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Fatty Acids Induce Leukotriene C₄ Synthesis in Macrophages in a Fatty Acid Binding Protein-Dependent Manner

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Abstract

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. Activated macrophages produce a variety of pro-inflammatory lipids such as leukotriene C₄ (LTC₄) and 5-, 12-, and 15-hydroxyeicosatetraenoic acid (HETE) suggesting the hypothesis that fatty acids may stimulate eicosanoid synthesis. To assess if eicosanoid production increases with obesity, adipose tissue of leptin deficient *ob/ob* mice was analyzed. In *ob/ob* mice, LTC₄ and 12-HETE levels increased in the visceral (but not subcutaneous) adipose depot while the 5-HETE levels decreased and 15-HETE abundance was unchanged. Since macrophages produce the majority of inflammatory molecules in adipose tissue, treatment of RAW264.7 or primary peritoneal macrophages with free fatty acids led to increased secretion of LTC₄ and 5-HETE, but not 12- or 15-HETE. Fatty acid binding proteins (FABPs) facilitate the intracellular trafficking of fatty acids and other hydrophobic ligands and in vitro stabilize the LTC₄ precursor leukotriene A₄ (LTA₄) from non-enzymatic hydrolysis. Consistent with a role for FABPs in LTC₄ synthesis, treatment of macrophages with HTS01037, a specific FABP inhibitor, resulted in a marked decrease in both basal and fatty acid-stimulated LTC₄ secretion but no change in 5-HETE production or 5-lipoxygenase expression. These results indicate that the products of adipocyte lipolysis may stimulate the 5-lipoxygenase pathway leading to FABP-dependent production of LTC₄ and contribute to the insulin resistant state.

Keywords

macrophage; fatty acids; leukotrienes; inflammation; LTC₄; fatty acid binding protein

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1. 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 A₂ (cPLA₂) and 5-lipoxygenase (5LO) to the perinuclear membrane resulting in liberation of arachidonic acid and formation of leukotriene A₄ (LTA₄) [15]. LTA₄ is metabolized by leukotriene A₄ hydrolase or leukotriene C₄ synthase (LTC₄S) to produce leukotriene B₄ (LTB₄) or leukotriene C₄ (LTC₄), respectively. Under some circumstances leukotriene synthesis may occur via a transcellular mechanism where formation may take place in a cell distinct from that producing LTA₄ [16]. LTB₄ and LTC₄ are secreted and LTC₄ undergoes extracellular proteolytic processing to leukotriene D₄ (LTD₄) and leukotriene E₄ (LTE₄) [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]. LTB₄ promotes chemotaxis and activation of various leukocytes [20, 21]. For example, LTB₄ stimulates MCP-1 synthesis by macrophages leading to monocyte recruitment [22]. Cysteinyl leukotriene (LTC₄, LTD₄, and LTE₄) bioactivity has been characterized primarily in bronchial diseases such as asthma and other chronic inflammatory disorders of the lung where LTC₄ and its metabolites LTD₄ and LTE₄ act as potent bronchoconstrictors, promote airway remodeling, mucus secretion and vascular permeability [19, 23–25].

While the role of leukotrienes in chronic lung inflammation is well characterized, a role for leukotrienes in adipose tissue inflammation is not well understood [26]. 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 [27–29]. Moreover, disruption of the receptor for LTB₄ renders experimental mice resistant to obesity-linked insulin resistance and reduces macrophage infiltration of adipose tissue [30]. Targeted deletion of 5-lipoxygenase leads to increased adiposity but protects mice from insulin resistance [31]. Finally, pharmacologic inhibition of 5-lipoxygenase activating protein (FLAP) results in reduced inflammation and improved insulin sensitivity in high-fat-fed mice [32].

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 [33, 34]. The two major FABPs in adipose tissue, adipocyte FABP (AFABP) and epithelial FABP (EFABP) are expressed in both adipocytes and macrophages [35]. 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 [36]. While the exact mechanisms of this protective effect are unknown, FABPs increase the half-life of the unstable epoxide-containing LTA₄, the precursor of LTB₄ and LTC₄, up to 20-fold in vitro [37]. This property suggests that FABPs in general may play a crucial role in leukotriene biosynthesis by stabilizing LTA₄ 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 LTA₄, we hypothesized that absence or inhibition of FABPs block leukotriene production by decreasing overall availability of LTA₄ 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 LTC₄ and 12-HETE relative to control mice. We demonstrate that fatty acid treatment of macrophages increased LTC₄ and 5-HETE levels and that inhibition of FABPs significantly abrogated the fatty acid-dependent LTC₄ production indicating that LTC₄ formation is dependent on FABPs.

