Fatty acid binding protein 4/aP2-dependent BLT1R expression and signaling

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Abstract Previous studies have shown that reduced levels of the adipocyte fatty acid binding protein (FABP)4 (AFABP/aP2), result in metabolic improvement including potentiated insulin sensitivity and attenuated atherosclerosis. Mechanistically, pharmacologic or genetic inhibition of FABP4 in macrophages upregulates UCP2, attenuates reactive oxygen species (ROS) production, polarizes cells toward the anti-inflammatory M2 state, and reduces leukotriene (LT) secretion. At the protein level, FABP4 stabilizes LTÀ toward chemical hydrolysis, thereby potentiating inflammatory LTC4 synthesis. Herein, we extend the FABP4-LT axis and demonstrate that genetic knockout of FABP4 reduces expression of the major macrophage LT receptor, LTÀ receptor 1 (BLT1R), via a ROS-dependent mechanism. Consistent with inflammation driving BLT1R expression, M1 polarized macrophages express increased levels of BLT1R relative to M2 polarized macrophages and treatment with proinflammatory lipopolysaccharide increased BLT1R mRNA and protein expression. In FABP4 knockout macrophages, silencing of UCP2, increased ROS levels and led to increased expression of BLT1R mRNA. Similarly, addition of exogenous H2O2 upregulated BLT1R expression, whereas the addition of a ROS scavenger, N-acetyl cysteine, decreased BLT1R levels. As compared with WT macrophages, LTÀ-BLT1R-dependent JAK2-phosphorylation was reduced in FABP4 knockout macrophages. In summary, these results indicate that FABP4 regulates the expression of BLT1R and its downstream signaling via control of oxidative stress in macrophages.—Hertzel, A. V., H. Xu, M. Downey, N. Kvalheim, and D. A. Bernlohr. Fatty acid binding protein 4/aP2-dependent BLT1R expression and signaling. J. Lipid Res. 2017, 58: 1354–1361.

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The prevalence of obesity and its associated pathologies, including insulin resistance, have increased in the past several decades (1–4). Numerous studies have implicated adipose tissue-recruited immune cells as a critical component of insulin resistance and significant attention has been focused on macrophages, whose malleable nature allows for alternative states of activation dependent on the local environment (5, 6). With the development of obesity, adipose tissue acquires a chronic low-grade proinflammatory state, along with increased reactive oxygen species (ROS), dysfunctional mitochondria, and whole body insulin resistance (7–9).

Leukotrienes (LTs) are lipid mediators derived from arachidonic acid via the actions of the 5-lipoxygenase pathway (10–13). LTÀ hydrolase activity results in the production of LTÀ, whereas LTC4 synthase initiates the glutathione conjugation of LTÀ to produce LTÁ, LTD4, and LTE4 (14, 15). These lipids bind with high affinity to a subfamily of G protein-coupled receptors, thus initiating signal cascades to direct functional consequences, typically of a proinflammatory nature (16–18). Lipopolysaccharide (LPS), zymosan, and calcium ionophores have all been demonstrated to increase LTÀ production and secretion in multiple cell types, including neutrophils and macrophages (19, 20). LTÀ binds with high affinity to the LTÀ receptor 1 (BLT1R), a member of the G protein-coupled receptor family (16, 21, 22). A second LTÀ receptor, BLT2R, binds LTÀ with a 20-fold higher Kd (21, 22). Several other lipoxygenase products have been demonstrated to bind to BLT2R with the thromboxane synthase product, 12-HHT, having a 10-fold higher affinity than LTÀ; thus 12-HHT is thought to be an endogenous ligand (22, 23). LTÀ is involved in host defense following infection in a number of ways, including increasing the recruitment of immune cells via increased chemotaxis, calcium mobilization, and...
altered gene expression, ultimately leading to an inflammatory state (13, 24, 25). Additionally, LTB$_4$ stimulates neutrophil secretion of proteins, including lysozyme and matrix metalloproteinases (26, 27). In macrophages, LTB$_4$ has been demonstrated to increase proliferation and play a role in phagocytosis (28–30). Chronic LTB$_4$ proinflammatory signaling has been implicated in several pathological states including atherosclerosis, asthma, arthritis and cancer (16, 24, 31–33).

On a high-fat diet, murine adipose tissue accumulates M1-activated macrophages, thereby producing an inflammatory state (34–36). However, BLT1R knockout mice demonstrate an anti-inflammatory adipose phenotype and are resistant to pathologies associated with a high-fat diet. Without BLT1R, adipose tissue accumulates M2 macrophages with a corresponding decrease in M1 macrophages. This ultimately results in decreased circulating proinflammatory chemokines and cytokines and reduced insulin resistance (37, 38). Additionally, BLT1R has been implicated in the insulin resistance derived by adipose tissue B2 cells (39). BLT1R deletion also protects from the progression of atherosclerosis in an ApoE-deficient mouse (37). In addition to the BLT1R, Olefsky and colleagues have demonstrated that high-fat diet-induced obesity results in increased LTB$_4$ production in metabolic tissues, including adipose, liver, and muscle (38).

