavity close to the helix-turn-helix domain. This residue undergoes a large conformational shift from a closed to an open form upon binding of certain ligands. It has been suggested that F57 can adopt different orientations in order to allow entry or exit of the ligand from the cavity (29,30) In the crystal structure of AFABP/aP2 with HTS01037, F57 exists in an open conformation, an orientation also observed in the AFABP/aP2 structure with bound oleic acid, while the apo form and AFABP/aP2 with linoleic acid (PDB ID 2Q9S) exist in the closed conformation (Figure 2B).

The helix-turn-helix motif of AFABP/aP2 has been implicated in the interactions with HSL and Jak2. Four charged residues located in this motif, referred to as the

charge quartet, form two ion pairs, D17-R30 and D18-K21, and are required for HSL-interaction (31) Compared to the apo form of AFABP/aP2, the distance between the D17 and R30 ion pair is slightly decreased in the crystal structure of AFABP/aP2 with bound HTS01037, from 3.26 Å to 2.95 Å, respectively. For the D18-K21 ion pair, the distance is 4.01 Å in the apo structure and increases to 4.78 Å in the structure with HTS01037 (Figure 2C).

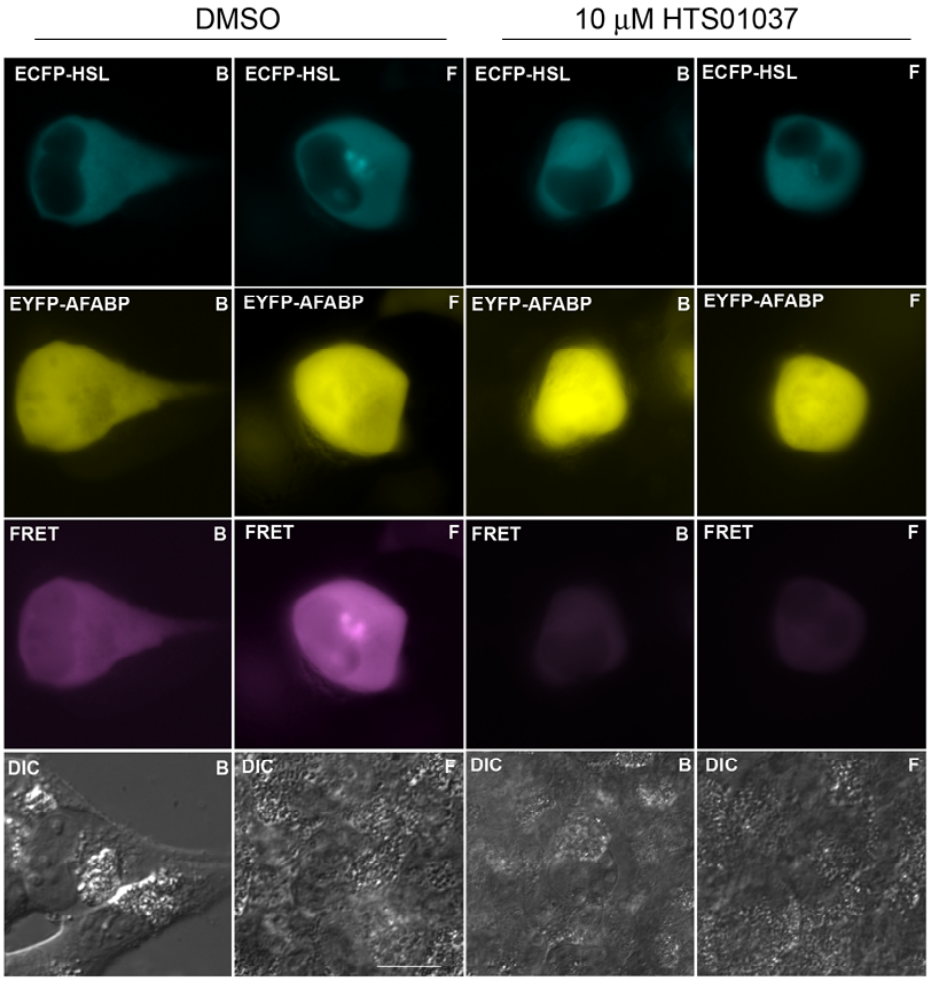
In some of the previously reported structures of AFABP/aP2, a rotation of the peptide bond between A36 and K37 has been observed compared to the apo form of the protein. Not all ligands, however, induce this peptide flip, such as linoleic acid and troglitazone (PDB ID 2QM9) bound in the ligand-binding cavity. The peptide flip is present in the structure of AFABP/aP2 with HTS01037 (Figure 2D).

We modeled the HTS01037 molecule from AFABP/aP2 into liver FABP using Modeler 9.5 (32) (data not shown). Upon analysis, it is not clear what molecular determinants result in HTS01037 binding with higher affinity to AFABP/aP2 than to LFABP. The carboxyl group of HTS01037 is coordinated through R122 and N111 in LFABP instead of R126 and Y128 in AFABP/aP2, and there is no obviously identifiable region that would sterically or electrostatically affect HTS01037 association with LFABP.

HTS01037 Inhibits the Interaction of AFABP/aP2 with HSL. Previous studies have indicated that AFABP/aP2 physically interacts with HSL (33-35) and that this interaction requires a ligand bound to AFABP/aP2 (15,16). To determine if HTS01037 mimicked the lipid bound form of AFABP/aP2, we tested whether it would positively or negatively influence the interaction with HSL, as measured via FRET. This evaluation was conducted under basal as well as forskolin stimulation and a DIC image included to verify the dispersal of the large lipid droplet upon forskolin treatment (Figure 4). As such, we treated C8PA cells with varying concentrations of HTS01037 and determined the effect on interaction of AFABP/aP2 with HSL (Figure 4). The lower concentration of HTS01037, 1 μM, decreased basal FRET but was ineffective in altering forskolin-stimulated FRET (data not shown). Cells treated with 10 μM HTS01037 exhibited reduced FRET, both under basal and forskolin stimulated conditions. These results demonstrate that HTS01037 functions as an antagonist of the protein-protein interaction of AFABP/aP2 with HSL.

HTS01037 Effects in Adipocytes and Macrophages. Our previous studies and those of others have determined that targeted deletion of AFABP/aP2 decreases lipolysis in adipocytes (7-9). Thus we repeated these experiments in 3T3L1 adipocytes to assess whether HTS01037 would modulate the levels of lipolysis. The results show that the efflux of fatty acids was reduced in both basal and forskolin stimulated adipocytes (Figure 5). These results are again consistent

Figure 4. HTS01037 functions as an antagonist of the interaction of AFABP/aP2 with HSL. (A) Fluorescence resonance energy transfer (colored magenta) between ECFP-HSL and EYFP-AFABP/aP2 in C8PA cells under basal (B) and forskolin (F) stimulation. The cells were treated either with DMSO or 10 μM HTS01037. The scale bar represents 5 μm. (B) NFRET was calculated as described in the Experimental Section. White bars, DMSO; gray bars, 1 μM HTS01037; black bars, 10 μM HTS01037. Error bars represent standard error of the mean (n=15). The * denotes statistical significance ($p<0.05$) as compared to the DMSO treated.


DMSO
10 μM HTS01037
ECFP-HSL B
ECFP-HSL F
ECFP-HSL B
ECFP-HSL F
EYFP-AFABP B
EYFP-AFABP F
EYFP-AFABP B
EYFP-AFABP F
FRET B
FRET F
FRET B
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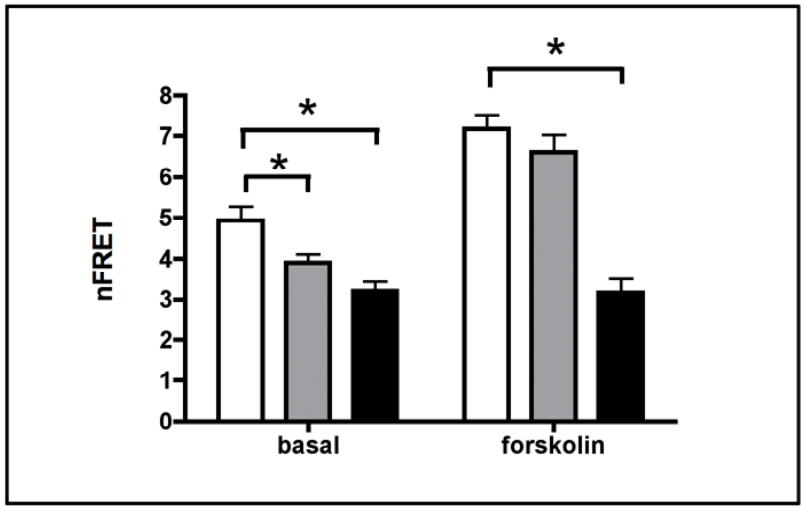
nFRET
0
1
2
3
4
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6
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8
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*
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basal
forskolin

with HTS01037 functioning as an antagonist of AFABP/aP2 in cultured adipocytes.

Through the study of macrophages deficient in AFABP/aP2, we have previously demonstrated that inflammatory cytokine production is reduced (10). To determine if HTS01037 mimicked the null phenotype in macrophages, we pretreated wild type macrophage cells with HTS01037 and quantified the production of IL-6, TNFα, and MCP-1/CCL2 in response to LPS stimulation. For each of these targets, LPS stimulated a robust increase in expression and secretion of the inflammatory cytokines. HTS01037 significantly reduced the LPS stimulated release of all three inflammatory cytokines measured (Figure 6), similar to the AFABP/aP2 knockout macrophages (10). These results are consistent with HTS01037 acting in macrophages as an antagonist of AFABP/aP2 function.

HTS01037 Does Not Directly Activate PPARγ. The AFABP/aP2 knockout mouse exhibits an overall phenotype of a reduced inflammatory state that has been suggested, at least in part, to arise from an increase in PPARγ activity. Because PPARγ and AFABP/aP2 both bind to hydrophobic ligands and have an overlapping set of molecules capable of binding in their respective cavities, we considered the possibility that HTS01037 was directly activating PPARγ. Therefore we measured the expression of PPARγ targets, CD36 and Cpt1α in

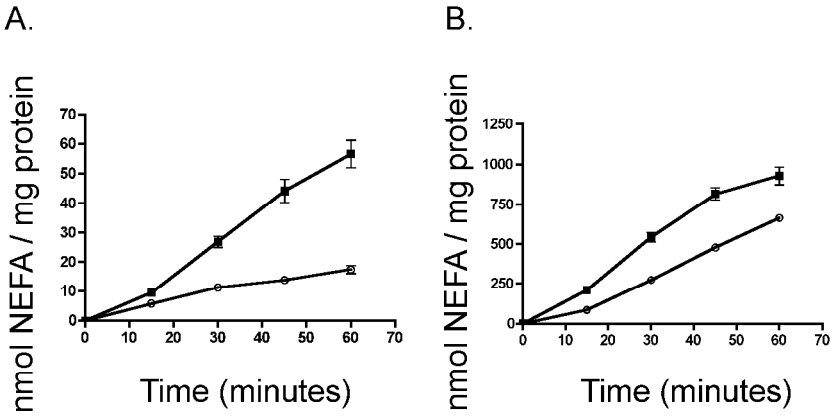

Figure 5. Treatment of 3T3L1 adipocytes with HTS01037 inhibits lipolysis. 3T3L1 adipocytes were treated with DMSO (closed squares) or HTS01037 (open circles) for 24 h. Aliquots of medium were taken and nonesterified fatty acids were quantified at various times and normalized to mg protein. (A) Basal lipolysis. (B) Forskolin-stimulated lipolysis.