2. Materials and Methods

2.1. Materials

PGE₂, PGD₂, PGE₂-d4, LTB₄, LTC₄, LTD₄, LTE₄, LTC₄-d5, 5S-HETE, 12S-HETE, 15S-HETE, and 15S-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. cPLA₂ (sc-438) and 5LO (sc-20785) antibodies were purchased from Santa Cruz Biotechnology.

2.2. Animals

C57Bl/6J and Lep^{ob} (*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 procedures were approved by the University of Minnesota Institutional Animal Care and Use Committee.

2.3. 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 1×10^6 cells per well, incubated overnight and used the next day.

2.4 Stromal Vascular and Primary Adipocyte Preparation

Murine epididymal fat pads were minced and digested with Krebs-Ringer Hepes (KRH) buffer (3.8 mM Hepes, 3.2 mM CaCl_2 , 2 mM sodium pyruvate, 1.2 mM MgSO_4 , 4.7 mM KCl, 120 mM NaCl, 5 mM NaHCO_3 , $1 \times$ glutamax, 1% BSA fatty acid free, 5 mM glucose, pH 7.4) containing 1 mg/ml collagenase (type II, Sigma) for 1 h at 37°C with constant gentle agitation. Digested tissue was filtered through a $100\text{ }\mu\text{m}$ mesh nylon cell strainer and centrifuged at $500 \times g$ for 10 min at room temperature. Adipocytes were recovered from the floating cell layer and stromal vascular cells from the pellet. Separated stromal vascular and adipocyte fractions were each incubated for 2 h in KRH and media collected for eicosanoid analysis describe in section 2.6.

2.5. Fatty acid stimulated eicosanoid synthesis

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, $20\text{ }\mu\text{M}$ fatty acid complexed to $5\text{ }\mu\text{M}$ 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 $25\text{ }\mu\text{M}$ palmitate complexed to $5\text{ }\mu\text{M}$ BSA for 2h before culture media were collected. Palmitate concentrations are reported as free palmitate when complexed to BSA at the indicated ratios as calculated according to Richieri et al, [38]. For studies with HTS01037, primary peritoneal macrophages were incubated with the FABP inhibitor for 18 h, washed and incubated with fresh medium containing HTS01037 plus fatty acids coupled to BSA. The medium was collected after 1 h and LTC_4 quantitated by LC-MS/MS.

2.6. Lipidomic analyses of eicosanoids

Internal standards ($4\text{ }\mu\text{L}$ of 250 nM $\text{LTC}_4\text{-d}_5$, $\text{PGE}_2\text{-d}_4$, and 15S-HETE-d_8) 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 $25\text{ }\mu\text{L}$ of methanol for LC-MS/MS analysis. For tissue analyses, samples were homogenized in 100mM sodium acetate pH 3.9, $500\text{ }\mu\text{M}$ diethylenetriaminepentaacetic acid and $250\text{ }\mu\text{M}$ 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 Shimadzu Prominence UPLC coupled to an AB/Sciex API 5500QTrap. Chromatographic separations were achieved using a $2.1 \times 100\text{ mm}$ 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 $400\text{ }\mu\text{L/minute}$ (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).

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.

2.7. 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: cPLA₂ Forward: 5'-CCTTTATGGCTCCTGACCTATTTG-3', cPLA₂ Reverse: 5'-CATTACTCACGATGTGCTTTGCT-3', LTC₄S Forward: 5'-AGCCCTGTGCGGACTGTTCTAC-3', LTC₄S Reverse: 5'-GCATCTGGAGCCATCTGAAGAG-3', TFIIE Forward: 5'-CAAGGCTTTAGGGGACCAGATAC-3', TFIIE Reverse: 5'-CATCCATTGACTCCACAGTGACAC-3'

2.8. 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).