Fatty acid binding proteins (FABPs) are small soluble fatty acid carriers and comprise a family of nine members (40). FABP4 (aP2/AFABP) plays a significant role in lipid metabolism; in the absence of FABP4, lipolysis is reduced (41–43). Furthermore, in the obese state, genetic deletion of FABP4 improves metabolism, resulting in reduced insulin resistance, atherosclerosis, and asthma (44–49). More specifically, FABP4-null macrophages have been shown to exert an anti-inflammatory phenotype, most closely resembling an M2 state. Interestingly, members of the FABP family, including FABP4, have been implicated in stabilizing short-lived LT intermediates, by binding the unstable epoxide containing LAT$_4$, functionally protecting it from water hydrolysis to the inactive 5,6- or 5,12-diHETEs (50, 51). Herein, we report that in macrophages lacking FABP4, the LT$_B_4$-induced signal cascade is dramatically reduced.

**RESEARCH DESIGN AND METHODS**

**Cell culture**

RAW264.7 macrophages were maintained in DMEM (Invitrogen) with 10% FBS. WT, FABP4/aP2 knockout (AKO), shGFP control, and shUCP2 knockout AKO macrophages were maintained in RPMI 1640 (Invitrogen) with 5% FBS. Concentrations and times of treatment of LPS, H$_2$O$_2$, N-acetyl cysteine, or LT$_B_4$ are listed in each figure legend.

**Bone marrow-derived macrophage isolation**

Bone marrow-derived macrophages (BMDMs) were isolated from 10-week-old female C57BL/6J mice as indicated in Ying et al. (52). Cells were plated and maintained in Iscove’s modified Dulbecco’s medium (Invitrogen) with 10% FBS and 10 ng/ml macrophage colony-stimulating factor (M-CSF) for 7 days to allow maturation of the cells into naïve macrophages. Polarization of cells to either M1 or M2 activation states occurred by treatment with either LPS (1 ug/ml)/IFN$\gamma$ (10 ng/ml) or IL-4 (10 ng/ml)/IL-13 (10 ng/ml) for 48 h, respectively. All experimental procedures using animals were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee.

**Quantitative RT-PCR**

Total RNA was isolated using TRizol reagent (Invitrogen) and cDNA synthesis was performed using iScript according to the manufacturer’s protocol (Bio-Rad). Quantitative (q)RT-PCR amplification utilized a Bio-Rad CFX 96 real-time system with a SYBR green Supermix (Bio-Rad). Transcription factor II E (TFII E) was used as the internal control to normalize expression. Primer sequences used are (forward; reverse): Arginase, (AACAGCAGGCTGGCTTTTAACC; 5CTTTTATGCTGGCATTAC); BLT1R, (GGCATCTGGGTGGTTGCTTTT; TGGCCTTTGTTGGATAGTGG); CD206, (CTCGTGATCCGTGACAC; GCAATGGAGCCCGTCTGTG); CXCL10, (CGTGTTGATGACATTGCAC; TTAAGGAGCCTTTTAAAC); IL-1β, (AAATACCTGTTGTGTGGGG; CTTGGGATCCACACTCTCC); iNOS, (AGGGAGGTTGTGGATTGTCC; TCTCTGCCTATCCGTCTCG); LCN2, (GGAGTGTGGCCGAAATAAG; TGCACCTCCATCTTTGTTTGTT); PPARγ, (GCCATGATGCTGGGATGCT; TGTTGATGGCCGCAATTTA); TFII E, (CAAGGCTTTAGGGACCTGATA; CATCCATTGACCTCAGT);

**Immunoblot analysis**

Cells were lysed with RIPA buffer supplemented with protease inhibitors (Calbiochem). Fifty micrograms of protein from each sample were separated by SDS-PAGE and transferred to a PVDF membrane. After blocking with Odyssey blocking buffer (Li-Cor Biosciences), membranes were incubated with primary antibody overnight at 4°C. Membranes were washed and incubated with secondary antibody conjugated to Li-Cor IRDye for 1 h and visualized using Odyssey infrared imaging (Li-Cor Biosciences). The primary antibodies used were anti-BLT1R (Cayman Chemical) and anti-actin (Sigma).