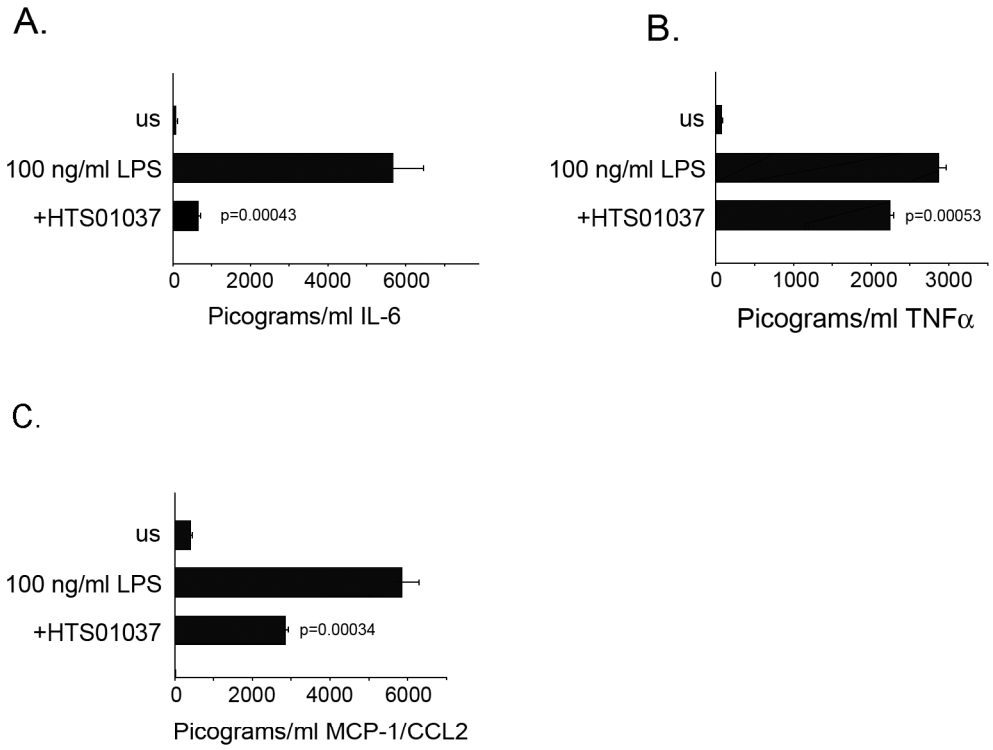

Figure 6. Treatment of macrophages with HTS01037 inhibits inflammatory cytokine production. Bone marrow-derived macrophages were cultured and pretreated with or without HTS01037, followed by LPS stimulation. Cytokine production was quantified by ELISA. (A) IL-6. (B) TNFα. (C) MCP-1. p Values <0.05 were considered significant. us denotes no LPS stimulation.

macrophages treated with HTS01037 (Figure 7A). Whereas the known PPARγ ligand, 15-deoxy-δ12,14-prostaglandin J2 (15dPGJ$_2$), was proficient in elevating the mRNA levels of CD36 and Cpt1α, HTS01037 treatment was not. Furthermore, we tested the ability of HTS01037 to activate PPARγ in a heterologous luciferase reporter system in CV-1 cells (Figure 7B). While the thiazolidinedione, troglitazone activated a PPARγ luciferase reporter, HTS01037 did not activate the PPARγ reporter even at concentrations as high as 25 μM. These results indicate that HTS01037 is not capable of direct activation of PPARγ.

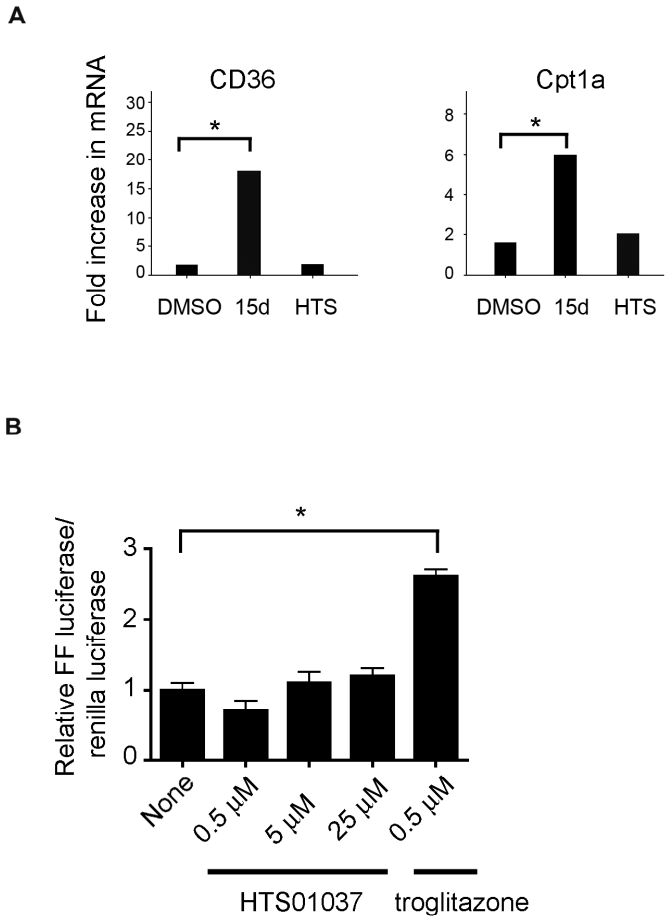

Figure 7. HTS01037 does not activate PPARγ in macrophages or CV-1 cells. (A) The expression of the PPARγ-regulated genes CD36 and Cpt1α was analyzed by real-time PCR in response to 5 μM 15dPGJ2, referred to as 15d, or 500 nM HTS01037, referred to as HTS (DMSO as control). Fold increase in mRNA was quantified using β-actin as a reference gene and an unstimulated sample as the baseline for expression using the relative expression tool. (B) PPARγ activation was measured in CV-1 cells using a luciferase reporter assay. Cells were exposed to increasing amounts of HTS01037. Troglitazone was used as a positive control. * p Values <0.05 were considered significant.

Discussion

AFABP/aP2 is an intracellular fatty acid carrier protein implicated in fatty acid metabolism through its ability to solubilize and traffic fatty acids. Various studies utilizing AFABP/aP2 null mice have established an important role for AFABP/aP2 in insulin sensitivity and overall metabolic homeostasis. More recently, AFABP/aP2 has been shown to function as a fatty acid sensor, responding to the levels of free fatty acids by affecting target protein function via direct protein-protein interactions. Herein, we described the screening and identification of a small molecule inhibitor of AFABP/aP2. Through the displacement of the fluorescent molecule 1,8-ANS we demonstrated that AFABP/aP2 binds HTS01037 with an affinity similar to fatty acids and thus it would be capable of functionally competing for binding in the ligand-binding cavity of AFABP/aP2. Furthermore, HTS01037 treatment of adipocytes and macrophages produced phenotypes that mimicked those observed in AFABP/aP2 null cells/animals. In this study, the adipocytes and macrophages not only express AFABP/aP2 but also EFABP and HFABP. Although we found that the affinity of EFABP and HFABP for HTS01037 was approximately 5- to 15-fold lower than for AFABP/aP2, it is unclear in these experiments whether binding of HTS01037 to these other FABPs contributed to the anti-inflammatory phenotypes observed.

Improvements in the affinity of AFABP/aP2 for HTS01037 could be realized by mutational engineering. Mutation of Y19 and/or F16 to arginine could stabilize the

carbonyl oxygen of the ester group in HTS01037 by functioning as a hydrogen bond donor. It may also be possible to stabilize the proximal thiofuran ring by mutating V25 to asparagine, where the side chain amide nitrogen could potentially form an interaction to stabilize the thioether. An alternate approach would be to chemically modify HTS01037 in order to increase affinity. We speculate that adding a carboxyl group to the distal thiofuran ring could increase the affinity by forming an interaction to the hydroxyl group of S55. Alternatively, exchanging the sulfur atom of the distal ring with a secondary amine could allow for an interaction with the main chain carbonyl of A75. Future experiments will be focused on design improvements in order to develop AFABP/aP2-specific inhibitor with higher affinity and selectivity.

The crystal structure of AFABP/aP2 with bound HTS01037 showed it located in the ligand-binding cavity with the carboxyl group coordinated through R126 and Y128 in a manner similar to the way in which fatty acids are bound. There were no obvious large structural changes in this high-resolution structure as compared to previous structures. This lack of structural change has been seen in apo structures as well as in several ligand-containing FABP structures. With this in mind, it remains a conundrum as to how the functional differences of holo-AFABP/aP2 are effectively communicated to other cellular proteins or processes. For example, it has been clearly demonstrated that holo-AFABP/aP2 efficiently forms protein-protein interactions with HSL and Jak2, as demonstrated through

the lack of interaction of these target proteins with a nonfatty acid binding mutant of AFABP/aP2, R126Q. Thus a mechanism allowing recognition of holo-AFABP/aP2 must exist. Interestingly, HTS01037 inhibits the interaction of AFABP/aP2 with HSL. It is not known if only exogenous ligands, such as HTS01037, or whether endogenous ligands exist that may also inhibit this protein-protein interaction.

In an attempt to identify the structural alterations that occur when an inhibitor (HTS01037) was bound, we analyzed regions of the protein that experience the largest difference upon binding. The four charged residues in the helix-turn-helix motif referred to as the charge quartet undergo small changes upon ligand binding. Even though the distance between the D17-R30 ion pair is slightly decreased with HTS01037 bound to AFABP/aP2 compared to the apo protein, the distance between the second ion pair, D18-K21 is increased (0.77 Å). Because this domain is involved in interaction with HSL (and potentially other proteins), the increase may destabilize the motif and affect binding to interaction partners. However, a similar increase in the D18-K21 ion pair distance is also observed in the structure of the activating ligand, troglitazone, making it unlikely that this feature by itself is responsible for the inhibitory effects of HTS01037.

The fact that F57 can adopt an opened or closed conformation makes it an interesting candidate as a switch, indicating that a specific ligand is bound in the

ligand-binding cavity. This shift could, however, merely be a secondary effect due to steric hindrance between the bound ligand and the side chain of F57. As seen in Figure 2B, the ligands oleic acid and HTS01037, which both give rise to a open conformation of F57, extend out of the ligand-binding pocket and hence would occupy the same space as the side chain of F57 in its closed orientation. On the other hand, F57 is in its closed orientation when linoleic acid is bound. Linoleic acid adopts a bent conformation in the cavity and therefore does not extend as far out of the cavity as do oleic acid or HTS01037. It is plausible that a closed conformation of F57 is the most energetically favorable and therefore the preferred orientation when possible.

The A36-K37 peptide flip observed in some structures while absent in others is also an interesting feature (Figure 2D). Residues A36 and K37 are located in a disordered region connecting α-helix 2 with β-strand B. In the apo form of AFABP/aP2, the carbonyl group of A36 does not form any interactions with other residues or solvent molecules. In HTS01037, as well as AFABP/aP2 with oleic acid, the carbonyl group of A36 is rotated almost 180° and can form a hydrogen bond to a water molecule positioned 3 Å away. Furthermore, this water forms three other hydrogen bonds, one with the carbonyl group of A33, one with the guanidinium group of R126, and one with another water molecule. This water molecule network can be extended from the A36 carbonyl group throughout a large portion of the ligand-binding cavity. The water molecule in proximity to the

carbonyl group of A36 is also present in the apo structure of AFABP/aP2, although it cannot form a hydrogen bond with this residue because it is facing the other direction. It is also shifted so it is positioned almost 0.5 Å farther away from R126. In addition, the structure of the apo form contains fewer water molecules so the hydrogen-bonding network extending into the ligand-binding pocket cannot be traced. One possibility is that a peptide rotation at A36 and K37 confers more stability to R126, which interacts directly with different ligands. Unfortunately, this alone cannot explain the differences specific ligands exert on the function of the protein because only some ligands induce this peptide flip.

