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5-Lipoxygenase Activating Protein Signals Adipose Tissue Inflammation and Lipid Dysfunction in Experimental Obesity

Raquel Horrillo,* Ana González-Pérez,* Marcos Martínez-Clemente,* Marta López-Parra,* Natália Ferre,* Esther Titos,† Eva Morán-Salvador,* Ramon Deulofeu,* Vicente Arroyo,*† and Joan Claria*†

The presence of the so-called low-grade inflammatory state is recognized as a critical event in adipose tissue dysfunction, leading to altered secretion of adipokines and free fatty acids (FFAs), insulin resistance, and development of hepatic complications associated with obesity. This study was designed to investigate the potential contribution of the proinflammatory 5-lipoxygenase (5-LO) pathway to adipose tissue inflammation and lipid dysfunction in experimental obesity. Constitutive expression of key components of the 5-LO pathway, as well as leukotriene (LT) receptors, was detected in adipose tissue as well as in adipocyte and stromal vascular fractions. Adipose tissue from obese mice, compared with that from lean mice, exhibited increased 5-LO activating protein (FLAP) expression and LTB₄ levels. Incubation of adipose tissue with 5-LO products resulted in NF-κB activation and augmented secretion of proinflammatory adipokines such as MCP-1, IL-6, and TNF-α. In addition, LTB₄, but not LTD₄, reduced FFA uptake in primary adipocytes, whereas 5-LO inhibition suppressed isoproterenol-induced adipose tissue lipolysis. In mice with dietary obesity, elevated FLAP expression in adipose tissue was paralleled with macrophage infiltration, increased circulating FFA levels, and hepatic steatosis, phenomena that were reversed by FLAP inhibition with Bay-X-1005. Interestingly, FLAP inhibition induced AMP-activated protein kinase phosphorylation in parallel with decreases in hormone-sensitive lipase activity and the expression and secretion of TNF-α and IL-6. Similar effects were observed in differentiated 3T3-L1 adipocytes incubated with either Bay-X-1005 or the selective LTB₄ receptor antagonist U-75302. Taken together, these findings indicate that the 5-LO pathway signals the adipose tissue low-grade inflammatory state and steatogenic potential in experimental obesity. The Journal of Immunology, 2010, 184: 3978–3987.

A state of mild subclinical inflammation in the adipose tissue has been associated with features of the metabolic syndrome, including obesity and insulin resistance. Unequivocal evidence has demonstrated that this state of chronic, “low-grade” inflammation is related to abnormal adipokine production and activation of proinflammatory signaling pathways in adipose tissue (1, 2). Moreover, the low-grade inflammatory state results in a deregulated release of free fatty acids (FFAs) from adipose tissue (1, 2). Accumulating data indicate that excessive release of adipokines and increased production of FFAs play an important role in the pathogenesis of obesity-related complications in peripheral tissues, including liver (3). In fact, one of the major metabolic consequences of obesity-driven inflammation and increased FFA efflux to the liver is hepatic steatosis, which is the accumulation of triglycerides (TGs) in the cytoplasm of hepatocytes (4, 5). Although generally asymptomatic, hepatic steatosis or fatty liver is no longer regarded as a neutral and innocent bystander but rather as a pre-morbid condition that increases the vulnerability of this organ to progress to steatohepatitis and to more advanced stages of liver disease (4, 5).

The 5-lipoxygenase (5-LO) pathway, which generates leukotrienes (LTs) from arachidonic acid, is one of the major proinflammatory systems in mammals (6, 7). Inflammatory stimuli elicit a sequence of events, including activation of the enzyme 5-LO and the accessory 5-LO activating protein (FLAP), a fatty acid transport protein that specifically binds and presents arachidonic acid to 5-LO and enhances the sequential oxygenation and dehydration of this FA into the highly unstable allylic epoxide LT₄. This epoxide is subsequently transformed either to LTB₄ via stereoselective hydration by LTA₄ hydrolase (LT₄H) or to LTC₄ through glutathione conjugation catalyzed by LTC₄ synthase (LT₄CS) (6, 7). LTB₄ is among the most potent chemoattractant mediators and represents a potential link between innate and adaptive immunological reactions (8). Within the arachidonate 5-LO pathway, the protein FLAP has recently been identified as an emerging target in metabolic disease. In fact, FLAP is over-expressed in the adipose tissue of patients with experimental animals.
with obesity and insulin resistance (8, 9). In addition, 5-LO products have pleiotropic actions on adipose fat accumulation and pancreatic function in mice (10, 11). Moreover, we recently identified a novel steatogenic role for 5-LO in the liver of obese ob/ob mice, through mechanisms involving the regulation of hepatic microsomal TG transfer protein activity and very low density lipoprotein-TG and apolipoprotein B secretion (12). However, the potential involvement of the 5-LO pathway in adipose tissue inflammation and adipocyte lipid dysfunction in experimental obesity has not been explored.

In the current investigation, we tested the influence of the 5-LO pathway in adipose tissue inflammation and lipid dysfunction. We first characterized the expression of key enzymes and receptors of this proinflammatory pathway in adipose tissue and isolated adipocytes and stromal vascular cells (SVCs). We also identified the most abundant 5-LO–derived eicosanoids in adipose tissue and explored the effects of these 5-LO products on NF-κB activity, on the production of inflammatory cytokines and chemokines by adipose tissue explants, and on lipid metabolism in isolated adipocytes. Finally, we assessed the effects of blocking the 5-LO pathway with a selective FLAP inhibitor in vivo in a mouse model of dietary obesity. Our findings support the hypothesis that the 5-LO pathway signals the adipose tissue low-grade inflammatory state and its steatogenic potential in experimental obesity.