**Immunoprecipitation**

For JAK2 phosphorylation experiments, following LTB$_4$ treatments, JAK2 antibody (Cell Signaling) was added to cell lysates and incubated overnight at 4°C. Subsequently protein A-agarose was added and after 2 h of rocking at 4°C, centrifuged and the pellet was washed three times. The immunoprecipitants were denatured by boiling and run on SDS-PAGE. Immunoblots utilized antibodies to phospho-tyrosine (4G10; Millipore) and total JAK2 (Cell Signaling).

**Statistical analysis**

All data in this work are expressed as ±SEM. Statistical significance was determined using an unpaired two-tailed Student t-test ($*P < 0.05$).

**RESULTS**

**Genetic or pharmacologic depletion of FABP4 reduces BLT1R expression**

Previously, genetic knockout or pharmacologic inhibition of FABP4 has been shown to reduce inflammation and
improve insulin sensitivity (44, 53). This inhibition of FABP4 results in accumulation of intracellular fatty acids in both adipocytes and macrophages (41, 42, 54). Recently we demonstrated that monounsaturated fatty acids, particularly C16:1 palmitoleic acid and C18:1 oleic acid, drive the upregulation of UCP2 in macrophages resulting in reduced ROS and proinflammatory cytokine production (54). Because the LTB4 pathway is intimately involved in promoting inflammation, we evaluated the expression of the receptor, BLT1R, in macrophages generated from C57BL/6J (WT) or FABP4 knockout mice (AKO). FABP4 knockout macrophages expressed approximately 50% the BLT1R mRNA levels of WT (Fig. 1A) with BLT1R protein levels similarly reduced in FABP4 knockout macrophages (Fig. 1B, C).

**Signaling by LTB4 is compromised in FABP4 knockout macrophages**

Because the expression of BLT1R is reduced in FABP4 knockout macrophages, we next determined whether this translated into a functional effect on signaling. LTB4 has been demonstrated to activate the JAK2 pathway in macrophages via coupling to Goi, which inhibits the known JAK2 inhibitor, SOCS1 (55). Therefore, we tested whether the LTB4-dependent activation of JAK2 differed in FABP4 knockout as compared with control macrophages. As expected, a time course of LTB4 treatment resulted in phosphorylation of JAK2 in WT macrophages; however this was dramatically blunted in FABP4 knockout macrophages (Fig. 1D).

**Macrophage polarization affects BLT1R expression**

Macrophages can be activated by a variety of autocrine or paracrine factors to yield either a classical inflammatory M1 state or an alternatively activated anti-inflammatory M2 state (35, 56, 57). To determine whether expression of BLT1R is altered depending on the activation state, we utilized BMDMs activated to the M1 proinflammatory state using LPS and IFNγ, or to the M2 anti-inflammatory state using IL-4 and IL-13. To evaluate macrophage polarization, we quantified the expression of several genes known to be markers of M1 or M2 macrophages (58). As shown in Fig. 2A, LPS and IFNγ resulted in robust expression of the M1 markers, iNOS, CXCL10, IL-1β, and LCN2, while reducing expression of the M2 markers, arginase, PPARγ, and CD206 (Fig. 2B). In contrast, IL-4 and IL-13 increased the M2 markers while reducing the expression of the M1 markers. Consistent with an inflammatory state regulating the expression of BLT1R, M1 macrophages expressed higher BLT1R mRNA levels than macrophages in the M2 state (Fig. 2C).

**BLT1R expression is regulated by UCP2 and ROS**

We have previously demonstrated that knockout or inhibition of FABP4 leads to decreased inflammation and ROS due to the upregulation of UCP2 (54). To determine whether downregulation of BLT1R in AKO cells was UCP2-dependent, we silenced UCP2 expression (approximately 80%) in AKO macrophages using stable shRNA (54). As
shown in Fig. 4A, loss of UCP2 in AKO cells resulted in increased expression of BLT1R mRNA. Although there is some controversy as to whether UCP2 has uncoupling activity, it is generally agreed that UCP2 expression is negatively correlated with ROS (64, 65). We too have provided evidence supporting this correlation, because we measured increased ROS levels when UCP2 was silenced in RAW264.7 macrophages (52). To determine whether ROS could independently regulate BLT1R, RAW264.7 macrophages were treated with hydrogen peroxide and BLT1R mRNA levels were evaluated. As shown in Fig. 4B, BLT1R mRNA levels were robustly increased following H2O2 treatment of RAW264.7 macrophages. Furthermore, if reduced oxidative stress in AKO macrophages was responsible for the decreased BLT1R expression, exogenous H2O2 should increase BLT1R in both WT and AKO macrophages. For this, we isolated and treated BMDCs from C57BL/6J (WT) and AKO mice, and treated both with exogenous H2O2. Figure 3C shows that basal expression of BLT1R in AKO is trending down, similar to our stable macrophage cell lines, but exogenous H2O2 results in a similar increase in BLT1R expression in both cell types. Finally, we tested whether the general ROS scavenger, N-acetyl cysteine, could decrease expression of BLT1R in WT and AKO macrophages. Consistent with the model of ROS being a primary regulator of BLT1R, mRNA levels of BLT1R decreased in both WT and AKO following treatment with N-acetyl cysteine (Fig. 4D).