The phenotypes demonstrated in this paper are consistent with HTS01037 acting as an antagonist of AFABP/aP2 because the effects mimicked those seen in the AFABP/aP2 knockout mice. Additionally, because PPARγ activation would mimic many of the observed phenotypes associated with reduced inflammatory states, we tested the direct and/or indirect modes of HTS01037 activation of PPARγ. In both cases, HTS01037 was incapable of demonstrating PPARγ activation. However, it is also possible that HTS01037 can bind and/or affect other cellular targets as well. One model proposed to explain the differences detected in AFABP/aP2 null macrophages involves the indirect action of unbound fatty acids. These fatty acids could activate PPARγ, resulting in increased target gene expression and inhibition of inflammatory cytokine production. The data included here do not support this model. While inflammatory cytokine production was

decreased, expression of PPARγ targets was not increased. Thus an alternative model that includes alteration of PPARγ activity through protein-protein interaction of AFABP/aP2 may at least partially explain the changes detected. Further work is necessary to evaluate this model.

Experimental Section

Purification and Ligand-Binding Analysis. Escherichia coli BL21 (DE3) cells, transformed with a gene encoding His-tagged AFABP/aP2 (or other FABPs), were grown in rich media and 1 mM IPTG was added for 4 h to induce protein expression. The cells were harvested by centrifugation and lysed in a French press. The debris was pelleted by centrifugation at 100000g for 60 min and the solubilized protein purified using a nickel affinity chromatography. The pooled eluate was subjected to Lipidex-1000 chromatography to remove any endogenously bound ligands and dialyzed into 25 mM Tris-HCl (pH 7.4), 50 mM NaCl plus 5% glycerol for storage at -80 °C. The purity of the isolated FABPs was verified by SDS-PAGE.

To analyze the ligand-binding properties of the FABPs, the fluorescent ligand 1-anilinonapthalene 8-sulfonic acid (1,8- ANS) was utilized as described by Kane and Bernlohr (21). Briefly, 1,8 ANS was dissolved in absolute ethanol and diluted with 25 mM Tris-HCl (pH 7.4) to a final concentration of 5 μM (final EtOH concentration of 0.05%). Protein was titrated into 500 μL 1,8-ANS and the fluorescence enhancement was measured using a Perkin-Elmer 650-10S fluorescence spectrophotometer with 4 nm excitation and emission slit widths. Quantitative analysis of ligand binding was evaluated using nonlinear regression using PRISM software.

Small Molecule Library Screening. A Maybridge chemical library (≥90-95% purity; Fisher Scientific International) was screened for compounds binding to AFABP/aP2 using a Biomek FX workstation integrated into a Saigen core system and a molecular devices Gemini XPS plate reader. Each well contained 12 μM AFABP/aP2 complexed with 400 nM 1-anilino-napthalene 8-sulfonic acid and the fluorescence reduction due to displacement of the bound fluorophore by the test compounds measured in duplicate. Each plate also contained a positive control (oleic acid) as well as a negative control (methyl octanoate) as internal standards and controls.

X-ray Crystallography. Non His-tagged AFABP/aP2 was purified as described previously with minor modifications (24). Briefly, AFABP/aP2 was expressed in E. coli, harvested by centrifugation, and the cells lysed with a French press. After centrifugation of the crude extract, protamine sulfate (5% final w/v) was added to the supernatant, followed by centrifugation. The pH of the protamine sulfate soluble protein was adjusted to 5 and proteins allowed to precipitate overnight at 4 °C. After centrifugation and neutralization, the sample was chromatographed through a Lipidex-1000 column followed by two rounds of gel filtration using Superdex G75 resin. The fractions containing AFABP/aP2 were pooled and applied to a long-chain fatty acid affinity column (36) to obtain pure AFABP/aP2, which was verified by SDS-PAGE. The purified AFABP/aP2 was concentrated to 10 mg/mL, dialyzed against 12.5 mM Hepes (pH 7.5), 1 mM DTT, and incubated

overnight with 4-fold molar excess of HTS01037 (dissolved in DMSO; 1% final DMSO concentration) at room temperature while stirring.

The sitting drop method (1.3-3.1 M sodium/potassium phosphate; pH 6.8-7.6) was used to obtain crystals (25) The plates were incubated at 18 °C, and rod shaped crystals grew within five days with the best crystal found in a well containing 2.5 M sodium/potassium phosphate (pH 7.0). Crystals were flash frozen in liquid nitrogen with 20% glycerol in the mother liquor and stored until data collection. Diffraction data was collected from a single crystal using a Rigaku MicroMax 007HF generator with a Cu anode and a Rigaku Raxis IV++ detector and was processed using the HKL2000 software (37). The space group was determined to be $C222_1$. Molecular replacement using the previously solved apo form (PDB ID 1LIB) of the protein was conducted with MolRep (38) in the CCP4 package (39) and the refinement was performed using Refmac5 (40) and WinCoot (41). The electron density in the F_o - F_c map clearly indicated the orientation and position of the HTS01037 ligand in the cavity of AFABP/aP2. Data collection and refinement statistics are shown in Table 2.

Fluorescence Resonance Energy Transfer. C8PA lipocytes used for FRET analysis were cultured as described previously (16,35) C8PA cells were grown on polylysine-coated 13 mm coverslips and transfected with pECFP-HSL and/or pEYFP-AFABP expression plasmids. After 24 h, the cells were lipid-loaded with

Table 2. Crystallographic Data Collection and Refinement Statistics[a]

space group	$C222_1$
cell parameters: *a, b, c* (Å)	77.0, 94.1, 49.7
resolution (Å)	1.70
$\langle I \rangle / \sigma \langle I \rangle$	49.1 (6.5)
completeness (%)	99.8 (98.4)
redundancy	6.8 (6.5)
R_{sym} (%)[b]	3.5 (27.7)
unique reflections	20158
R_{work}/R_{free} (%)[c]	17.5/19.5
No. of atoms	
protein/ligand/water	1022/22/225
Average B-factors (Å^2)	
protein/ligand/water	17.2/16.6/33.5
rmsd bond length (Å)	0.011
rmsd bond angles (º)	1.855

[a] Data in parentheses are for the highest resolution bin. [b] $R_{sym} = \Sigma| I_i - \langle I \rangle |/\Sigma I_i$, where I_i indicates the intensity of the *i*th observation and $\langle I \rangle$ indicates the mean intensity of the reflection. [c] $R_{work} = \Sigma|F_{obs} - F_{calcd}|/\Sigma F_{obs}$, where F_{obs} and F_{calcd} are the observed and calculated structure factors, respectively. R_{free} were calculated using the same equation as for R_{work} but using the reflections in the test set instead of the working set.

300 μM oleic acid and 100 μM bovine serum albumin for 24 h, followed by treatment of 1 or 10 μM of HTS01037 complexed with 100 μM bovine serum albumin for an additional 24 h. Lipolytic conditions were initiated by the addition of 20 μM forskolin and digital images captured between 2 and 4 h later. During microscopy, cells were kept in medium at 37 °C with 5% CO2 until imaging at room temperature. Images were captured and real-time FRET measurements were performed as previously reported (16,35). Normalized FRET (NFRET) was calculated from 15 to 20 cells as previously reported (31).

Adipocyte Lipolysis. Adipocytes were differentiated from confluent 3T3-L1 preadipocytes by the addition of methylisobutylxanthine, dexamethasone, and insulin for two days, insulin for an additional two days, and maintained in DMEM plus 10% FBS (42). After eight days, the adipocytes were incubated in Krebs Ringer Hepes with 2% BSA for 60 min in the presence or absence of 20 μM forskolin. Total NEFA secreted was quantified by the NEFA quantification kit (Wako).

Macrophage Growth and Treatments. Bone marrow-derived macrophages were isolated from FABP+/+ C57Bl/6J animals and plated at 10^5 cells per well in 96-well plates (Nalge-Nunc International, Rochester, NY). Cells were preincubated with the indicated concentrations of HTS01037 or carrier (DMSO) for 48 h. Additionally, cells were left untreated or stimulated with the indicated

concentrations of LPS (Sigma-Aldrich) for 24 h. Supernatants were harvested and assayed by ELISA for IL-6, MCP-1/CCL2, and TNFα (BD Biosciences-OptEIA).

Real-Time PCR. Wild-type bone marrow-derived macrophages were plated at $2*10^6$ cells/well in 6-well plates (Corning) and stimulated with 5 μM $15dPGJ_2$ (BIOMOL) overnight following 48 h preincubation with HTS01037. mRNA was isolated and cDNA synthesized using μMACS One-Step cDNA columns (Miltenyi Biotech). Real-time PCR was performed on a DNA Opticon 2 monitor (MJ Research, currently BioRad) using SYBR Green (New England Biolabs). CD36 and carnitine palmitoyltransferase 1A (Cpt1α) were analyzed by Quantitect Primer Assays (Qiagen). β-Actin was used as the reference gene with primers purchased from Clontech (Mountain View, CA). Relative mRNA expression was quantified using the relative expression software tool.

Reporter Assays. CV-1 cells were maintained in DMEM with 10% fetal bovine serum at 37 °C. Transient transfections were performed using lipofectamine 2000 as per the manufacturer's directions (Invitrogen). Cotransfected were pCMV expression plasmids for PPARγ and RXRα and an AFABP/aP2 promoter-luciferase reporter plasmid. All transfections were normalized to the expression of a cotransfected pCMV-renilla luciferase plasmid. Cells were treated 18 h after transfection with varying concentrations of ligand and after another 24 h were

washed, harvested, and luciferase activity assayed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI).

X-ray coordinates have been deposited in the protein data bank for AFABP/aP2 complexed with HTS01037 (PBD ID 3HK1).

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CHAPTER 4

FATTY ACIDS INDUCE LEUKOTRIENE C_4 SYNTHESIS IN MACROPHAGES IN A FATTY ACID BINDING PROTEIN-DEPENDENT MANNER

This chapter has been submitted for publication to Biochimica et Biophysica Acta – Molecular and Cell Biology of Lipids: Eric K. Long*, Kristina Hellberg*, Rocio Foncea, Ann V. Hertzel, Jonathan S. Marchant, Jill Suttles, and David A. Bernlohr

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Kristina Hellberg performed experiments in Figure 1, 2 and 6, wrote part of the paper and edited the manuscript.

Obesity results in increased macrophage recruitment to adipose tissue that promotes a chronic low-grade inflammatory state linked to increased fatty acid efflux from adipocytes. Fatty acids such as palmitate stimulate the production of pro-inflammatory cytokines by macrophages that contribute to obesity-induced insulin resistance. In other inflammatory states, macrophages produce a variety of pro-inflammatory eicosanoids such as leukotriene C_4 (LTC_4) and 5S-, 12S-, and 15S-hydroxyeicosatetraenoic acid (HETE). To assess if eicosanoid production increases with obesity, adipose tissue of leptin deficient *ob/ob* mice was analyzed. In *ob/ob* mice, LTC_4 and 12S-HETE levels increased in the visceral adipose depot while the 5S-HETE levels decreased and 15S-HETE abundance was unchanged. Since macrophages produce the majority of inflammatory cytokines in adipose tissue, RAW264.7 or primary peritoneal macrophages were treated with free fatty acids leading to a sustained increase in intracellular calcium, secretion of LTC_4 and 5S-HETE, but not 12S- or 15S-HETE. Fatty acid binding proteins (FABPs) facilitate the intracellular trafficking of fatty acids and other hydrophobic ligands and *in vitro* stabilize the LTC_4 precursor leukotriene A_4 (LTA_4) from non-enzymatic hydrolysis. Consistent with a role for FABPs in LTC_4 synthesis, treatment of macrophages with HTS01037, a specific FABP inhibitor, resulted in a drastic decrease in both basal and fatty acid-stimulated LTC_4 secretion but no change in 5S-HETE production or 5-lipoxygenase expression. These results indicate that in macrophages, fatty acids

stimulate the 5-lipoxygenase pathway leading to FABP-dependent production of LTC_4 that may contribute to the insulin resistant state.