Materials and Methods

Materials

Murine 3T3-L1 fibroblasts were purchased from the European Collection of Cell Cultures (Salisbury, U.K.). Arachidonic acid, LTβ4, LTβ2, U-75302, MK-571, the primary Ab against hormone-sensitive lipase (HSL), and LTβ2 and cytokeratin ET-746 Abs were purchased from German Chemical (Aubor, MI), TRZol was obtained from Invitrogen (Carlsbad, CA), and RNAqueous and DNA-free kits were from Ambion (Austin, TX). Sep Pak Plus C18 cartridges were from Waters Associates (Milford, MA). Primary Abs against AMP-activated protein kinase (AMPK), phospho-AMPK, and phospho-HSL were from Cell Signaling Technology (Beverly, MA). The primary F4/80 Ab was from Serotec (Oxford, U.K.). The ECL Detection System and secondary donkey anti-rabbit HRP Ab Technology (Beverly, MA). The primary F4/80 Ab was from Serotec (Oxford, U.K.). The ECL Detection System and secondary donkey anti-rabbit HRP Ab, were from GE Healthcare (Chalfont St. Giles, U.K.). Bay-X-1005 was kindly provided by Dr. R. Müller-Peddinghaus (Pharma Research Center, Bayer AG, Wuppertal, Germany). Oleic acid (9,10-3H(N)) was from Perkin Elmer (Waltham, MA).)

Isolation of adipocytes and SVCs

Adipocytes and SVCs were isolated from epididymal fat pads of C57BL6 mice. Epididymal tissue was excised, collected in cold carbogen-gassed Krebs-Ringer-bicarbonate buffer at pH 7.4 with 2% BSA, washed, and minced (using an ultraspec насадка) to a size suitable for suspension. Adipose tissue was then centrifuged at 500 g for 5 min to remove erythrocytes and other blood cells. Subsequently, tissue was digested in Krebs-Ringer-bicarbonate buffer with 1 mg/ml collagenase I at 37°C for 40 min with gentle shaking. The suspension was filtered through a 100-μm nylon mesh and centrifuged at 500 g for 5 min. Floating cells (adipocytes) were collected and washed, whereas pelleted cells (SVCs) were incubated with erythrocyte lysis buffer (NHCl, 155 mM; KHCO3, 10 mM; and EDTA, 0.1 mM) for 5 min and centrifuged. Adipocytes were cultured in carbogen-gassed DMEM with FBS (10%), glutamine (2 mM), penicillin (50 U/ml), streptomycin (5 mg/ml), and HEPES (20 mM). Cells were maintained at 37°C in a 5% CO2 atmosphere, and the medium was changed every 24 h.

Ex vivo experiments in adipose tissue explants

Adipose tissue explants were obtained from murine epididymal fat pads. Under sterile conditions, samples of adipose tissue were placed in a 96-well plate with Dulbecco’s PBS (DPBS) containing penicillin (100 U/ml) and streptomycin (100 mg/ml), prewarmed at 37°C. Connective tissue and blood vessels were removed by dissection before cutting the tissue into small pieces (<10 mg). Explants were washed with DPBS at 37°C by centrifugation for 1 min at 400 g to remove blood cells and pieces of tissue containing insufficient adipocytes to float. Thereafter, explants were cultured in DMEM with 10% FBS, 2% BSA, L-glutamine (2 mM), penicillin (50 U/ml), streptomycin (5 mg/ml), and 2% FA-free BSA. To assess FFA secretion, explants were cultured in 12-well plates (40 mg/well) in vehicle (<0.1% DMSO) or iso- proterenol (10 μM), with or without the presence of a 5-LO inhibitor (1 μM), for 1 h. FFA levels in supernatants were determined by the NEFA-C KIt (Wako Pure Chemicals, Osaka, Japan).

Experiments in 3T3-L1 adipocytes

3T3-L1 cells were maintained in DMEM containing 10% bovine calf serum, L-glutamine (4 mM), penicillin (50 U/ml), and streptomycin (50 mg/ml). To differentiate these cells into adipocytes, cells were seeded in 12-well plates (150,000 cells/well) 2 d after reaching confluence. Two days later, the medium was changed to DMEM with 10% FBS, 5 μg/ml insulin, 0.25 μg/ml dexamethasone, and 170 μg/ml isobutylmethylxanthine. After 2 d, cells were maintained in DMEM with 5 μg/ml insulin for an additional 3 d. Finally, the medium was replaced with DMEM containing only 10% FBS, L-glutamine, and penicillin-streptomycin. At least 90% of the cell population exhibited the adipocyte phenotype with evident accumulation of lipid droplets. Adipocytes were incubated with IL-1β (10 ng/ml; Peprotech, Rocky Hill, NJ) and vehicle (<0.1% DMSO), the FLAP inhibitor Bay-X-1005 (20 μM), the LTβ4 receptor antagonist U-75302 (1 μM), or the cysteinyl-LT receptor antagonist MK-571 (1 μM) for 24 h at 37°C. RNA was extracted using the RNAqueous kit, and real-time quantitative PCR was performed as described below.