2.9. Statistical analysis

Data are presented as mean $\pm$ S.E. Statistical comparisons were performed pairwise by Student's t-test with statistical significance defined by $p < 0.05$.

3. Results

3.1. LTC₄ and 12-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 (Fig. 1A). After a 4-hour fast, serum glucose levels were significantly elevated in *ob/ob* mice compared to controls (Fig. 1B). The increase in body weight was accompanied by an increase in epididymal fat pad weight (Fig. 1C), as well as increased inguinal adipose tissue weight (Fig. 1D). Eicosanoid levels in visceral and subcutaneous adipose tissue, represented by the epididymal and inguinal depots respectively, were measured by targeted lipidomic profiling in *ob/ob* and control mice (a representative chromatogram of synthetic standards is displayed in Fig. 2A). LTC₄ levels in epididymal adipose tissue were increased in *ob/ob* animals compared to control mice (Fig. 2B). Similarly, LTC₄ levels were elevated in inguinal adipose tissue of *ob/ob* mice compared to control mice (Fig. 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 5-HETE were decreased, 12-HETE levels were increased and 15-HETE were unaltered in epididymal adipose tissue from *ob/ob* mice versus control mice (Fig. 2D). Prostaglandins were not quantified, as chromatograms from adipose tissue preparations exhibited a more complex multiple reaction monitoring signature resulting in an inability to adequately resolve PGE₂ and PGD₂ likely due to the presence of other isomers such as isoprostanes

[41]. In addition, LTB₄, 6-trans-LTB₄, 5,6-diHETE, LTD₄ and LTE₄ were not detected in adipose tissue from chow fed C57BL/6J or *ob/ob* mice. These data indicate that in conditions where fatty acids are elevated, LTC₄ and 12-HETE production are also increased in adipose tissue.

3.2 Eicosanoid production by primary adipocytes and stromal vascular fraction

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, 42, 43]. 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 [44, 45]. To further characterize the types of cells that produce eicosanoids, adipocytes and stromal vascular (SV) cells were separated by collagenase digestion and eicosanoids evaluated. The SV fraction produced LTB₄, LTC₄, and LTE₄, while primary adipocytes produced only LTC₄ (Fig. 3A). The presence of LTB₄ and LTE₄ in the isolated SV fraction, but not whole tissue, may be due to LTA₄ metabolism via LTA₄ hydrolase and gamma-glutamyl transpeptidase and dipeptidases, respectively [15]. In addition to greater leukotriene production in the SV fraction, 12-HETE was also found at elevated levels in the SV fraction. 5-HETE was only produced in equivalent amounts by the SV and adipocyte fractions while 15-HETE was only produced by adipocytes (Fig. 3B). Finally, PGE₂ was produced at greater levels by SV cells while PGD₂ was produced equivalently by both SV cells and adipocytes (Fig. 3C).

3.3. Fatty acid-dependent production of eicosanoids in macrophages

As immune cells were the primary producers of a number of eicosanoids such as leukotrienes and 12-HETE, macrophages may produce pro-inflammatory lipids in a fatty acid-dependent manner. 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. Treatment of RAW264.7 macrophages with palmitate led to a dose-dependent increase in LTC₄ secreted into the medium (Fig. 4A). Furthermore, treatment with palmitate, stearate, oleate, or linoleate for 2h led to a significant increase in LTC₄ production as compared to a fatty acid-free control, while palmitoleate showed a trend toward an increase (Fig. 4B). LTC₄ was not found intracellularly in any cell type examined (results not shown). LTB₄, LTD₄ and LTE₄ were not detected in media from RAW264.7 macrophages under any treatment condition. PGD₂ and PGE₂ were detected in all analyses, but levels were not altered by fatty acid treatment. Furthermore, 5-HETE, 12-HETE, and 15-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 LTC₄ levels than fatty acid-free control (Fig. 5A). Most of the change was observed within the first hour of treatment. PGE₂ levels were also elevated in response to all fatty acids, but this happened at a slower time scale compared to LTC₄ and in most cases PGE₂ was only significantly increased after four hours of fatty acid stimulation (Fig. 5B). Fatty acid treatments also lead to greater levels of 5-HETE (Fig. 5C). In general, these changes were observed within the first hour of treatment. Finally, treatment with fatty acids did not result in increased production of 12-HETE, with the exception of oleate, after 4h treatment (Fig. 5D). LTD₄, LTE₄, and LTB₄ were not detected under any treatment condition, and 15-HETE levels did not differ from control under any conditions (data not shown). Although cultured and primary macrophages both produce LTC₄ in a fatty acid-

dependent manner, primary adipocytes do not. Adipocytes are capable of producing LTC₄, but the levels were not altered in response to palmitate stimulation (results not shown).