Introduction

Obesity-induced insulin resistance is characterized by increased basal lipolysis resulting in elevated levels of circulating free fatty acids and a state of chronic low-grade local inflammation (1-4). A hallmark of the inflammatory state is infiltration of macrophages into adipose tissue resulting in release of a variety of pro-inflammatory molecules that affect insulin action and systemic energy metabolism (5-7). Most studies examining inflammatory processes in adipose tissue have focused on the production and signaling effects of pro-inflammatory cytokines such as interleukin 6, tumor necrosis factor α, and monocyte chemoattractant protein-1 (MCP-1) (8-12). However, the role of pro-inflammatory lipid mediators has received less attention.

Pro-inflammatory lipid mediators result from enzymatic oxygenation and/or cyclization of arachidonic acid, that is present in abundance at the sn-2 position of phospholipids in many cell types including macrophages and other immune cells. Arachidonic acid is metabolized by the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes resulting in formation of prostaglandins and leukotrienes, respectively (13,14). Leukotriene production is dependent on calcium-induced translocation of cytosolic phospholipase A_2 ($cPLA_2$) and 5-lipoxygenase (5LO) to the perinuclear membrane resulting in liberation of arachidonic acid and formation of leukotriene A_4 (LTA_4) (15). LTA_4 is metabolized by leukotriene A_4 hydrolase or leukotriene C_4 synthase (LTC_4S) to

produce leukotriene B_4 (LTB_4) or leukotriene C_4 (LTC_4), respectively. Under some circumstances leukotriene synthesis may occur via a transcellular mechanism where formation may take place in a cell distinct from that producing LTA_4 (16). LTB_4 and LTC_4 are secreted and LTC_4 undergoes extracellular proteolytic processing to leukotriene D_4 (LTD_4) and leukotriene E_4 (LTE_4) (15).

Leukotriene production is an important component of the immune response. After synthesis and secretion from the cell, leukotrienes bind to cell surface G-protein coupled receptors on target cells and elicit a variety of biological outputs (17-19). LTB_4 promotes chemotaxis and activation of various leukocytes (20,21). For example, LTB_4 stimulates MCP-1 synthesis by macrophages leading to monocyte recruitment (22). Cysteinyl leukotriene (LTC_4, LTD_4, and LTE_4) bioactivity has been characterized primarily in bronchial diseases such as asthma and other chronic inflammatory disorders of the lung where LTC_4 and its metabolites LTD_4 and LTE_4 act as potent bronchoconstrictors, promote airway remodeling, mucus secretion and vascular permeability (19,23-26).

While the role of leukotrienes in chronic lung inflammation is well characterized, a role for leukotrienes in adipose tissue inflammation is not well understood (27). In the chronic inflammatory state observed in obesity-induced insulin resistance, eicosanoid biology may play a role in the onset and maintenance of the inflammatory response. Indeed, several recent reports focus on the role of

hydroxyeicosatetraenoic acids (HETEs) in adipose tissue suggesting their importance in obesity related disorders. Lipoxygenases catalyze synthesis of HETEs via an oxygenation reaction, resulting in monohydroxylated products. Studies have shown that the 12/15-lipoxygenase product 12S-hydroxyeicosatetraenoic acid (12S-HETE) is associated with obesity and insulin resistance (28-30). Moreover, disruption of the receptor for LTB_4 renders experimental mice resistant to obesity-linked insulin resistance and reduces macrophage infiltration of adipose tissue (31). Targeted deletion of 5-lipoxygenase leads to increased adiposity but protects mice from insulin resistance (32). Finally, pharmacologic inhibition of 5-lipoxygenase activating protein (FLAP) results in reduced inflammation and improved insulin sensitivity in high-fat-fed mice (33).

Fatty acid binding proteins (FABPs), a family of 15 kDa polypeptides, bind fatty acids and other hydrophobic molecules in a ligand-binding cavity to facilitate solubilization and intracellular transport (34,35). The two major FABPs in adipose tissue, adipocyte FABP (AFABP) and epithelial FABP (EFABP) are expressed in both adipocytes and macrophages (36). While AFABP is more abundantly expressed in adipocytes compared to EFABP the opposite is true in macrophages. In addition, low levels of the heart FABP (HFABP) is expressed in both adipocytes and macrophages. Targeted disruption of AFABP and/or EFABP results in reduced inflammation and protection against obesity-induced

insulin resistance in mouse models (37). While the exact mechanisms of this protective effect are unknown, FABPs increase the half-life of the unstable epoxide-containing LTA_4, the precursor of LTB_4 and LTC_4, up to 20-fold *in vitro* (38). This property suggests that FABPs in general may play a crucial role in leukotriene biosynthesis by stabilizing LTA_4 against hydrolysis, resulting in increased inflammatory signaling.

Based on increased adipose tissue inflammation observed in the obese, insulin resistant state, we hypothesized that inflammatory eicosanoid levels are increased in adipose tissue of *ob/ob* mice. In conjunction, we hypothesized that macrophages produce eicosanoids in a fatty acid-dependent manner. Finally, considering the stabilizing effect of FABPs on LTA_4, we hypothesized that absence or inhibition of FABPs block leukotriene production by decreasing overall availability of LTA_4 for conversion to more stable leukotrienes. To that end, we used targeted lipidomic profiling of eicosanoids and found that visceral adipose depots from *ob/ob* mice contain elevated levels of inflammatory LTC_4 and 12S-HETE relative to control mice. We showed that fatty acid treatment of macrophages stimulated increased LTC_4 and 5S-HETE levels and that inhibition of FABPs significantly abrogated the fatty acid-dependent LTC_4 production indicating that LTC_4 formation is dependent on FABPs.

Materials and Methods

Materials. PGE_2, PGD_2, PGE_2-d4, LTB_4, LTC_4, LTD_4, LTE_4, LTC_4-d5, 5S-HETE, 12S-HETE, 15S-HETE, and15S-HETE-d8 were obtained from Cayman Chemical (Ann Arbor, MI). Fluo4-AM and red blood cell lysing buffer was purchased from Sigma-Aldrich (St. Louis, MO). Palmitate (16:0), stearate (18:0), palmitoleate (16:1n-7), oleate (18:1n-9), linoleate (18:2n-6), and α-linolenate (18:3n-3) were obtained from Nu-Chek Prep, Inc. (Elysian, MN). Strata-X solid phase extraction cartridges (200 mg/3mL) were purchased from Phenomenex (Torrance, CA). HTS01037 was a kind gift provided by Maybridge Ltd, UK. $cPLA_2$ (sc-438) and 5LO (sc-20785) antibodies were purchased from Santa Cruz Biotechnology.

Animals. C57Bl/6J or *ob/ob* mice were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were sacrificed and tissues harvested at 12 weeks of age and blood glucose levels were measured immediately using a one-touch ultra glucose meter. All animal procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Primary Peritoneal Macrophage Isolation. Female C57Bl/6J mice were injected with 2 mL 4% thioglycollate to induce macrophage infiltration into the peritoneal cavity. After four days, animals were sacrificed using carbon dioxide asphyxiation and macrophages harvested by peritoneal lavage with sterile phosphate-buffered saline with 3% fetal bovine serum (FBS). The lavage fluid

was centrifuged at 1500 rpm at 4° C for 10 minutes and the resultant cell pellet was resuspended in red blood cell lysing buffer and incubated for 15 minutes on ice. The cells were centrifuged at 1500 rpm for 10 minutes and then resuspended in RPMI 1640 medium supplemented with 10% FBS. Cells were plated in 6- or 12-well plates at a density of 1 x 10^6 cells per well, incubated overnight and used the next day.

Fatty Acid Treatment Conditions. RAW264.7 macrophages were cultured in DMEM containing 10% FBS prior to treatment with free fatty acids. RAW264.7 or primary peritoneal macrophages were washed twice prior to treatment with fatty acids, 20 μM fatty acid complexed to 5 μM BSA (4:1 ratio) in Krebs-Ringer Hepes (KRH) buffer containing 4 mM calcium. After 2h the culture media were collected for eicosanoid analysis. For time course experiments, primary peritoneal macrophages were incubated with free fatty acids and culture media were collected after 1, 2, and 4h. For dose-response experiments, RAW264.7 macrophages were treated with 10, 15, 20, or 25 μM palmitate complexed to 5 μM BSA for 2h before culture media were collected. For experiments using HTS01037, cells were pre-incubated with 20μM HTS01037 for 18h prior to treatment with fatty acids as explained above except that fatty acid treatment media contained HTS01037.

Lipidomic Analyses of Eicosanoids. Internal standards (4 μL of 250 nM LTC_4-d5, PGE_2-d4, and 15S-HETE-d8) were added to culture media immediately following harvest. Samples were vortexed briefly and loaded on Strata-X columns conditioned with 4 mL methanol and equilibrated with 4 mL water. The columns were washed with 4 mL water and eicosanoids eluted with 4 mL methanol. The eluate was dried under nitrogen and resuspended in 25 μL of methanol for LC-MS/MS analysis. For tissue analyses, samples were homogenized in 100mM sodium acetate pH 3.9, 500 μM diethylenetriaminepentaacetic acid and 250 μM butylated hydroxytoluene containing internal standards and centrifuged at 3800 rpm for 10 minutes. The aqueous phase was transferred to Strata-X solid phase extraction cartridges and eicosanoids recovered.

LC-MS/MS analyses for cell culture experiments were performed using an Agilent 1100 HPLC coupled to an AB/Sciex API 4000QTrap mass spectrometer with methods similar to Buczynski *et al.* (39). Analyses of adipose tissues were performed using a Shimamdzu Prominence UPLC coupled to an AB/Sciex API 5500QTrap. Chromatographic separations were achieved using a 2.1 x 100 mm Agilent Zorbax Eclipse C18 column with a 3.5 micron particle size using a gradient elution. Solvent A consisted of 70:30:0.1 water:acetonitrile:acetic acid and solvent B consisted of 50:50 acetonitrile:isopropanol. A gradient was employed at a flow rate of 400 μL/minute (0-1 min 0% B, 1-10 min, 0-100% B, 10-15 min 100% B, 15-16 min 100-0% B, 16-23 min 0% B).

Table 1. Multiple Reaction Monitoring (MRM) Conditions

Analyte	MRM 1 (m/z)	MRM 2 (m/z)
LTB_4	335.3/195.0	335.3/203.0
LTC_4	624.5/272.0	624.5/254.2
LTD_4	495.0/177.0	495.0/143.0
LTE_4	438.2/333.0	438.2/351.2
5S-HETE	319.4/115.0	319.4/203.1
12S-HETE	319.4/179.1	319.4/203.1
15S-HETE	319.4/175.0	319.4/203.1
5,6-diHETE	335.3/115.0	335.2/219.0
PGE_2	351.2/271.0	351.2/189.1
PGD_2	351.2/271.0	351.2/189.1
PGE_2-d4	355.2/275.2	355.2/193.2
LTC_4-d5	629.5/272.0	629.5/254.2
15S-HETE-d8	327.4/226.2	327.4/309.5

Mass spectrometry analyses were performed using electrospray ionization with an ionspray voltage of -4500V and a curtain gas flow rate of 10 L/min at a source temperature of 600° C. All analytes were detected using multiple reaction monitoring (MRM) conditions listed in Table 1 and quantified by stable isotope dilution.