FA uptake

FA uptake by isolated adipocytes was measured using the QBT™ Fatty Acid Uptake assay Kit (Molecular Devices, Sunnyvale, CA) according to the manufacturer’s instructions. The kit uses a dipyromethene boron difluoride (BODIPY)–dodecanoic fatty fluorescent analog that remains quenched until it is internalized by the cell. The BODIPY analog is a known substrate for FA transporters because its uptake by adipocytes can be nonlabeled FAs (13, 14). Briefly, 50,000 cells/well were plated in 96-well black fluorescence plates and different [3H]-FA uptake for 30 min at 37°C with FBS-containing media, and the uptake by adipocytes can be completed by nonlabeled FAs (13, 14). Briefly, 50,000 cells/well were plated in 96-well black fluorescence plates and exposed to vehicle (<0.5% ethanol), LTB4 (100 nM), or LTD4 (100 nM) for 30 min at 37°C in a 5% CO2 atmosphere. In some experiments, cells were pretreated for 10 min with U-75302 (1 μM) or MK-571 (1 μM). Following the incubation period, QBT dye was added, and the plate was read in a Fluostar Optima fluorescence plate reader (BMG Labtech, Offenburg, Germany) at 490-nm excitation and 520-nm emission wavelengths every 20 s for 60 min. In addition, FA uptake was determined using a radioactive FA by the rapid filtration method (15, 16). Briefly, 50,000 primary adipocytes were incubated in 1 ml DMEM containing 2% FA-free BSA and different [3H] oleic acid concentrations (oleate/BSA ratios, 0.25:1 and 2:1) for up to 30 min at 37°C. At different time points, FA uptake was stopped by the addition of 5 ml ice-cold stop solution (400 μM phloretin, 0.1% BSA). Subsequently, cells were filtered, and the radioactivity was counted by a liquid scintillation analyzer. Insulin (160 nM) and BSA-pherorin (200 μM) were used as positive and negative controls, respectively.

NF-κB activity

NF-κB activity was assessed in nuclear extracts from adipose tissue explants cultured in 6-well plates (200 mg/well) in the presence of vehicle (0.5% ethanol), LTB4 (100 nM), or LTD4 (100 nM) for 2 h. Nuclear extracts were prepared using the Nuclear Extraction Kit from Millipore (Bedford, MA), following the manufacturer’s protocol with slight modifications. Briefly, 200 mg adipose tissue was homogenized in 1 ml cold cytoplasmatic lysis buffer in a Dounce homogenizer, and the suspension obtained was filtered through a 250-μm nylon mesh and centrifuged for 5 min at 250 g at 4°C. The pellet obtained was resuspended in ice-cold cytoplasmatic lysis buffer and lyzed using a small-gauge needle (26 G). Thereafter, lysates were placed on an orbital shaker for 1 h at 4°C and centrifuged at 8,000 g for 20 min at 4°C to obtain the nuclei. Nuclear extracts proteins were quantified by the MicroBCA™ Protein Assay Kit (Pierce, Rockford, IL), and NF-κB activity was determined by the NF-κB EZ-Transcription Factor Assay (Millipore). A total of 5 μg protein/nuclear well was assayed, and the binding of NF-κB transcription factor subunits p50 and p65 was detected in a microplate luminometer (Fluostar Optima).

Cytokine array

Cytokines secreted by adipose tissue were screened using the RayBio Mouse Inflammation Antibody Array 1 (RayBiotech, Norcross, GA), which allows the simultaneous detection of 40 different adipokines, cytokines, and chemokines related to inflammation. Briefly, 200 mg adipose tissue was exposed to vehicle (0.5% ethanol) or LTB4 (1 μM) for 24 h at 37°C in 5% CO2 atmosphere. In some experiments, cells were pretreated for 10 min with LTB4 (1 μM). At the end of the incubation period, supernatants were collected, supplemented with protease inhibitors, and frozen at –80°C until analysis. A total of 1 ml supernatant of cultured adipose tissue was added to the Ab-coated membrane and incubated according to
the manufacturer’s instructions. The intensity of signals was quantified by densitometry, and the positive control was used to normalize the results from different membranes. Cytokine production in adipose tissue was also assessed in fat explants cultured in 12-well plates (40 mg/well) in the presence of vehicle (<0.5% ethanol), LTB4 (1, 10, and 100 nM), or LDT4 (1, 10, and 100 nM) for 24 h. TNF-α, IL-6, and MCP-1 levels in the supernatants were determined by specific enzyme immunoassay (EIA) kits from R&D Systems (Minneapolis, MN). Some experiments were performed in the presence of Bay X-1005 (0.5 μM) for 48 h.

Experimental models of obesity-induced hepatic steatosis

Male C57BL6 mice were fed a high-fat diet (HFD) (45% kcal from fat, Harland Teklad, Madison, WI) for 16 wk. After 12 wk of feeding, animals were randomly distributed into two groups that received a daily dose of Bay X-1005 (n = 10, 100 mg/kg body weight, by mouth) or placebo (n = 10, 0.5% carboxymethylcellullosellose, by mouth) for 4 wk. At the end of the intervention period, mice were euthanized, blood was collected, and serum obtained by centrifugation at 3000 g for 10 min. Epipdydal adipose tissue and liver were excised; rinsed in DPBS; fixed in 10% formalin; and embedded in paraffin or placed in optimal cutting temperature compound, immersed in cold 2-methylbutane on dry ice, and kept at −80°C. In addition, portions of adipose tissue were snap-frozen in liquid nitrogen for further analysis. Samples of adipose tissue, liver, and serum were also collected from groups of C57BL6 and olibo (B6.V-Lepob/J) mice (The Jackson Laboratory, Bar Harbor, ME) fed on a control diet (11% kcal from fat). All animal studies were conducted in accordance with the criteria of the Investigation and Ethics Committee of the Hospital Clinic (University of Barcelona, Barcelona, Spain) and the European Community laws governing the use of experimental animals.