**DISCUSSION**

Herein, we demonstrate that the expression of macrophage BLT1R is upregulated by LPS and ROS, and that FABP4 knockout (AKO) macrophages have significantly blunted BLT1R expression and activity. Previous studies have demonstrated that the reduced proinflammatory activity in the FABP4 knockout macrophages is due in large part to increased UCP2 expression and, in the present study, we extend that analysis to link UCP2 expression and ROS to the expression of BLT1R (40, 62, 63). Increased expression of UCP2 in FABP4 knockout macrophages reduces inflammatory cytokine synthesis and secretion (54). Moreover, FABP4 protein stabilizes LTA4 to chemical degradation and stimulates LT synthesis and secretion. These findings are consistent with our current data demonstrating the inverse relationship of elevated BLT1R expression and reduced UCP2 expression.

FABP4 knockout mice are protected from chronic inflammation, insulin resistance, atherosclerosis, and experimental autoimmune encephalomyelitis (44–49, 66). The LTB4-BLT1R axis has been demonstrated to play a significant role in promoting each of these conditions (16, 24, 37, 67, 68) and has been identified as a potential therapeutic target (69). Furthermore, the phenotype of the BLT1R knockout mice shares similarities to that of the FABP4 knockout mice. These include increased alternative activation (M2) of macrophages, decreased MCP-1 expression, and JNK activation, as well as decreased liver triglycerides (37). Additionally, inhibition of BLT1R in the
leptin-deficient obesity model, ob/ob, increased the amount of M2 adipose tissue macrophages and decreased M1 macrophages (38). Consistent with this, inhibition of BLT1R also led to decreased expression and circulation of proinflammatory cytokines (37, 55). Therefore, the reduction of BLT1R in the FABP4-null macrophages may underlie the reduced susceptibility to metabolic diseases in the FABP4-null mice.

Fig. 3. LPS regulates BLT1R expression. A: Time course (2–24 h) of BLT1R mRNA expression in RAW264.7 macrophages treated with LPS (100 ng/ml). B: Expression of BLT1R mRNA in response to a 24 h concentration course of LPS. C: Expression of BLT1R protein in response to short-term LPS (100 ng/ml) treatment (0–3 h). D: Expression of BLT1R protein in response to 24 h of LPS (100 ng/ml) treatment. E: BLT1R mRNA expression in WT and AKO macrophages under basal conditions and in response to LPS (100 ng/ml) treatment for 24 h. Data were analyzed by Student's t-test. *P<0.05 as compared with no treatment (A–D). *P<0.05 AKO compared with WT (E).
Upon ligand binding, G protein-coupled receptors typically become desensitized, internalized, and degraded (70, 71) and this regulatory mechanism has been demonstrated for LTB4/BLT1R in an atypical phosphorylation-independent manner via β-arrestin association (72). In this study, LPS treatment resulted in a short-term increase in protein expression of BLT1R; however, chronic LPS treatment resulted in decreased BLT1R protein levels (Fig. 3C, D). The apparent discordance between BLT1R mRNA and protein expression may be linked to desensitization and subsequent degradation of the receptor protein. However, preliminary work thus far has not supported this assertion (data not shown). Thus the mechanism that leads to the dramatic decrease in LTB4 signaling in the FABP4-null macrophages, in addition to the reduction of the receptor, is not currently known. Additional work is necessary to determine the mechanism underlying this observation.

The LTB4 precursor, LTA4, has been shown to be highly unstable due to the water hydrolysis of its epoxide ring, eliminating its biological activity (73). The half-life of LTA4 is markedly increased by members of the FABP family, including FABP4 (50), and pharmacologic inhibition of FABP4 in macrophages markedly reduces LTC4 secretion (74). As such, FABP4 regulates LT signaling on multiple levels. First, it directly affects the stability and secretion of LTA4 and LTs and, second, it indirectly regulates BLT1R via an UCP2-dependent ROS-based mechanism. Together these effects significantly influence the activity of LTB4/BLT1R proinflammatory signaling and suggest that decreased LTB4 signaling in FABP4 knockout mice would contribute to the anti-inflammatory phenotype of the animals.

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