3.4. FABPs play a role in LTC₄ 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 LTA₄, the unstable precursor of the biologically active leukotrienes [37]. Since macrophages express AFABP, EFABP and HFABP, and all FABPs have been shown to stabilize LTA₄ in vitro, we utilized a pharmacologic approach towards blocking FABP function. To characterize FABP-dependent eicosanoid production we utilized a specific FABP inhibitor, HTS01037, that binds avidly to all FABP isoforms [46]. Pre-treatment of primary peritoneal macrophages with HTS01037 prior to fatty acid stimulation resulted in reduced LTC₄ levels in a time (Fig. 6A) and concentration (Fig. 6B) dependent manner. Furthermore, pretreatment with HTS01037 resulted in blunted fatty acid-dependent LTC₄ production (Fig. 6C). Although LTC₄ was consistently reduced upon HTS01037 treatment, the basal abundance of other eicosanoids (5-HETE, 12-HETE and 15-HETE) was not lower and the level of 12-HETE was increased (Fig. 6D).

HTS01037 may affect LTC₄ production directly by inhibiting FABP lipid binding resulting in release and destabilization of LTA₄. It is also possible that HTS01037 indirectly reduced LTC₄ production by affecting the expression of enzymes involved in LTC₄ production. To assess if HTS01037 altered the expression of enzymes in the leukotriene pathway, quantitative RT-PCR was performed. cPLA₂ and LTC₄S mRNA expression were not changed in response to HTS01037 treatment (Fig. 6E). Furthermore, western blot analysis confirmed that 5LO and cPLA₂ protein levels were unaltered upon stimulation with HTS01037 (Fig. 6F).

4. 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, 47]. 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. *Ob/ob* mice on the C57BL/6J genetic background have been characterized as a model of obesity-induced insulin resistance [48]. LTC₄ levels were increased in both the epididymal and inguinal adipose depot in *ob/ob* mice. We found 5-HETE levels reduced, 12-HETE levels increased and 15-HETE levels unaltered in the epididymal white adipose tissue from *ob/ob* mice. A previous study by Gonzalez-Periz and colleagues also reported levels of a number of eicosanoids by LCMS/MS analysis [49]. However, our study differs in that cysteinyl leukotrienes were analyzed in addition to a representative sampling of HETEs and prostaglandins. Based on the finding that LTC₄ levels differed in *ob/ob* vs control mice, further experiments were designed to examine fatty acid dependent LTC₄ synthesis. An additional intriguing possibility is that since the levels of

both LTC₄ and 12-HETE are increased in adipose tissue from obese models that the two lipids are somehow linked or that one leads to the production of the other.

To address which cell types in adipose tissue were responsible for eicosanoid synthesis, we digested adipose tissue and separated the stromal vascular fraction from adipocytes. Profiling of eicosanoids in separated cells revealed that the SV fraction was responsible for the majority of leukotriene, 12-HETE, and PGE₂ production. Interestingly, the eicosanoid profile of the SV fraction was quite different from whole tissue. Specifically, LTB₄ and LTE₄ were not detected in whole tissue homogenate, but were produced by the SV fraction. Leukotriene synthesis and metabolism occurs, in part, via transcellular mechanisms [16, 50]. It is likely that the unique composition of a collagenase digested SV fraction allows LTA₄ and LTC₄ access to enzymes that convert them to LTB₄ and LTE₄, respectively, while this access may be restricted in whole tissue where vascular cells are morphologically separated from macrophages and other immune cells. Further study would provide greater mechanistic evidence as to the nature of the differences between whole tissue and collagenase digested SV fractions. However, the results of this experiment support the hypothesis that leukotrienes are produced in greater quantity by immune cells as opposed to adipocytes.