Glucose and insulin tolerance tests

To perform the glucose tolerance test, overnight-fasted mice received an i.p. injection of 20% glucose (2 g/kg body weight), and blood samples were collected from the tail vein 0, 15, 22.5, 30, 45, 60, 90, and 120 min later for serum glucose measurement. Liver sections were stained with Oil Red O in 60% isopropanol for 10 min. Epididymal adipose tissue and liver were excised and stained with hematoxylin. Sections were visualized at magnification ×200, and the results expressed as the percent of F4/80-expressing cells. At minimum, 18 independent fields per sample were evaluated.

Analysis of eicosanoids by EIA and reversed phase-HPLC

LBT4 and Cys-LT levels were determined in samples of adipose tissue of olibo mice obtained from wild-type and olibo animals. Each sample was indi
dividually homogenized with an Ultra-Turrax T25 (Ika, Werke Staufen, Germany) in 5 ml cold MeOH-H2O (65/35, v/v) and extracted with Sep Pak C18 columns, prior to EIA analysis. 5-L0 products were also determined by reversed phase (RP)-HPLC analysis. Briefly, adipose tissue explants from olibo mice were incubated with 5 ml Ringer buffer containing arachidonic acid (50 μM) and ionophore A23187 (5 μM) for 90 min at 37°C. An internal standard, PGB2, was added to the samples, and the tissue was extracted as explained above. The final eluate was collected, dried under a stream of N2, and dissolved in solvent A (methanol/H2O/acetic acid; 65:35:0.01, v/v/v, pH 5.7). Reversed phase-HPLC (RP-HPLC) analysis was performed with a C18 column (Spherisorb ODS, 5 μm, 4.6 × 250 mm; Supelco, Bellefonte, PA). After sample loading, the column was developed with a solvent gra
dient consisting of 36 min of solvent A, 1 min of gradient to 45% (v/v) solvent B (methanol/acetic acid; 100:0.01, v/v), and 2 min of 45% (v/v) solvent B at a flow rate of 1.2 ml/min. The eluate from the column was monitored by UV absorption at 234 and 270 nm, and identification of the compounds was performed by comparing peak retention times with that of the standards.

Gene expression profiling

Total RNA was isolated using the TRIzol reagent. RNA concentration was assessed in a UV spectrophotometer, and its integrity was tested in a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Samples were treated with DNase (DNA-free) and reverse-transcribed with the High-Capacity cDNA Ar
chive Kit (Applied Biosystems, Foster City, CA). PCR amplification of 5-L0, FLAP, LTC4S, LTA4H, 12/15-LO, LTB4 type 1 (BLT1) and type 2 (BLT2) receptors, and cytokinin-LT type 1 (Cys(LT1) and type 2 (Cys(LT2) receptors was performed with specific oligonucleotides (Table I) (19, 20). The specificity of primers was confirmed in the GenBank database, using the basic local alignment search tool, and by direct sequencing of the amplified PCR products in an ABI Prism 3130xl Genetic Analyzer using a Big Dye Terminator (version 3.1) Cycle Sequencing Kit (Applied Biosystems). PCR products were analyzed by electrophoresis in 1.5% agarose gels and visualized by ethidium bromide staining, using a 100-bp DNA ladder (Invitrogen) as an m.w. marker.

Quantitative analysis of gene expression was performed by real-time PCR in an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Ready-to-use primer and probe sets (TaqMan Gene Expression Assays; Applied Biosystems) were used to quantify acetyl-CoA carboxylase (ACC) (ID: Mm03134285_m1), adiponectin (ID: Mm0056425_m1), fatty acid synthase (FASN) (ID: Mm00662319_m1), FLAP (ID: Mm00821001_m1), LCT4S (ID: Mm00521864_m1), GLUT-4 (ID: Mm00436615), IL-6 (ID: Mm00446461-90_m1), insulin receptor substrate-1 (IRS-1) (ID: Mm1278327_m1), lipoprotein lipase (LPL) (ID: Mm00434764_m1), MCP-1 (ID: Mm0044124_2_m1), peroxisome proliferator-activated receptor (PPARs) (ID: Mm0044-0939_m1), PPARy (ID: Mm00449045_m1), resistin (ID: Mm00445641_m1), and TNF-α (ID: Mm0043258_m1) gene expression, using β-actin (ID: Mm00607939_s1) as an endogenous control. PCR products were analyzed with Sequence Detector Software (version 2.1; Applied Biosystems). The amount of target gene, normalized to β-actin and relative to a calibrator, was de
termined by the arithmetic Equation 2^−ΔΔCt described in the comparative Cm method.

AMPK and HSL phosphorylation

Total proteins from adipose tissue and adipocytes were extracted in ho
mogenizing buffer containing 50 mM HEPES, 20 mM β-glycerophosphate, 2 mM EDTA, 1% Igepal, 10% glycerol, 1 mM MgCl2, 1 mM CaCl2, 150 mM NaCl, 10 mM NaF, 20 mM sodium pyrophosphate decahydrate, 2 mM sodium orthovanadate, and protease inhibitors. Homogenates were incubated on ice for 15 min with frequent vortexing. Thereafter, homogenates were centrifuged at 16,100 g for 20 min at 4°C, and supernatants were collected. AMPK, phosphoThr(172)-AMPK, HSL, and phosphoSer(563)-HSL protein expression was analyzed by Western blot. A total of 80 μg adipose tissue protein (determined by the Micro BCA Protein Assay Kit; Pierce) was resuspended in SDS-containing Laemmli sample buffer, heated for 5 min at 95°C, and separated by SDS-PAGE (12% and 10% for AMPK and HSL, respectively). Proteins were electrophoresed for 120 min at 100 V at 4°C onto polyacrylamide gels and transferred to a nitrocellulose membrane and 

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dry milk. Blots were washed three times for 5 min each with 0.1% T-TBS and subsequently treated overnight at 4°C with primary rabbit anti-mouse Abs against phospho-AMPK or phospho-HSL (dilution 1:1,000) in 0.1% T-TBS, and bands were visualized using an ECL Detection System. Total AMPK and HSL proteins were detected after blot stripping. Briefly, membranes were incubated 20 min at 50°C with Tris/HCl, pH 6.7; 100 mM β-mercaptoethanol; and 2% SDS. After washing, membranes were treated overnight at 4°C with rabbit antibody Abs against AMPK or HSL (1:2,000) in 0.1% T-TBS containing 5% nonfat dry milk.