Fatty Acid-Induced Calcium Flux. RAW264.7 macrophages or primary peritoneal macrophages were plated at 5 x 10^4 cells per well in 96-well plates. The cells were allowed to adhere overnight, washed with KRH buffer containing 4 mM calcium and loaded with 5 μM Fluo-4 AM for 30 minutes at room temperature. Cells were washed and incubated in KRH for 30 minutes to allow for intracellular de-esterification of Fluo-4 AM. To evaluate changes in cytoplasmic calcium the cells were incubated with 20 μM free fatty acids complexed to 5 μM BSA in the presence or absence of 1 mM EGTA and calcium influx fluorometrically assessed for 20 minutes using excitation/emission wavelengths of 492 nm and 535 nm, respectively.

Quantitative RT-PCR. Expression of mRNAs was measured by quantitative RT-PCR. Total RNA was isolated from cells using Trizol (Invitrogen) and cDNA was made using iScript cDNA synthesis kit (BioRad). Relative transcript measurements were performed with a MyiQ detection system (BioRad) using relevant primers and iQ SYBRgreen Supermix. Data was normalized to TFIIE.

Primers: $cPLA_2$ Forward: 5'-CCTTTATGGCTCCTGACCTATTTG-3', $cPLA_2$ Reverse: 5'-CATTACTCACGATGTGCTTTGCT-3', LTC_4S Forward: 5'-AGCCCTGTGCGGACTGTTCTAC-3', LTC_4S Reverse: 5'-GCATCTGGAGCCATCTGAAGAG-3', TFIIE Forward: 5'-CAAGGCTTTAGGGGACCAGATAC-3', TFIIE Reverse: 5'-CATCCATTGACTCCACAGTGACAC-3'

Immunoblotting. Cells were lysed in RIPA buffer, sonicated and centrifuged at 14000 rpm for 10 min. Proteins were subjected to SDS-PAGE, transferred to PVDF membranes and incubated with the indicated primary antibodies. Membranes were incubated with secondary antibody conjugated to LI-COR IRDye for 45 minutes and visualized using LI-COR Odyssey infrared imager (LI-COR biotechnologies, Lincoln, NE).

Statistical Analysis. Data are presented as mean ± S.E. Statistical comparisons were performed pair-wise by Student's t-test with statistical significance defined by $p<0.05$.

Results

LTC_4 and 12S-HETE are Elevated in Adipose Tissue from ob/ob mice. To evaluate the eicosanoid profile in adipose tissue of lean and obese mice, we utilized male C57Bl/6J or leptin deficient *ob/ob* mice maintained on a normal chow diet. At 12 weeks of age, *ob/ob* mice had significantly increased body weight compared to lean mice as would be expected (Figure 1A). After a 4-hour fast, serum glucose levels were significantly elevated in *ob/ob* mice compared to controls (Figure 1B). The increase in body weight was accompanied by an increase in epididymal fat pad weight (Figure 1C), as well as increased inguinal adipose tissue weight (Figure 1D). Eicosanoid levels in visceral and subcutaneous adipose tissue, represented by the epididymal and inguinal depots respectively, were measured by lipidomic profiling in *ob/ob* and control mice (a standard chromatogram is displayed in Figure 2A). LTC_4 levels in epididymal adipose tissue were increased in *ob/ob* animals compared to control mice (Figure 2B). Similarly, LTC_4 levels were elevated in inguinal adipose tissue of *ob/ob* mice compared to control mice (Figure 2C). This result agrees with previously data establishing that epididymal adipose tissue, as well as subcutaneous adipose tissue, from obese mice fed a high-fat diet produce increased levels of cysteinyl leukotrienes compared to mice fed a normal chow diet (40). Furthermore, levels of 5S-HETE were decreased, 12S-HETE levels were increased and 15S-HETE were unaltered in epididymal adipose tissue from *ob/ob* mice versus control mice (Figure 2D). Prostaglandins were not quantified,

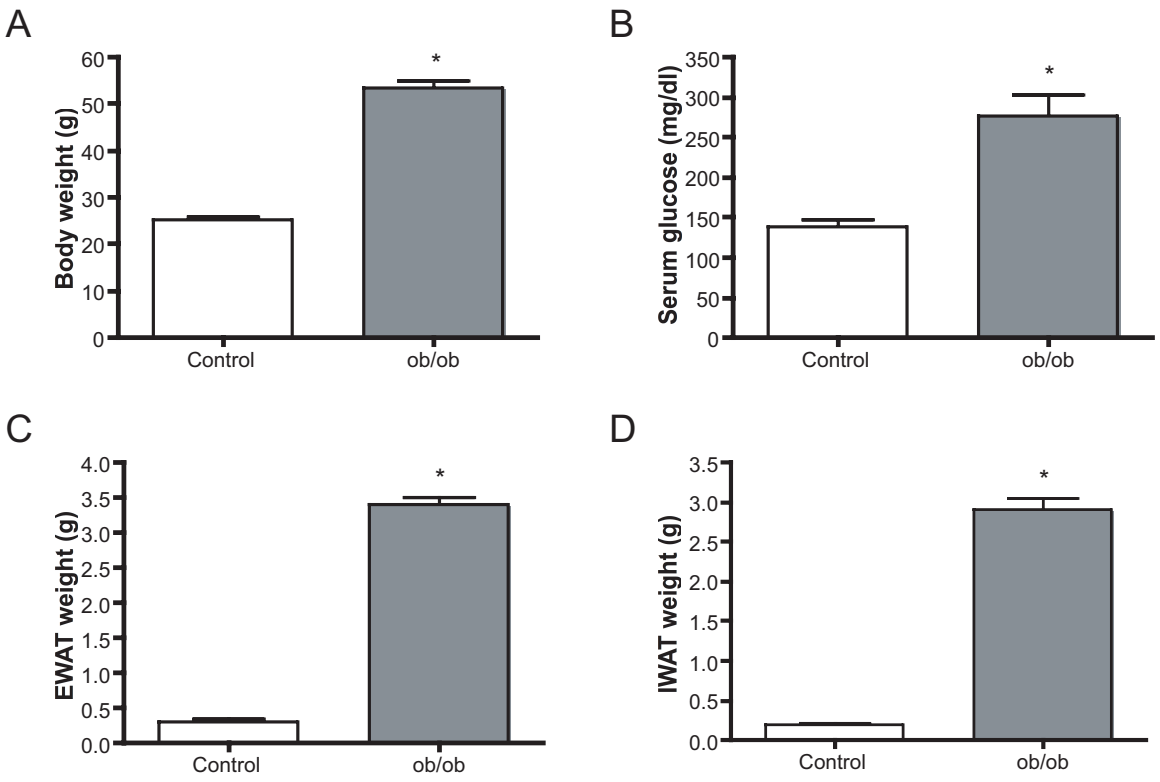

Figure 1. Characterization of *ob/ob* and C57Bl/6J mice. A) Body weight, B) fasted glucose levels, C) epididymal and D) inguinal white adipose tissue weights of 12-week old control and *ob/ob* mice. n=18 for control and n=12 for *ob/ob*.

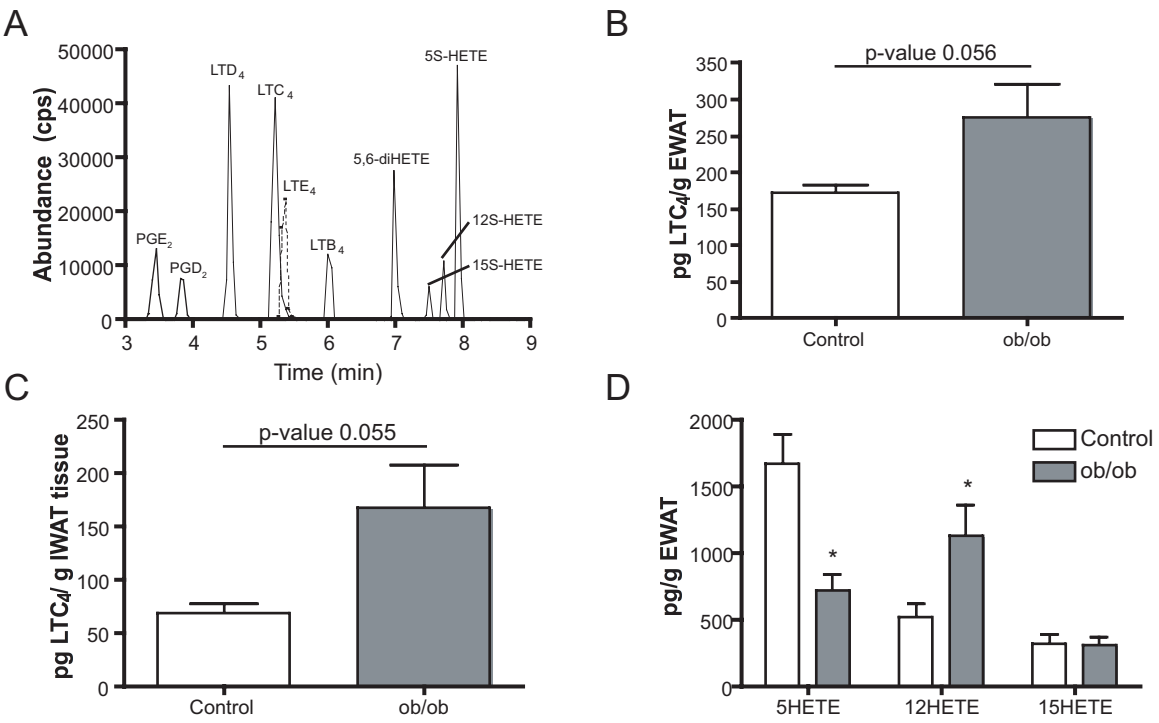

Figure 2. Eicosanoid levels in adipose tissue of *ob/ob* and C57Bl/6J mice. A) Standard chromatogram of the eicosanoids measured via LC-MS/MS. LTC_4 levels in B) epididymal and C) inguinal white adipose tissue of 12-week old *ob/ob* or control C57Bl/6J mice profiled via LC-MS/MS. D) Levels of HETEs in epididymal white adipose tissue. n=3-12 for control and n=6-12 for *ob/ob*.

as the method employed did not adequately separate prostaglandins chromatographically in tissue samples. In addition, LTD_4 and LTE_4 were not detected in adipose tissue from chow or *ob/ob* mice. These data indicate that in conditions where fatty acids are elevated, LTC_4 and 12S-HETE production is increased in adipose tissue.

Fatty Acid-Dependent Production of Eicosanoids in Macrophages. Adipose tissue is comprised of a variety of cell types including adipocytes, preadipocytes, endothelial cells, macrophages and other immune cells such as T cells and dendritic cells (6,41,42). Although adipocytes are capable of secreting leukotrienes, cells within the stromal vascular fraction produce the majority of leukotrienes in visceral adipose tissue (40). Furthermore, in respiratory disorders such as asthma and chronic obstructive pulmonary disease (COPD), immune cells are the primary producers of eicosanoids (43,44). To test the hypothesis that macrophages in adipose tissue carry out fatty acid-dependent eicosanoid synthesis, we evaluated the capacity of RAW264.7 macrophages and primary peritoneal macrophages to produce leukotrienes and other eicosanoids. In RAW264.7 macrophages, treatment with palmitate led to a dose-dependent increase in LTC_4 levels in the media (Figure 3A). Furthermore, treatment with palmitate, stearate, palmitoleate, oleate, or linoleate for 2h led to a significant increase in LTC_4 production as compared to a fatty acid-free control (Figure 3B). LTC_4 was not found intracellularly in any cell type examined (results not shown).

