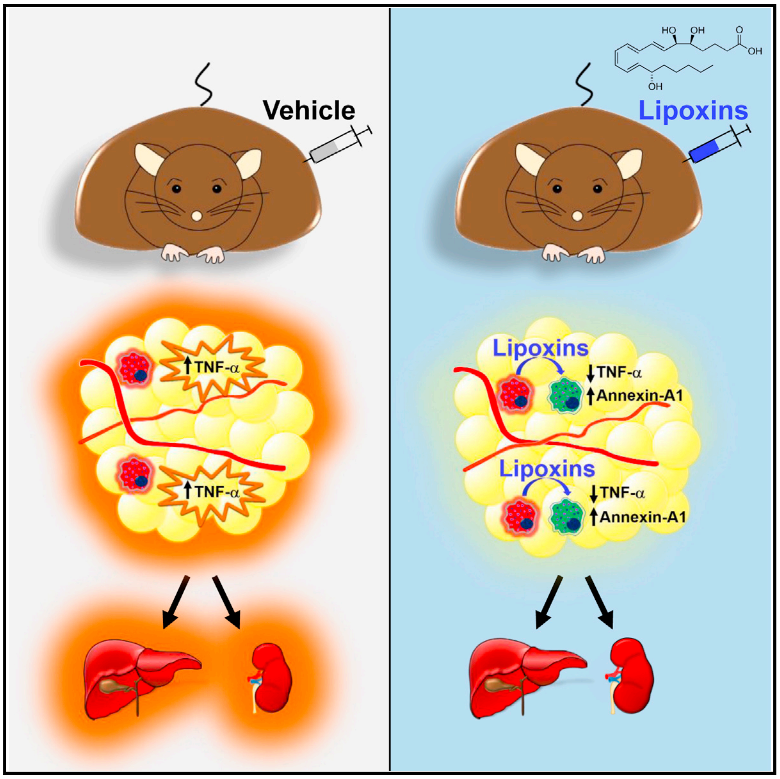
Cell Metabolism

Article

Lipoxin A_4 Attenuates Obesity-Induced Adipose Inflammation and Associated Liver and Kidney Disease

Graphical Abstract



Highlights

- Lipoxins attenuated high-fat diet-induced liver and kidney disease
- LXA_4 attenuated adipose inflammation, promoting a macrophage M1-to-M2 switch
- LXA_4 restored obesity-induced attenuation of autophagy markers LC3-II and p62
- LXA_4-mediated protection was adiponectin independent, but restored Annexin-A1

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In Brief

Börgeson et al. investigated the role of anti-inflammatory lipid mediators in obesity. LipoxinA_4 and a synthetic lipoxin analog protected against obesity-induced kidney and liver disease. Lipoxins mediated protection by decreasing adipose inflammation and promoting a macrophage M1-to-M2 switch. Lipoxin-mediated protection was adiponectin independent, but correlated with restored adipose Annexin-A1 levels.

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Lipoxin A_4 Attenuates Obesity-Induced Adipose Inflammation and Associated Liver and Kidney Disease

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SUMMARY

The role of inflammation in obesity-related pathologies is well established. We investigated the therapeutic potential of LipoxinA$_4$ (LXA$_4$:5(*S*),6(*R*),15(*S*)-trihydroxy-7E,9E,11Z,13E,-eicosatetraenoic acid) and a synthetic 15(*R*)-Benzo-LXA$_4$-analog as interventions in a 3-month high-fat diet (HFD; 60% fat)-induced obesity model. Obesity caused distinct pathologies, including impaired glucose tolerance, adipose inflammation, fatty liver, and chronic kidney disease (CKD). Lipoxins (LXs) attenuated obesity-induced CKD, reducing glomerular expansion, mesangial matrix, and urinary H_2O_2. Furthermore, LXA$_4$ reduced liver weight, serum alanine-aminotransferase, and hepatic triglycerides. LXA$_4$ decreased obesity-induced adipose inflammation, attenuating TNF-α and CD11c$^+$ M1-macrophages (MΦs), while restoring CD206$^+$ M2-MΦs and increasing Annexin-A1. LXs did not affect renal or hepatic MΦs, suggesting protection occurred via attenuation of adipose inflammation. LXs restored adipose expression of autophagy markers LC3-II and p62. LX-mediated protection was demonstrable in adiponectin$^{-/-}$ mice, suggesting that the mechanism was adiponectin independent. In conclusion, LXs protect against obesity-induced systemic disease, and these data support a novel therapeutic paradigm for treating obesity and associated pathologies.