Statistical analysis of the results was performed by one-way or two-way ANOVA and by unpaired Student’s t test. Results are expressed as mean ± SEM, and differences were considered significant at p < 0.05.

Results

In a first series of experiments, we examined the expression of key components of the 5-LO pathway in adipose tissue by PCR (Table I). As shown in Fig. 1A, whole adipose tissue from lean wild-type mice expressed all enzymes necessary for LT biosynthesis (5-LO, FLAP, LTA4H, and LTC4S), as well as all four LT receptors (BLT1, BLT2, CysLT1, and CysLT2). Adipose tissue also expressed 12/15-LO (Fig. 1A). Separation of the adipose tissue into the adipocyte and SFC fractions demonstrated that both fractions expressed the enzymes and receptors involved in the 5-LO pathway (Fig. 1B, lanes 2 and 3). To examine whether the 5-LO pathway is modulated by obesity, we analyzed gene expression in whole adipose tissue from obese mice. As shown in Fig. 1C, FLAP mRNA levels were significantly increased in adipose tissue from ob/ob mice, compared with that from lean wild-type mice. Consistent with the presence of this FLAP overexpression, adipose tissue from obese mice had increased levels of the proinflammatory 5-LO product LTB4, without changes in Cys-LT levels (Fig. 1D). These differences were more evident following the incubation of adipose tissue samples with exogenous arachidonic acid (50 μM) and stimulated with calcium ionophore (5 μM) (data not shown). To profile the eicosanoids from the 5-LO pathway produced by obese adipose tissue, we performed RP-HPLC analysis. As shown in Fig. 1E, products eluting with synthetic LTB4 were detected after the incubation of adipose tissue with arachidonic acid (Fig. 1E, lower panel). LTB4 was not detected in these incubations, although a small peak eluted with synthetic LTC4 (Fig. 1E, lower panel). The addition of exogenous arachidonic acid was needed because the picogram amounts of endogenous 5-LO products isolated from adipose tissue precluded their direct detection by RP-HPLC (Fig. 1E, middle panel). Together, these findings indicate the presence of increased FLAP expression and LTB4 levels in adipose tissue from obese mice.

Because the presence of low-grade inflammation is linked to the transcriptional activation of NF-kB, we next examined the effects of the most representative 5-LO products on NF-kB activity. As shown in Fig. 2A, nanomolar concentrations of LTB4 were able to induce, in a concentration-dependent manner, NF-kB activity in adipose tissue, as revealed by a significant increase in the nuclear translocation of the p65 subunit. LTD4 was also able to induce NF-kB activity in the adipose tissue, but to a lower extent (Fig. 2A). Similar results were obtained with the nuclear translocation of the NF-kB p50 subunit (data not shown). Given that activation of NF-kB stimulates the production of proinflammatory cytokines, we next assessed by EIA the effects of LTB4 and LTD4 on the production of MCP-1, IL-6, and TNF-α by adipose tissue. As shown in Fig. 2B, the addition of LTB4, especially at 10-nM concentration, induced the production of these adipokines characteristic of inflamed adipose tissue, an effect that was not reproduced by the addition of LTD4 to the explants. Because LTB4 influences the secretion of adipokines by adipose tissue, we analyzed the profile of adipokines secreted by this tissue either under baseline conditions or following the addition of this 5-LO product. Under baseline conditions, among the 40 cytokines tested in a cytokine array test, 24 were secreted into the medium by adipose tissue (Fig. 2C). The most abundant adipokines secreted by resting adipose tissue were MCP-1, MIP-1γ, and IL-6 (Fig. 2C). Remarkably, following prolonged incubation (24 h) with LTB4 there was a significant induction of cytokine and chemokine release by adipose tissue (Fig. 2D). For instance, LTB4 induced a 3- and 6-fold increase in IL-6 and MCP-1 secretion, respectively (Fig. 2D). In addition, LTB4 induced the release of eotaxin-2, TNFRSF8L, and IL-4, which were not detected under resting conditions (Fig. 2D). These changes in adipokine secretion appeared to be LTB4 specific because they were prevented by the addition of the BLT1 receptor antagonist U-75302 (Fig. 2D). Interestingly, the results of the cytokine array were consistent with those obtained by EIA.

Given that the state of low-grade inflammation influences lipid metabolism in the adipocyte, in the next series of experiments we examined the role of the 5-LO pathway in FFA uptake and release by adipocytes and adipose tissue explants. For this purpose, primary