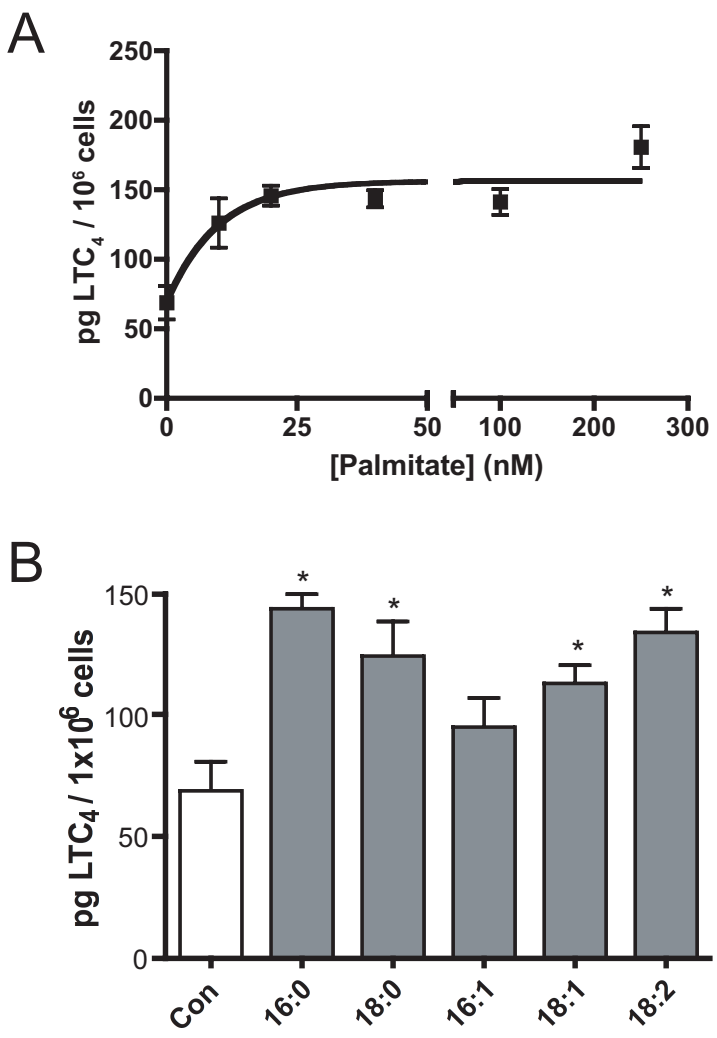

Figure 3. Quantitation of eicosanoids in RAW264.7 macrophages. A) Dose-dependent palmitate-induced LTC_4 production by RAW264.7 macrophages was quantified by LC-MS/MS (n=4 at each concentration). B) RAW264.7 macrophages were treated with fatty acids for 2h and LTC_4 levels were measured by LC-MS/MS (n=4).

LTB_4, LTD_4 and LTE_4 were not detected in media from RAW264.7 macrophages under any treatment condition. PGD_2 and PGE_2 were detected in all analyses, but levels were not altered by fatty acid treatment. Furthermore, 5S-HETE, 12S-HETE, and 15S-HETE were not detected under any condition using RAW264.7 macrophages.

Treatment of primary peritoneal macrophages with palmitate, stearate, palmitoleate, oleate, or linoleate led to dose-dependent changes in eicosanoid production. All fatty acids resulted in significantly greater LTC_4 levels than fatty acid-free control (Figure 4A). Most of the change was observed already within the first hour of treatment. PGE_2 levels were also elevated in response to all fatty acids, but this happened at a slower time scale compared to LTC_4 and in most cases PGE_2 were only significantly increased after four hours of fatty acid stimulation (Figure 4B). Fatty acid treatments also lead to greater levels of 5S-HETE (Figure 4C). In general, these changes were observed within the first hour of treatment. Finally, treatment with fatty acids did not result in increased production of 12S-HETE, with the exception of oleate, after 4h treatment (Figure 4D). LTD_4, LTE_4, and LTB_4 were not detected under any treatment condition, and 15S-HETE levels did not differ from control under any conditions (data not shown). Although cultured and primary macrophages both produce LTC_4 in a fatty acid-dependent manner, primary adipocytes do not. Adipocytes are capable

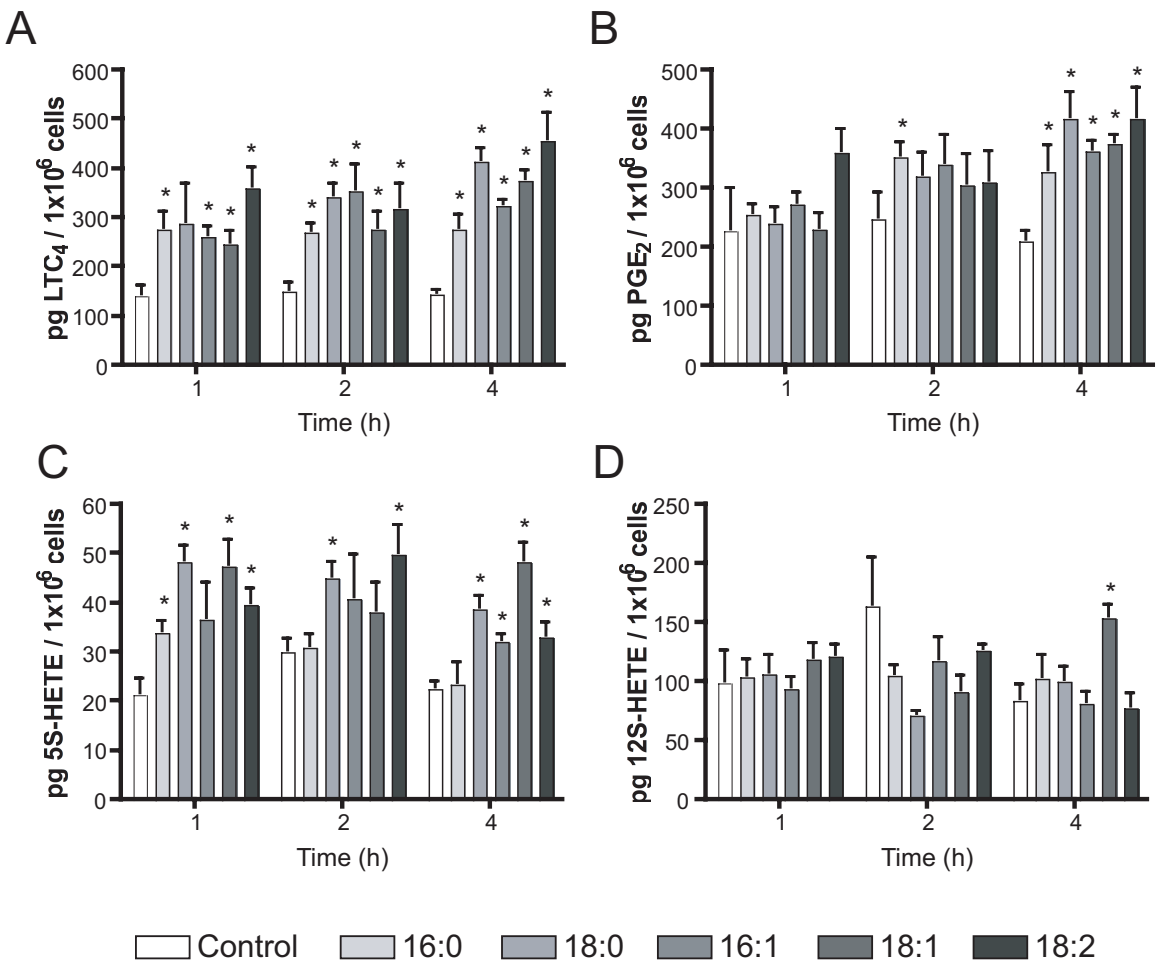

Figure 4. Fatty acid-induced eicosanoid synthesis in primary peritoneal macrophages. Primary peritoneal macrophages were treated with 20 μM of the indicated fatty acid complexed to 5μM BSA and the production of A) LTC_4, B) PGE_2, C) 5S-HETE, and D) 12S-HETE measured by LC-MS/MS after 1, 2, and 4 hour incubations (n=4).

of producing LTC_4, but the levels were not altered in response to palmitate stimulation (results not shown).

Calcium Flux in Macrophages in Response to Fatty Acid Treatment. To assess the events linked to fatty acid-stimulated eicosanoid production in macrophages, calcium flux was evaluated in response to free fatty acids. In RAW264.7 macrophages, palmitate, stearate, palmitoleate, oleate, and linoleate induced a sustained increase in calcium levels as determined using the calcium-sensitive indicator, Fluo-4 AM (Figure 5A, B). In the absence of extracellular calcium (calcium-free media including 1 mM EGTA), the change in calcium was markedly attenuated. In primary peritoneal macrophages, palmitate, linoleate, and α-linolenate induced a similar sustained increase in cytoplasmic calcium (Figure 5C) whereas in the absence of extracellular calcium, the increase in intracellular calcium in response to free fatty acids was blunted in a manner similar to that seen in RAW264.7 macrophages.

FABPs Play a Role in LTC_4 Production in Macrophages. To further characterize the mechanism of fatty acid-dependent eicosanoid formation, we evaluated the role of FABPs in eicosanoid production. FABPs stabilize LTA_4, the unstable precursor of the biologically active leukotrienes (38). Since macrophages express AFABP, EFABP and HFABP, and all FABPs have been shown to stabilize LTA_4 *in vitro*, we utilized a pharmacologic approach towards blocking

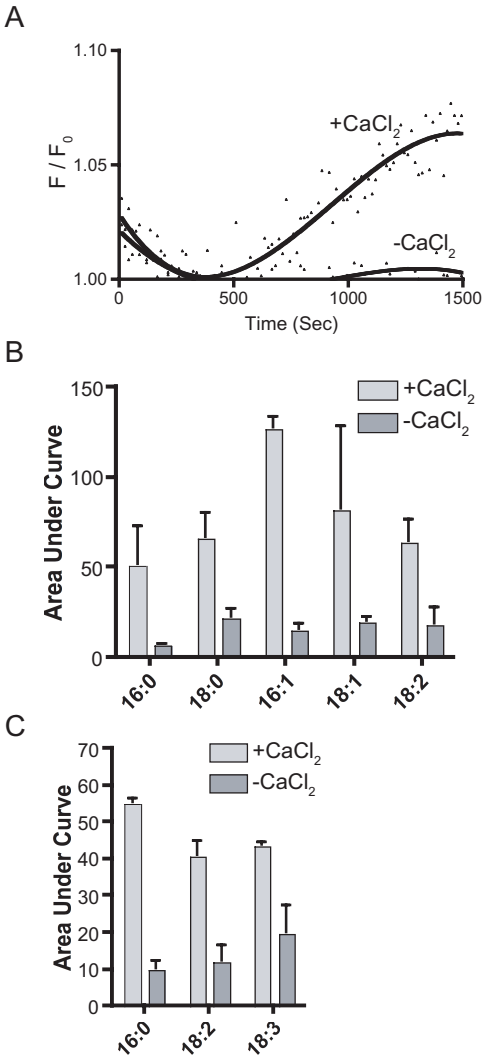

Figure 5. Fatty acid-induced calcium influx in macrophages. A) RAW264.7 macrophages were incubated with 20μM palmitate complexed to 5μM BSA in the presence or absence of calcium for 1500 seconds and calcium flux was monitored by fluorescence of Fluo-4 AM at 535nm. Quantitation of free fatty acid-induced calcium flux in B) RAW264.7 and C) primary peritoneal macrophages was performed using area under the curve analysis. Data are presented from one representative experiment of three that were carried out.