INTRODUCTION

Obesity and the metabolic syndrome represent a global health problem, particularly due to associated co-morbidities. Obesity is an independent risk factor for systemic diseases, including diabetes, liver cirrhosis, and chronic kidney disease (CKD) (Börgeson and Sharma, 2013; Ix and Sharma, 2010). Metabolism is closely linked to the immune system, and chronic, non-resolving inflammation is considered a driving force of obesity-related pathologies. Prolonged and excessive nutrient overload results in chronic activation of the immune system and associated inflammation (Donath et al., 2013).

In addition to low-grade systemic inflammation, obesity is associated with significant adipose inflammation (Donath et al., 2013; Wen et al., 2011). The initiating processes for adipose inflammation are not entirely understood, but hypoxia due to adipose hypertrophy and a shift of macrophage (MΦ) phenotype from anti-inflammatory M2 to pro-inflammatory M1 likely play critical roles (Masoodi et al., 2015; McNelis and Olefsky, 2014). M1 MΦs produce significant amounts of pro-inflammatory cytokines and chemokines, as do adipocytes due to FFA ligation or as a result of adipocyte apoptosis. There is a growing recognition that adipose inflammation culminates in systemic disease, as it exaggerates systemic inflammation and reduces the production of the protective adipokine adiponectin (Börgeson and Sharma, 2013). Reduced adiponectin has been found to be associated with organ dysfunction in mice and humans and contributes directly to liver (Finelli and Tarantino, 2013) and kidney diseases (Sharma, 2009; Sharma et al., 2008).

Results of recent studies highlight the possibility that failed resolution of inflammation may underlie the pathogenesis of chronic inflammatory disorders, such as in metabolic syndrome