Table I. PCR primer sequences, product sizes, and gene identification numbers

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*Previously reported in Horillo et al. (19).
"Previously reported in Hui et al. (20).
adipocytes were incubated with nanomolar concentrations of 5-LO products and subsequently exposed to a BODIPY-labeled fluorescent FA, and the uptake was monitored in the assay. As shown in Fig. 3A, FFA uptake by adipocytes was significantly decreased by LTB4. Furthermore, the effects of LTB4 on the uptake of FFAs were partially prevented by the addition of the BLT1 receptor antagonist U-75302 (Fig. 3A). In contrast, FA uptake by adipocytes was not significantly modified by LTD4 and its selective receptor antagonist MK-571 (Fig. 3A). The results obtained in the BODIPY-labeled fluorescent FA uptake assay were validated using radioactive-labeled oleic acid. As shown in Fig. 3B, addition of LTB4 to primary adipocytes significantly reduced [3H] oleic acid uptake in a time-dependent manner. Interestingly, the inhibitory effect of this 5-LO product was more prominent at 30 s of incubation when the uptake of radioactive-labeled FA was maximal. The uptake of [3H] oleic acid was also concentration dependent and reached a maximum when the oleate/albumin molar ratio was 2:1 (data not shown). Consistent with the effects of LTB4 on lipid transport, 5-LO inhibition was able to abrogate the lipolysis induced by the β-adrenergic agonist isoproterenol in adipose tissue explants (Fig. 3C). In these experiments, 5-LO inhibition induced a 65% reduction in the levels of LTB4 in the adipose tissue explants (Fig. 3C, inset). Together, these results support the notion that in addition to the inflammatory response, the 5-LO pathway is also involved in the dysregulation of lipid metabolism in adipose tissue.

To investigate the relevance of these in vitro findings in vivo, we next assessed the effects of blocking the 5-LO pathway with a selective and potent FLAP inhibitor in a mouse model of dietary obesity. At 12 wk under an HFD (45% kcal from fat), and in comparison with a group receiving a control diet (11% kcal from fat), mice showed increased adiposity characterized by statistically significant differences in terms of body weight, white adipose tissue (WAT) weight, serum glucose and insulin levels, and glucose tolerance (Fig. 4A). In this dietary model of obesity, we observed elevated FLAP expression.

FIGURE 1. Characterization of key components of the 5-LO pathway in adipose tissue. A, Representative PCR analysis of mRNA expression for 5-LO, FLAP, LTC4S, LTA4H, and 12/15-LO, as well as the two LTB4 (BLT1 and BLT2) and the two cys-LT (CysLT1 and CysLT2) receptors in adipose tissue from lean wild-type mice. A 100-bp DNA ladder was used as a size standard (M). B, Representative PCR analysis of 5-LO, FLAP, LTC4S, LTA4H, BLT1, BLT2, CysLT1, CysLT2, and GADPH mRNA expression in adipocytes (lane 2) and SVCs (lane 3) from lean wild-type mice. Lanes 1 (water) and 4 (Raw 264.7) were used as negative and positive controls, respectively. C, FLAP expression in adipose tissue samples from wild-type (lean, n = 5) and ob/ob (obese, n = 5) mice was assessed by real-time PCR and normalized to β-actin levels. D, LTB4 and cys-LT levels in whole adipose tissue samples from lean and obese mice were assessed by EIA after solid extraction with Sep-Pak C18 columns. Results are expressed as mean ± SEM. *p < 0.05 versus wild-type (C, D). E, Representative RP-HPLC chromatograms of 5-LO products in WAT from obese mice. Materials were extracted with Sep-Pak C18 cartridges and injected into an RP-HPLC system, as described in Materials and Methods. The upper panel shows a representative profile of synthetic standards. The middle panel shows a representative profile of materials from unstimulated WAT explants. The lower panel shows a representative profile of materials from WAT explants incubated with arachidonic acid (50 μM) and A23187 (5 μM) for 90 min at 37˚C. PGB2 (50 ng) was added as an internal standard. Arrows indicate the retention times of appropriate standards. AA, arachidonic acid.
in adipose tissue, which was paralleled with more F4/80-positive cells, indicative of more macrophages in the adipose tissue, and an increased area stained with Oil Red O in the liver, indicative of accelerated hepatic steatosis (Fig. 4B). The expression of LTF4S was also found to be increased in adipose tissue from HFD-fed mice (data not shown). Most importantly, the administration of the FLAP inhibitor Bay-X-1005 to these mice exerted a remarkable protective effect, inducing a significant reduction in F4/80 immunostaining in adipose tissue as well as a significant reduction of the area stained with Oil Red O in the liver (Fig. 5A). As shown in Fig. 5B, inhibition of the 5-LO pathway with the selective FLAP inhibitor Bay-X-1005 achieved a 60% reduction in the formation of 5-LO products (LTB4 and Cys-LTs) in the adipose tissue of obese mice. Furthermore, the anti-inflammatory and antisteatotic effects exerted by FLAP inhibition were associated with markedly lower circulating levels of FFAs and insulin and with an improvement in insulin sensitivity (Fig. 5B). No changes in body, liver, and adipose tissue weight or serum biochemistry values were observed in mice receiving Bay-X-1005, compared with those receiving placebo (Table II).

To understand the molecular mechanisms underlying the protective effects exerted by the FLAP inhibitor Bay-X-1005, we assessed changes in the activity of AMPK, a key fuel-sensing enzyme that responds to changes in cellular energy state. As shown in Fig. 6A (upper panel), Western blot analysis revealed that AMPK phosphorylation was significantly increased in adipose tissue from mice treated with Bay-X-1005. Consistent with this finding, the addition of LTB4 to isolated adipocytes resulted in a reduction in AMPK phosphorylation (Fig. 6A, middle panel). In addition, Western blot analysis also showed that the activity of HSL, the enzyme responsible for the secretion of FFAs from adipocytes, was markedly decreased in adipose tissue from mice treated with Bay-X-1005 (Fig. 6A, lower panel). Analysis by quantitative real-time PCR of a selection of relevant genes in glucose/lipid metabolism revealed significant downregulation of adipokines under the control of AMPK activity such as TNF-α and IL-6 (Fig. 6B). This finding was further confirmed by measuring the levels of these two cytokines in the medium, using ELISA (Fig. 6C). Consistent with these findings, TNF-α and IL-6 were downregulated by incubation of 3T3-L1 adipocytes, either with Bay-X-1005 or with the selective BLT1 receptor antagonist U-75302 (Fig. 6D). Incubation of 3T3-L1 adipocytes with the selective CysLT1 receptor antagonist MK-571 also decreased TNF-α and IL-6 expression (from 1.05 to 0.15 and from 1.15 to 0.10, respectively). In contrast, PPARγ, which is a nuclear receptor regulating FA metabolism, was significantly upregulated in adipose tissue samples from mice receiving Bay-X-1005 (Fig. 6E). No changes in MCP-1, adiponectin, resistin, PPARγ, LPL, ACC, FASN, IRS-1, or GLUT-4 were observed (Fig. 6B, 6D, 6E).