FABP function. To characterize FABP-dependent eicosanoid production we utilized a specific FABP inhibitor, HTS01037 that binds avidly to all FABP isoforms (45). Pre-treatment of primary peritoneal macrophages with HTS01037 prior to fatty acid stimulation resulted in greatly reduced LTC_4 levels both basally and in response to fatty acids (Figure 6A). Although LTC_4 was consistently reduced upon HTS01037 treatment, the basal abundance of other eicosanoids (5S-HETE, 12S-HETE and 15S-HETE) was not lower and in fact 12S-HETE was increased (Figure 6B). HTS01037 may affect LTC_4 production directly by inhibiting FABPs, resulting in destabilization of LTA_4. It is also possible that HTS01037 indirectly causes reduced LTC_4 production by reducing the expression of enzymes involved in LTC_4 production. To assess if HTS01037 alters expression of enzymes in the leukotriene machinery, quantitative RT-PCR was performed. $cPLA_2$ and LTC_4S mRNA expression were not changed in response to HTS01037 treatment (Figure 6C). Furthermore, western blot analysis confirmed that 5LO and $cPLA_2$ protein levels were unaltered upon stimulation with HTS01037 (Figure 6D). It would be interesting to assess LTC_4 synthesis in cells from triple knockout mice lacking AFABP, EFABP and HFABP, if and when such resources become available.

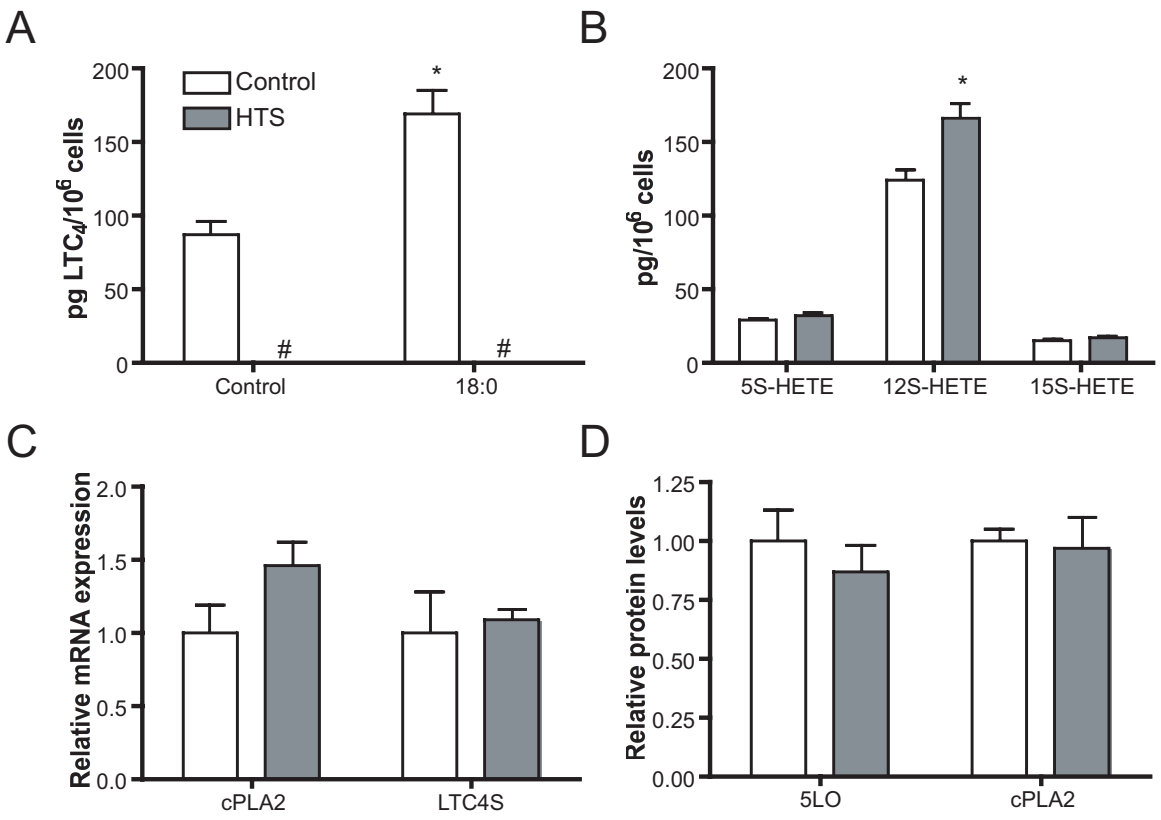

Figure 6. HTS01037 treatment of macrophages. Primary peritoneal macrophages were treated with 20μM HTS01037 (HTS) for 18h prior to 2h fatty acid treatment and analysis of A) LTC_4 (n=6) or B) HETEs (n=16) via LC-MS/MS. C) RT-PCR analysis of $cPLA_2$ and 5LO in primary peritoneal macrophages after 18h HTS01037 treatment (n=6). D) $cPLA_2$ and 5LO protein levels in primary peritoneal macrophages were analyzed by immunoblotting after 18h HTS01037 stimulation (n=3). #, under limit of quantification.

Discussion

Inflammation is often characterized by formation of potent bioactive eicosanoids, such as leukotrienes that act on G-protein coupled receptors to initiate a signaling cascade linked to altered cellular function (19). In the lung, cysteinyl leukotrienes contribute significantly to bronchoconstriction and vascular permeability in asthma and other pulmonary inflammatory diseases. To that end, antagonists of leukotriene receptors are used to counteract the pathology of these diseases (17,24,46). Obesity has been characterized in part by a chronic low-grade inflammatory state yet the involvement of leukotrienes in adipose biology has not been fully delineated. The present study was undertaken to evaluate eicosanoid synthesis in adipose tissue and in particular, fatty acid-dependent eicosanoid synthesis by macrophages. In this context, fatty acids are not substrates for eicosanoid synthesis but rather function as regulatory ligands that stimulate macrophage signaling and elicit the formation and secretion of eicosanoids.

In this study, we evaluated the levels of eicosanoids in visceral and subcutaneous adipose tissue of control or *ob/ob* mice. LTC_4 levels were increased in both the epididymal and inguinal adipose depot in *ob/ob* mice. We found 5S-HETE levels reduced, 12S-HETE levels increased and 15S-HETE levels unaltered in the epididymal white adipose tissue from *ob/ob* mice.

To address which cell type in adipose tissue was responsible for eicosanoid synthesis, we focused on fatty acid-dependent eicosanoid formation in macrophages. The fatty acids chosen for this study represent the five most abundant fatty acids found in serum of humans and animals consuming a western high-fat diet. Interestingly, saturated, monounsaturated, and polyunsaturated fatty acids were all capable of inducing LTC_4 synthesis in RAW264.7 macrophages. A major characteristic of obesity is increased lipolysis resulting in increased levels of free fatty acids (47,48). The free fatty acid composition of adipose tissue and serum mirrors the fatty acid composition of the diet ingested (49,50). These results suggest that the major fatty acids comprising a western high-fat diet are all capable of inducing LTC_4 production in macrophages. High-fat diets, consisting of 60% calories from fat derived from lard, are commonly used to induce the metabolic syndrome in mouse models. This diet is high in both saturated and monounsaturated fatty acids and induces pro-inflammatory effects *in vivo* (51). The data presented herein suggest that these pro-inflammatory events may, at least in part, be mediated through LTC_4 production. However, RAW264.7 macrophages did not produce 5S-HETE, 12S-HETE, or 15S-HETE. Previous work has indicated that immortalized and primary macrophages display different eicosanoid production and metabolism (52). Due to this heterogeneity, we also evaluated the eicosanoid profiles in response to fatty acids in primary murine peritoneal macrophages.

In primary peritoneal macrophages, fatty acid treatment revealed a more complex eicosanoid profile than in immortalized murine cell lines. All fatty acid treatments led to increased production of LTC_4, PGE_2, and 5S-HETE. Interestingly, the time course of formation varied for each eicosanoid. LTC_4 and 5S-HETE levels were increased relatively quickly as measured at the 1h time point. These results are consistent with previous studies showing that 5S-HETE and LTC_4 production occur in an acute manner in response to sustained increases in calcium flux (39). In contrast, PGE_2 levels were not significantly increased until 4h. Increased PGE_2 production relies primarily on increased expression of COX-2, resulting in a slower onset of stimulation-induced PGE_2 production. Extant literature suggests that both LTC_4 and free fatty acids induce COX-2 expression and subsequent PGE_2 production (53,54). From our data, it is unclear whether LTC_4 formation induces PGE_2 production or if increased PGE_2 is a result of fatty acid treatment. Interestingly, we did not observe a fatty acid-dependent increase in 12S-HETE levels in macrophages indicating that adipocytes may be responsible for production of this inflammatory mediator (28). Taken together, our results suggest a primary role for macrophages in adipose tissue LTC_4 production, although other cells found in the stromal vascular fraction may also contribute.

To characterize the mechanisms by which free fatty acids induce eicosanoid formation, we demonstrated that fatty acid treatment of macrophages resulted in

a sustained increase in intracellular calcium, which is required for leukotriene synthesis. The fatty acid-evoked calcium increase was significantly attenuated, but not eliminated, in buffers lacking extracellular calcium in the presence of EGTA. These data, and the protracted time course of the calcium signal, suggest that the increase in calcium is due to both release of intracellular calcium stores and subsequent extracellular calcium influx (55). Similar properties are exhibited by embryonic stem cells treated with linoleic acid (56).

LTA_4 is a highly unstable molecule in aqueous solutions due to an epoxide moiety susceptible to spontaneous water hydrolysis, resulting in formation of 5,6-diHETE or 5,12-diHETE (57). Based on the observation that FABPs can stabilize LTA_4, we examined the role of FABPs in leukotriene formation. Pharmacologic inhibition of FABPs using HTS01037 reduces lipolysis in adipocytes and reduces inflammatory cytokine production in macrophages (45). Macrophages express EFABP > AFABP >> HFABP as assessed using microarray analysis and mono-specific antibodies directed towards each FABP isoform (unpublished). The use of HTS01037 does not allow us to distinguish which, if any, isoform of FABP expressed in macrophages plays the major role in leukotriene production. HTS01037 binds to AFABP inside the ligand-binding cavity in a manner similar to other ligands, coordinating the carboxyl group to Arg126 and Tyr128. Such residues are conserved in both EFABP and HFABP. HTS01037 treatment of macrophages ablated LTC_4 levels in the media indicating that FABPs play a

crucial role in leukotriene production. While HTS01037 can block LTA_4 binding to FABPs, it is also possible that HTS01037 has indirect effects on the expression of fatty acid-dependent transcription factors such as PPARγ and PPARδ. Such changes in gene expression could affect other steps in the leukotriene production pathway. However, mRNA and protein levels of enzymes in the leukotriene synthesizing machinery were not altered upon HTS01037 stimulation (Figure 6).