To further characterize immune cell production of eicosanoids, we evaluated fatty acid-dependent eicosanoid production 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 LTC₄ synthesis in RAW264.7 macrophages. A major characteristic of obesity is increased lipolysis resulting in increased levels of free fatty acids [51, 52]. The free fatty acid composition of adipose tissue and serum mirrors the fatty acid composition of the diet ingested [53, 54]. These results suggest that the major fatty acids comprising a western high-fat diet are all capable of inducing LTC₄ 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 [55]. The data presented herein suggest that these pro-inflammatory events may, at least in part, be mediated through LTC₄ production. However, RAW264.7 macrophages did not produce 5-HETE, 12-HETE, or 15-HETE. Previous work has indicated that immortalized and primary macrophages display different eicosanoid production and metabolism [56]. 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 LTC₄, PGE₂, and 5-HETE. Interestingly, the time course of formation varied for each eicosanoid. LTC₄ and 5-HETE levels were increased relatively quickly as measured at the 1h time point. These results are consistent with previous studies showing that 5-HETE and LTC₄ production occur in an acute manner in response to sustained increases in calcium flux [39]. In contrast, PGE₂ levels were not significantly increased until 4h. Increased PGE₂ production relies primarily on increased expression of COX-2, resulting in a slower onset of stimulation-induced PGE₂ production. Extant literature suggests that both LTC₄ and free fatty acids induce COX-2 expression and subsequent PGE₂ production [57, 58]. From our data, it is unclear whether LTC₄ formation induces PGE₂ production or if increased PGE₂ is a result of fatty acid treatment. Interestingly, we did not observe a fatty acid-dependent increase in 12-HETE levels in macrophages indicating that adipocytes may be responsible for production of this inflammatory mediator [27]. It is important to note that the time course of fatty acid-dependent leukotriene production is slower than previously described. Other studies have relied heavily on calcium ionophores such as A23187 or ADP to induce a

rapid increase in calcium that results in increased leukotriene production within minutes, or even seconds [59]. In contrast, fatty acid treatment results in a sustained increase in calcium flux that occurs much more slowly than ionophore treatment, accounting for the delayed increase in leukotriene production (results not shown). Taken together, our results suggest a primary role for macrophages in adipose tissue LTC₄ production, although other cells found in the stromal vascular fraction may also contribute.

LTA₄ 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 [60]. Based on the observation that FABPs can stabilize LTA₄, 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 [46]. 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 LTC₄ levels in the media in a time and concentration-dependent manner. Interestingly, LTC₄ levels transiently increased in response to HTS01037 but decreased at longer time points. Furthermore, LTC₄ production in response to fatty acid treatment was blocked by pretreatment with HTS01037 indicating that FABPs play a crucial role in leukotriene production. While HTS01037 can block LTA₄ binding to FABPs, it is also possible that HTS01037 has indirect effects on the expression of fatty acid-dependent transcription factors such as PPAR γ and PPAR δ . 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, 61, 62]. 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 κ B (NF- κ B) and subsequent formation of pro-inflammatory cytokines and prostaglandins via increased expression of cyclooxygenase-2 [63, 64]. 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 [65]. 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 [66, 67]. CD36 facilitates uptake of long chain fatty acids but is also involved in transduction of signaling events [61, 66, 68–70]. CD36 knockout mice have increased insulin sensitivity, improved insulin signaling and reduced inflammation within the adipose tissue [69]. CD36 contributes to inflammation by promoting macrophage infiltration into adipose tissue and pro-inflammatory cytokine production as well as production of prostaglandins [66]. GPR120 selectively binds omega-3 fatty acids such as α -linolenate and docosahexaenoate [71]. GPR120 signaling mediated by n-3 fatty acids leads to blockade of NF- κ B activation by preventing TAK1-dependent phosphorylation of IKK β resulting in an attenuated inflammatory response [62]. While our studies herein do not address which cell surface fatty

acid receptor mediates eicosanoid production, future studies will focus on the signaling cascades activated by free fatty acids.