**Discussion**

The current study provides evidence for the expression of the 5-LO pathway in adipose tissue and outlines a previously unrecognized role for this pathway in mediating the obesity-associated low-grade inflammation.
inflammatory state in adipose tissue and adipocyte dysfunction. Our findings demonstrate that lipid mediators derived from the 5-LO pathway regulate inflammation (i.e., NF-κB activity and the secretion of the proinflammatory adipokines MCP-1, IL-6, and TNF-α) in adipose tissue, as well as directly control FA metabolism in adipocytes. At the in vivo level, we demonstrate that pharmacological inhibition of the 5-LO pathway by means of a selective FLAP inhibitor in a mouse model of dietary obesity results in decreased macrophage infiltration in the adipose tissue, in addition to reduced circulating levels of FFAs, which are essential for the progression of obesity-associated complications such as insulin resistance and hepatic steatosis. Beneficial responses to inhibition of the 5-LO pathway appear to be mediated by induction of AMPK activity in adipose tissue, with concomitant regulation of HSL activity and TNF-α and IL-6 secretion, direct targets of AMPK in this organ. To our knowledge, this is the first study reporting the impact of 5-LO–derived lipid mediators on adipose tissue inflammation and lipid dysfunction.

Our study provides evidence that the 5-LO pathway is involved in adiposity, namely, regulation of the inflammatory process and lipid dysfunction in adipose tissue. Indeed, we provide data demonstrating that 5-LO products significantly enhanced nuclear translocation of p50 and p65 subunits and therefore induced NF-κB activity in adipose tissue. Although activation of this nuclear receptor by LTs has been reported in monocytes and vascular smooth muscle cells (21, 22), we believe this is the first time that an NF-κB–dependent signaling pathway has been demonstrated for 5-LO products in adipose tissue. Transcriptional activation of NF-κB induces low-grade inflammation and stimulates production of proinflammatory molecules, leading to insulin resistance and hepatic steatosis (23, 24). In our study, we also detected an induction of the secretion of several cytokines and chemokines to the medium in adipose tissue explants in which NF-κB activity was increased by 5-LO products. Among these molecules, we detected adipokines directly involved in the pathogenesis of the metabolic syndrome, such as MCP-1, IL-6, and TNF-α, which connect adipose tissue inflammation with insulin resistance and hepatic steatosis (25). Our findings also provide evidence that the 5-LO pathway, in addition to contributing to mounting inflammation in adipose tissue, contributes to regulating the flux of FFAs from adipose tissue. According to the conventional explanation, increased adipocyte mass and increased lipolysis (i.e., hydrolysis of TGs) contribute to elevated circulating levels of FFAs, which play a critical role in obesity-associated insulin resistance and hepatic steatosis through increased HSL activity (26). In our study, LTB₄, but not LTD₄, reduced the uptake of FFAs by adipocytes, whereas inhibition of the 5-LO pathway decreased HSL activity and the lipolytic rate of adipose tissue. Similar effects on lipolytic activity and HSL phosphorylation were previously reported by Gowri et al. (27), using masprocol, a nonselective LO inhibitor. The physiological consequences of these changes in adipose tissue function were corroborated in vivo by the observation that inhibition of the 5-LO pathway reduced circulating FFA concentrations and alleviated insulin resistance and hepatic steatosis in
mice with dietary obesity. These findings are consistent with the antisteatotic effects exerted by a 5-LO inhibitor in a murine model of obesity-induced hepatic steatosis (12).

The results of the current study also provide a mechanism by which the 5-LO pathway may exert regulatory effects on adipose tissue function. According to our data, involvement of the 5-LO pathway in adipose tissue inflammation and lipid dysfunction is linked to the activation of AMPK, which is a key regulatory component of energy homeostasis in various cell types, including adipocytes (28, 29). AMPK responds to changes in the cellular energy state; thus, when the AMP/ATP ratio is increased, this enzyme is phosphorylated and becomes active to restore energy levels by inhibiting ATP-consuming pathways and activating ATP-producing pathways (28, 29). In our experiments, inhibition of the 5-LO pathway with a FLAP inhibitor resulted in an induction of AMPK phosphorylation in adipose tissue. Conversely, the 5-LO product LTB4 reduced AMPK phosphorylation in adipocytes. The mechanisms underlying this finding are not fully delineated, but our results are consistent with previous reports demonstrating the inhibition of AMP production by LTB4 (30, 31). In addition, the AMPK pathway has profound effects on the regulation of lipid metabolism. In our study, AMPK activation induced by FLAP inhibition was accompanied by a decrease in the activity of HSL, which controls the release of FFAs from adipose tissue (28, 29). Furthermore, FLAP inhibition downregulated the

FIGURE 5. Protective effects of FLAP inhibition in mice with dietary obesity. A, Histomorphometrical analysis of adipose tissue sections stained with F4/80 and liver sections stained with Oil Red O from mice with diet-induced obesity treated with placebo (n = 10) or with the FLAP inhibitor Bay-X-1005 (100 mg/kg body weight) (n = 10) for 4 wk (original magnification ×200). B, Effects of Bay-X-1005 on 5-LO production, serum FFA, and insulin levels and insulin sensitivity in mice with HFD-induced obesity. Levels of 5-LO products in adipose tissue samples were assessed by EIA after solid extraction with Sep-Pak C₁₈ columns. Insulin tolerance tests were assessed by calculating the area under the curve after insulin injection. Results are expressed as the mean ± SEM. *p < 0.05; **p < 0.001 versus mice treated with placebo. AUC, area under the curve.