Macrophages express a variety of free fatty acid receptors including TLR4, CD36 and GPR120 (39,58,59). Toll-like receptor 4 (TLR4) plays a role in the inflammatory response induced by invading pathogens and saturated fatty acids such as palmitate and stearate resulting in the activation of nuclear factor κB (NF-κB) and subsequent formation of pro-inflammatory cytokines (60). TLR4 is incapable of binding fatty acids directly but recent data identified Fetuin-A as an endogenous ligand of TLR4 and as a necessary component for activation of TLR4 in response to fatty acids (61). Fetuin-A is produced and secreted from the liver and would therefore not be available in our primary or cultured macrophages. However, it remains to be evaluated if cell types other than hepatocytes can secrete Fetuin-A or an unidentified protein with a similar function or if the effect that has been attributed to TLR4 activation by fatty acids in culture models in fact represents activation of another fatty acid receptor. Macrophages abundantly express the class B scavenger receptor CD36 (62,63). CD36 facilitates uptake of long chain fatty acids but is also involved in

transduction of signaling events (58,62,64-66). CD36 knockout mice have increased insulin sensitivity, improved insulin signaling and reduced inflammation within the adipose tissue (65). CD36 contributes to inflammation by promoting macrophage infiltration into adipose tissue and pro-inflammatory cytokine production. GPR120 selectively binds omega-3 fatty acids such as α-linolenate and docosahexaenoate (67). GPR120 signaling mediated by n-3 fatty acids leads to blockade of NF-κB activation by preventing TAK1-dependent phosphorylation of IKKβ resulting in an attenuated inflammatory response (59). Future studies will focus on the signaling cascades activated by free fatty acids that result in formation of LTC_4 and other eicosanoids.

In sum, our study shows that free fatty acids induce production of pro-inflammatory LTC_4 by macrophages and that the genetically obese mouse model, *ob/ob*, contains elevated levels of LTC_4 and 12S-HETE in adipose tissue. This production of LTC_4 is dependent on the presence of FABPs, presumably via stabilization of LTA_4. Previous studies examining the role of lipid metabolites in obesity-induced inflammation and insulin resistance have been limited to examination of 12S-HETE (28,29). Our results provide evidence that other lipid metabolites such as LTC_4 could be a potential mediator of obesity-induced inflammation and may be linked to obesity-linked insulin resistance. Future studies will focus on the molecular actions of such signaling lipids.

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CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

Kristina Hellberg wrote this chapter.

Obesity is reaching epidemic proportions and more adults, adolescents, and young children become obese at an alarming rate. Although genetic factors contribute to obesity and associated pathologies in some individuals, others become obese as a result of poor lifestyle choices. With an increased availability of inexpensive calorie-dense food, a sedentary routine, and a stressful environment it is difficult to find the time to exercise and eat healthy and as a consequence the incidence of obesity is continuing to rise. Efforts to combat the obesity epidemic with exercise and diets have not proven efficacious enough and as a result more people than ever before have type 2 diabetes. Part of the problem is that the general population do not perceive type 2 diabetes as a dangerous condition when in fact more people die from diabetes and its complications every year than from breast cancer and AIDS combined. Hopefully with increased public awareness and a clear incentive, the progression of this epidemic can be prevented (1). Since life style changes have proven difficult it is important to understand the underlying mechanisms whereby obesity promotes a cluster of pathologies in order to develop therapeutics to intersect with disease progression.

AFABP deletion provides beneficial phenotypes to an organism or a cell, however, the molecular mechanisms are not completely understood. Several lines of evidence suggest that it is through actions in both adipocytes and macrophages (2) but the paramount question as to what these functions actually

are is still under investigation. Apart from passively being involved in FA transport in the cell, AFABP affects cellular events through protein-protein interactions with peroxisome proliferator-activated receptor γ (PPARγ) (3,4), hormone sensitive lipase (HSL) (5), Janus kinase 2 (Jak2) (6), and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (7). Both functions may affect enzymes important for glucose and lipid metabolism either directly or via altered transcriptional control. The protein-protein interactions require AFABP to have a bound FA and the interaction partner to contain an ExYK motif. This implies that AFABP function as a FA sensor and depending on the cellular FA status, AFABP interacts with proteins to affect activity and signaling. The FA requirement makes interaction studies more complex since detergents, which would extract the FA from AFABP, cannot be used. In addition, FABPs are inherently sticky proteins, hence interaction studies without proper detergents may lead to false positive results. As an alternative approach, cross-linking experiments have been successfully performed to preserve an interaction even in the presence of detergent (6). Since existing antibodies towards AFABP are not optimal for immunoprecipitation (IP), it would be interesting to express AFABP with an affinity tag in adipocytes, IP AFABP using the tag and evaluate the interactome using mass spectrometry. Again, in such experiment caution has to be used to decipher functional interactions from non-specific sticking.

Further research is important to clearly define the mechanism of action of this protein. Most likely it is a combination of different functions and not one single action that lead to protection from obesity-related disease.

We set out to analyze the three dimensional structure of AFABP bound to different ligands, either activating (4-HNE) or inhibitory (HTS01037), to provide clues as to how the protein is altered upon binding to identify a structure-function relationship.

4-HNE modification of AFABP increases in obesity, however the significance of this event is unknown. It has been hypothesized that FABP function as an antioxidant protein, scavenging 4-HNE to prevent modification of other proteins (8). The fact that AFABP knock out mice are protected from disease in response to obesity, which results in oxidative stress, argues against an antioxidant function of AFABP. Another hypothesis is that 4-HNE modification simply renders AFABP in a holo- and signaling-capable state. AFABP requires a bound FA to be able to interact with other proteins indicating that structural changes occur upon lipid binding to allow interaction. It is possible that 4-HNE binding to AFABP results in similar structural alterations as a FA and due to its covalent nature, 4-HNE modification support uninterrupted interaction between AFABP and its interactions partners discussed above. Using x-ray crystallography we

demonstrated that there are no distinct structural differences between AFABP modified with 4-HNE and previously solved structures of AFABP (Chapter 2).

Surprisingly, there were no observable structural alterations when AFABP bind the inhibitor HTS01037 either (Chapter 3). The AFABP-HTS01037 structure was superimposable with previously reported apo and holo forms. One possible explanation is that FA binding alters dynamic fluctuations in the protein, affecting interaction abilities, and that HTS01037 influences the time scale of these fluctuations. If this is the case, HTS01037 binding may keep AFABP in state resembling the apo form, preventing the protein from interacting with various partners. Dynamic changes are not detectable by x-ray crystallography since you only capture one single conformation of the protein. In light of this, Nuclear Magnetic Resonance (NMR) techniques might be useful in the future. Alternatively, subtle changes in structure upon ligand binding could affect electrostatic surface potential to control interaction (9). However, interactions would have to be exceptionally sensitive to the electrostatic surface potential since the structures, with inhibitor and FA, are extremely similar.

The fact that AFABP knockout mice experience an anti-inflammatory and insulin sensitive phenotype has spurred the interest to identify AFABP antagonists. Indeed, several academic laboratories have described AFABP inhibitors and pharmaceutical companies are targeting AFABP in the search for anti-diabetic

therapeutics (10-12). However, there are currently no AFABP inhibitors on the market. One downside for the available AFABP inhibitors used for research is their lack of specificity, as most of them also bind to other isoforms of the protein. Hence, more specific AFABP inhibitors need to be developed.

Plasma FA levels rise in obesity contributing to insulin resistance. Our data presented in Chapter 4 suggest that FA stimulate macrophages to produce and release certain eicosanoids, particularly LTC_4. There is a growing body of evidence that leukotrienes exist in adipose tissue of obese subjects. However, there are many unanswered questions remaining regarding their involvement in adipose tissue. Are other cell types in adipose tissue capable of producing leukotrienes? What cell types in adipose tissue express leukotriene receptors? And what are the consequences of leukotriene signaling in this organ? Does leukotriene initiate inflammation in adipose tissue? Leukotrienes can stimulate recruitment of immune cells to the site of their release, as well as promote pro-inflammatory cytokine and chemokine production. It is therefore possible that leukotrienes contribute to inflammation in obese adipose tissue, by recruiting macrophages and/or by maintaining an inflammatory microenvironment. The downstream effects of leukotrienes on adipocytes are currently not known but preliminary data generated in our laboratory led us to hypothesize that in response to CysLT, insulin signaling is compromised. Clearly, a lot of research

has to be invested in this project to understand the effects of leukotrienes in obesity-induced insulin resistance.

Some key experiments that would be insightful are evaluation of insulin signaling, inflammation and obesity in mice with genetic disruption of 5LO, LTC_4S or leukotriene receptors that have been challenged with a high-fat diet. To assess if macrophages are the primary producers of leukotrienes in adipose tissue it would be interesting to generate mice with macrophage-specific deletion of 5LO or LTC_4S. In addition, mice with macrophage or adipocyte-specific disruption of leukotriene receptors should be informative in pinpointing the downstream target(s) and effect(s) of leukotrienes. Even though macrophage-specific knock out mice can be generated, the fact that immune cells reside in virtually every organ and in the circulation poses a major problem and may complicate interpretation. However, if loss of leukotriene production or their downstream signaling lead to improved insulin sensitivity it might be possible to repurpose already existing FDA approved drugs targeting the leukotriene synthesizing machinery (zileuton (Zyflo™)) or CysLT signaling (montelukast (Singlulair™) and zafirlukast (Accolate™)) in asthma for obesity-induced insulin resistance. There is also an interest in developing new therapeutics interfering with leukotrienes for use in asthma and such drugs, when available, should be evaluated for effects on obesity-induced insulin resistance.

Our mass spectrometry experiments use a targeted approach to detect a limiting set of inflammatory lipids. Other inflammatory lipids and certain degradation products of the currently detected molecules could also have biological effects and it would therefore be interesting to expand the list of analytes. This would require purchase of standards and further optimization of the existing mass spectrometry method. It is, however, possible to increase the number of analytes using multiple reaction monitoring (MRM) as other laboratories detect more than 100 analytes in a single LC-MS/MS run (13).

Interestingly, HTS01037 treatment dramatically attenuated LTC_4 production by macrophages but not the other inflammatory lipids analyzed (Chapter 4). One question is whether the reduced LTC_4 observed upon FABP inhibition is a direct or indirect effect. FABPs stabilize LTA_4 *in vitro*, however, if this happens *in vivo* has not been confirmed since LTA_4 levels cannot be measured directly due to the unstable nature of this lipid species. It is possible that HTS treatment results in altered calcium homeostasis or affect upstream events dependent on $cPLA_2$ or 5LO. However, this is unlikely since HTS treatment doesn't significantly affect HETE production. The specific reduction of LTC_4 is consistent with a direct effect of HTS01037, inhibiting AFABP to prevent LTA_4 stabilization. Nevertheless, it is possible that LTC_4S activity is compromised by HTS01037. To address this, leukotriene levels should be evaluated in adipose tissue and primary macrophages from wt and Ako mice. As mentioned above, there are other

pathways, not influenced by inflammatory lipids, which may be responsible for increased insulin sensitivity in AFABP knock out mice. The question is how much of the phenotype seen in AFABP KO mice can be attributed to leukotrienes?

To test if the mechanism of reduced leukotrienes in response to HTS01037 treatment is due to impaired stabilization of LTA_4, we aimed to generate a AFABP mutant incapable of stabilizing LTA_4 but with unaltered affinity for FA. Expressing such a mutant in macrophages lacking AFABP and analyzing the ability to produce leukotrienes would indicate if AFABP-dependent LTA_4 stabilization is required for leukotriene formation. After analyzing the structure and the probable localization of the unstable epoxide moiety of LTA_4 in the ligand-binding cavity of AFABP we generated a Q95L mutant of AFABP. However, this mutant did not alter LTA_4 stability but, surprisingly, did show an altered FA binding profile. In the future other mutants may be generated and evaluated for reduced LTA_4 stabilizing effects.

Although it is generally accepted that AFABP sequesters and delivers FA, and function as a FA sensor to regulate lipid metabolism and insulin sensitivity, questions regarding AFABP functions still remain. The emerging involvement of AFABP in adipose tissue leukotriene production provides several new interesting directions for future research.

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