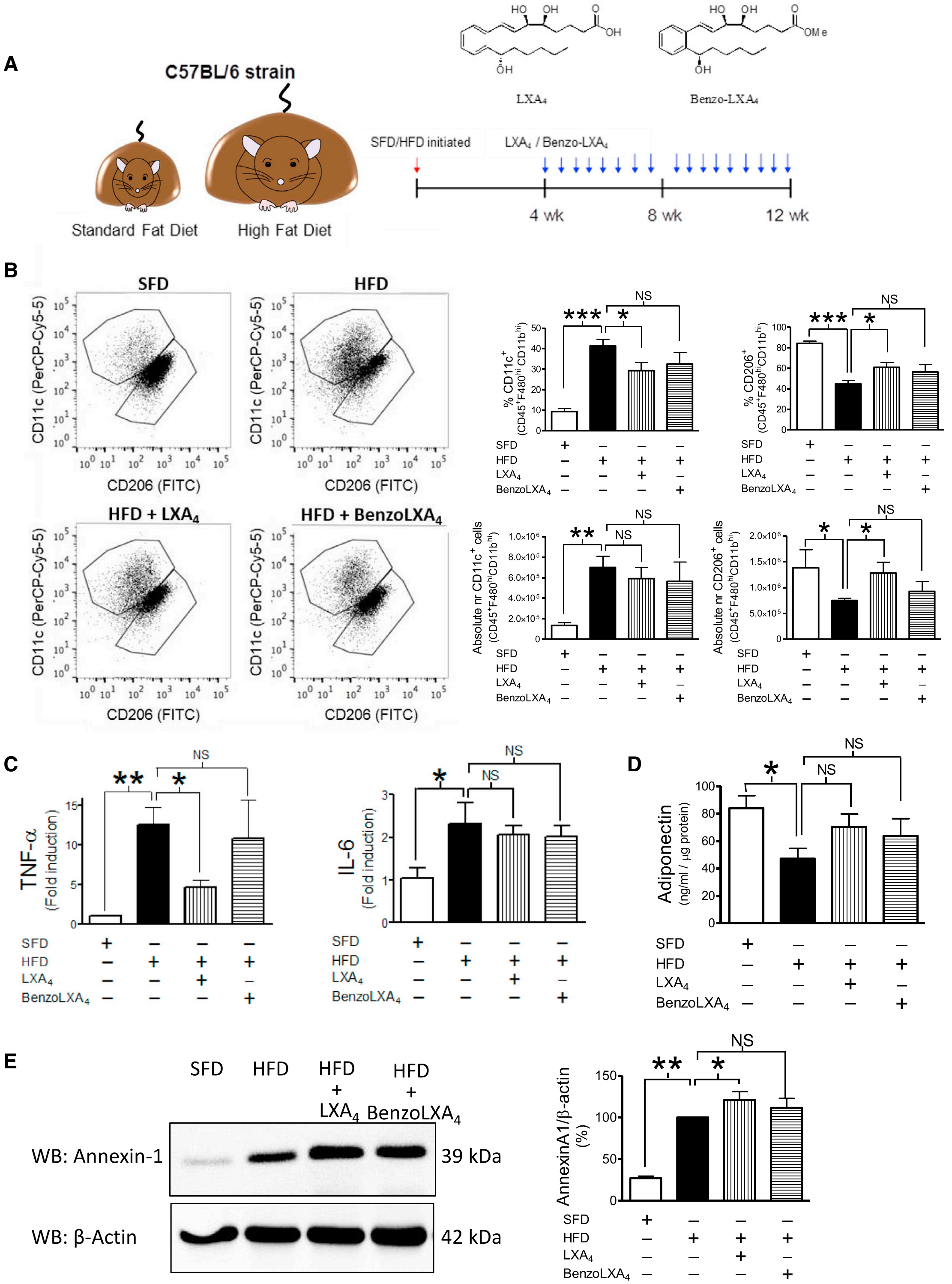

Figure 1. Lipoxins Attenuated Adipose Inflammation and Shift Adipose Macrophage Phenotype toward Resolution in Vivo

(A) Schematic illustration of the protocol: C57BL/6 mice fed a standard-fat diet (SFD: 10% fat) or a high-fat diet (HFD: 60% fat) for 12 weeks were treated with vehicle, LXA_4 (5 ng/g), or $BenzoLXA_4$ (1.7 ng/g) three times per week between weeks 5 and 12.

(B) WAT macrophage (MΦ) phenotype was analyzed by flow cytometry. Leukocytes were identified as inflammatory M1 MΦs ($CD11c^+$ of $CD45^+F480^{hi}CD11b^{hi}$ cells) versus anti-inflammatory M2 MΦs ($CD206^+$ of $CD45^+F480^{hi}CD11b^{hi}$ cells). Representative dot plots are shown as well as quantification of both percent positive cells and absolute cell numbers; n = 4.

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and diabetes (for recent review see Spite et al., 2014). Immunomodulation and specifically immunoresolvents are suggested as a therapeutic strategy to overcome chronic inflammation and disease (Börgeson and Godson, 2012; Donath, 2014; Donath et al., 2013; Serhan, 2007; Serhan and Savill, 2005; Tabas and Glass, 2013). Acute inflammation is orchestrated in part by chemical autacoids in the form of peptides (cytokines, chemokines) and lipid mediators (i.e., prostaglandins and leukotrienes), which induce edema and polymorphonuclear leukocyte (PMN) recruitment to inflammatory loci. In a physiologic state, this initial phase is followed by resolution, characterized by the cessation of PMN infiltration, MΦ-mediated efferocytosis, and the return to tissue homeostasis (Maderna and Godson, 2009). The resolution of inflammation is regulated by specialized pro-resolving mediators (SPMs). These include the ω3-derived protectins, resolvins (Rvs), and maresins as well as the ω6-derived Lipoxin A_4 (LXA_4; 5(*S*),6(*R*),15(*S*)-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid) and Lipoxin B_4 (LXB_4; 5(*S*),14(*R*),15(*S*)-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid). SPMs attenuate PMN recruitment and induce a pro-resolving M2 phenotype. These M2 MΦs produce more SPMs compared to M1 MΦs (Dalli and Serhan, 2012), thus further sustaining resolution, and ω3-derived SPMs also facilitate production of ω6-derived SPMs (Fredman et al., 2014). LXA_4 potently attenuates acute inflammation (Maderna and Godson, 2009) and age-associated adipose inflammation ex vivo (Borgeson et al., 2012). Of note SPMs have been identified in a number of human tissues and fluids including spleen, lymph nodes (Colas et al., 2014), urine (Sasaki et al., 2015), and white adipose tissue (WAT) (Clària et al., 2013). Importantly, whether LXA_4 actively attenuates chronic inflammation remains to be addressed.