In sum, our study shows that free fatty acids induce production of pro-inflammatory LTC₄ by macrophages and that the genetically obese mouse model, *ob/ob*, contains elevated levels of LTC₄ and 12-HETE in adipose tissue. This production of LTC₄ is dependent on the presence of FABPs, presumably via stabilization of LTA₄. Previous studies examining the role of lipid metabolites in obesity-induced inflammation and insulin resistance have been limited to examination of 12-HETE [28, 29]. Our results provide evidence that other lipid metabolites such as LTC₄ (and LTB₄) could be potential mediators of obesity-induced inflammation and may be linked to obesity-linked insulin resistance. Consistent with this, Mothe-Satney et al. [72] have shown that mice deficient in 5-lipoxygenase exhibit reduced levels of inflammatory macrophages and T cells. Moreover, Claria et al. [73] have recently demonstrated that human adipose tissue produces a variety of lipid mediators (including resolvins and leukotrienes) implying that such molecules may play a significant role in insulin resistance in humans. Future studies will focus on the molecular actions of such signaling lipids.

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Abbreviations

LT	leukotriene
FABP	fatty acid binding protein
AFABP	adipocyte fatty acid binding protein
EFABP	epithelial fatty acid binding protein
HFABP	heart fatty acid binding protein
HETE	hydroxyeicosatetraenoic acid
PG	prostaglandin
MCP-1	monocyte chemoattractant protein-1
SV fraction	stromal vascular fraction
MRM	multiple reaction monitoring
cPLA₂	cytosolic phospholipase A ₂
5LO	5-lipoxygenase
LTC₄S	leukotriene C ₄ synthase
COX-2	cyclooxygenase 2
PPAR	peroxisome proliferator-activated receptor
TLR4	Toll-like receptor 4
NF-κB	nuclear factor κB

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Highlights

- Adipose tissue from obese mice contains elevated levels of LTC₄ and 12S-HETE.
- Macrophages produce LTC₄ and 5S-HETE in response to fatty acid stimulation.
- Inhibition of Fatty Acid Binding Proteins (FABPs) block LTC₄ production.

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* animals.

Figure 2. Eicosanoid levels in adipose tissue of *ob/ob* and C57Bl/6J mice

A) Chromatogram of synthetic standards of eicosanoids measured via LC-MS/MS. LTC₄ 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*.

Figure 3. Eicosanoid production by primary adipocytes and stromal vascular fraction
Minced adipose tissue was collagenase digested and separated into primary adipocyte and stromal vascular fractions and A) leukotrienes, B) HETEs and C) prostaglandins were measured by LC-MS/MS. n=3 for stromal vascular fractions, while n=2 for adipocyte fractions.

Figure 4. Quantitation of eicosanoids in RAW264.7 macrophages

A) Dose-dependent palmitate-induced LTC₄ 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 LTC₄ levels were measured by LC-MS/MS (n=4).

Figure 5. Fatty acid-induced eicosanoid synthesis in primary peritoneal macrophages
Primary peritoneal macrophages were treated with 20 μ M of the indicated fatty acid complexed to 5 μ M BSA and the production of A) LTC₄, B) PGE₂, C) 5-HETE, and D) 12-HETE measured by LC-MS/MS after 1, 2, and 4 hour incubations (n=4).

Figure 6. HTS01037 treatment of macrophages

To evaluate inhibition of eicosanoid production by HTS01037, primary peritoneal macrophages were; A) treated with 20 μM HTS01037 (HTS) for the indicated times and then evaluated for LTC₄ secretion into the cell culture medium during the final 60 min (n=3), B) treated with the indicated levels of HTS01037 for 18 h and then analyzed for LTC₄ secretion during the final 60 min (n=3), C) incubated for 18h with HTS01037 prior to 2h fatty acid treatment and analysis of LTC₄ (n=6) or D) HETEs (n=6). E) RT-PCR analysis of cPLA₂ and 5LO in primary peritoneal macrophages after 18 h HTS01037 treatment (n=6). F) cPLA₂ and 5LO protein levels in primary peritoneal macrophages were analyzed by immunoblotting after 18 h HTS01037 treatment (n=3). * denotes significant difference from 0h or 0 μM, ** indicates significant difference from 0 and 1h or 0 and 0.2 μM and *** denotes significant difference from 0, 1, and 2h. #, below the limit of quantification.

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