FIGURE 4. Increased adiposity, altered insulin sensitivity, increased FLAP expression, and inflammation in adipose tissue and augmented hepatic steatosis in mice with dietary obesity. A, Body and epididymal WAT weight, serum glucose and insulin levels, and glucose tolerance tests in mice fed with either an HFD (45% kcal from fat, n = 10) or a control diet (n = 8) for 12 wk. B, FLAP expression in adipose tissue and histomorphometrical analysis of adipose tissue sections stained with F4/80 (original magnification ×200) and liver sections stained with Oil Red O (original magnification ×200) from mice fed an HFD or a control diet. Results are the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 versus mice receiving a control diet.
expression and diminished the secretion of IL-6 and TNF-α, two adipokines involved in insulin resistance and hepatic steatosis and directly modulated by AMPK (32). Similar reductions of IL-6 and TNF-α release have been demonstrated during the incubation of adipose tissue explants with AICAR, a synthetic AMPK activator (32).

The 5-LO pathway catalyzes the oxygenation of arachidonic acid into a series of proinflammatory lipid mediators called LTs (6, 7). In this study, we detected constitutive expression of all the enzymes implicated in the formation of LTB₄, cyst-LTs, and 5-hydroxyeicosatetraenoic acid in adipose tissue, as well as in the adipocyte and SVC fractions isolated from this tissue. Moreover, in these samples we detected expression of the two receptors recognizing LTB₄ (i.e., BLT1 and BLT2) and the two receptors recognizing cyst-LTs (i.e., CysLT1 and CysLT2). Consistent with previous studies showing the presence of FLAP and 5-LO protein expression in adipocytes and the induction of their expression in obese adipose tissue (8, 9), we report in this paper that FLAP expression and LTB₄ levels increase with obesity, suggesting a potential novel function for the 5-LO pathway in obesity-associated inflammatory and metabolic changes in adipose tissue. It should be pointed out that we found similar endogenous levels of LTB₄ and cyst-LTs in lean adipose tissue, although LTB₄ but not cyst-LTs, levels significantly increased in adipose tissue from obese mice. We should also mention that the levels of 5-LO products in adipose tissue detected in our study by both ELA and RP-HPLC analysis were in the picogram range per milligram of tissue. These levels are similar to those reported previously in other tissues by Zhao et al. (33), in mouse aortas (0.3 pg/mg tissue); by Qiu et al. (34), in human carotid plaques (2.2 pg/mg tissue); and by López-Parra et al. (12), in mouse liver (0.6 pg/mg tissue). These data, together with the finding that nanomolar concentrations of 5-LO products were able to induce NF-kB activity and cytokine production (i.e., MCP-1, IL-6, and TNF-α) in adipose tissue, strongly support the concept that 5-LO products, mainly LTB₄, are involved in the inflammatory state of this tissue in obesity.

To summarize, in the current study we characterize the 5-LO pathway, a potent proinflammatory pathway of the arachidonic acid cascade, as a novel target in prevention of the low-grade inflammatory state in adipose tissue of mice with dietary obesity. In addition, we provide data supporting a role for the 5-LO pathway in signaling the dysfunction of lipid metabolism in adipose tissue that may represent a link between obesity, inflammation, and hepatic steatosis.

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** FLAP inhibition modulates AMPK activity. A. AMPK phosphorylation was determined by Western blot in adipose tissue samples from mice treated with placebo (n = 10) or Bay-X-1005 (n = 10) (upper panel) and in adipocytes exposed to increasing concentrations of LTB₄ (0, 0.1, and 1 μM) for 30 min at 37°C in a 5% CO₂ atmosphere (middle panel). HSL phosphorylation was determined by Western blot in adipose tissue samples from mice treated with either placebo or Bay-X-1005 (lower panel). The densitometric analysis of phosphorylated AMPK/total AMPK and phosphorylated HSL/total HSL ratios is shown in the right panels. B. The expression of TNF-α, IL-6, MCP-1, adiponectin, and resistin in adipose tissue samples from mice treated with placebo (n = 10) or Bay-X-1005 (n = 10) was determined by real-time PCR. C. Adipose tissue explants from lean wild-type mice were incubated in the presence of vehicle (0.5% ethanol) or Bay-X-1005 (30 μM) for 48 h. The levels of TNF-α and IL-6 in the supernatants were determined by specific ELISAs. Results are expressed as the mean ± SEM of two different experiments performed in duplicate. D. The expression of adipokines in 3T3-L1 adipocytes incubated with vehicle, Bay-X-1005 (20 μM), or U-75302 (1 μM) for 24 h was assessed by real-time PCR. E. The expression of genes involved in lipid and glucose metabolism (i.e., PPARα, PPARγ, LPL, ACC, FASN, IRS-1, and GLUT-4) was determined by real-time PCR in adipose samples from mice treated with placebo or Bay-X-1005. Results are expressed as the mean ± SEM. *p < 0.05; **p < 0.01 versus placebo (A, B, E) or versus vehicle (C, D). Adip, adiponectin.
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Disclosures

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