Here, we explored the therapeutic potential of LXA_4 in experimental obesity-induced systemic disease. Because native LXs are chemically labile and undergo inactivation in vivo via either dehydrogenation and/or omega-oxidation (depending on the local environment), we also evaluated the actions of a stable benzo-fused (15*R*)-stereoisomer analog, referred to as $BenzoLXA_4$ (Borgeson et al., 2011). We report that LXA_4 and $BenzoLXA_4$ attenuate obesity-induced adipose inflammation and alter the adipose M1/M2 ratio, while modulating adipose autophagy, a driver of adipose inflammation (Martinez et al., 2013; Stienstra et al., 2014). These actions resulted in adiponectin-independent protection against obesity-induced liver and kidney disease, demonstrating the therapeutic potential of LXs in obesity-induced complications.

RESULTS

Lipoxins Attenuate Obesity-Induced Adipose Inflammation and Alter the M1/M2 Ratio

Mice were subjected to a 3-month dietary regime to induce obesity and associated liver and kidney disease (Figure 1A). LXs were provided as interventional therapeutics, introduced from weeks 5 to 12. Animals tolerated all treatments well. LXs did not affect high-fat diet (HFD)-induced weight gain, WAT hypertrophy, or adipocyte size (Figures S1A–S1C). HFD was not associated with an increase in total WAT F4/80$^+$ MΦs, as analyzed both by flow cytometry and immunohistochemistry (IHC) (Figure S3). It is noteworthy that this is in contrast to other studies (Oh et al., 2012) and may possibly be due to the control diets. Rather than using vivarium chow, this study is conducted using a standard fat diet (SFD) control diet containing equal protein content and matched sucrose content compared to the HFD, which may incur important differences in MΦ infiltration. Importantly, HFD-induced obesity did cause a significant increase in M1/M2 ratio in visceral adipose tissue, as previously reported (Lumeng et al., 2007). LXs shifted the MΦs toward a resolution phenotype. Specifically, LXA_4 attenuated the HFD-induced increase of the percent pro-inflammatory CD11c$^+$ M1 MΦs ($p < 0.05$), and LXA_4 partially restored the percent anti-inflammatory CD206$^+$ MΦ population ($p < 0.05$) (Figure 1B). In addition, we calculated the absolute cell numbers, as the obesity-induced expansion of WAT and increase in overall leukocyte infiltration may mask percentage shifts of cellular populations. Interestingly, the effect of LXA_4 on CD206$^+$ M2 MΦs is amplified when comparing the absolute cell numbers, whereas the attenuation of CD11c$^+$ M1 is not apparent (Figure 1B). In addition to switching MΦ phenotype, LXA_4 attenuated obesity-induced expression of the pro-inflammatory cytokine TNF-α (Figure 1C). WAT expression of IL-10 remained unaltered (data not shown), and IL-6 was elevated with HFD, but was not reduced with treatment (Figure 1C). No significant changes were found in the CD8$^+$ and CD4$^+$ T cell populations, nor in CD19$^+$ B cell infiltration (data not shown). However, it is important to note that we were unable to include intracellular markers in this flow cytometry panel, which prevented T cell subset characterization, e.g., T-regs, T_H1, and T_H2 ratio. Visceral adipose tissue adiponectin levels were measured by ELISA and in accordance with previous reports (Neuhofer et al., 2013); the HFD led to reduced secretion of adiponectin ($p < 0.05$). Treatment with LXs partially restored adiponectin in comparison with the control groups (SFD, 84 ± 9; HFD, 47 ± 7; HFD+LXA_4, 70 ± 9; HFD+$BenzoLXA_4$, 64 ± 12 ng/ml) (Figure 1D). However, there was no effect of LXs on the degree of WAT hypertrophy (Figures S1B and S1C). Annexin-A1 (AnxA1) is a glucocorticoid effector (Perretti and D'Acquisto, 2009), and AnxA1 deficiency promotes HFD-induced adiposity and insulin resistance (Akasheh et al., 2013). Our study confirms that WAT AnxA1 expression is increased in obese mice (Akasheh et al., 2013), and LXA_4 treatment significantly increased AnxA1 expression (Figure 1E).

Adipose tissue is heterogeneous and comprised of numerous cell types, where adipocytes and MΦs are the major mediators of inflammation and disease (McNelis and Olefsky, 2014). Both cell types express the LXA_4 receptor (ALX/FPR2) and are susceptible to the anti-inflammatory actions of LXA_4 (Borgeson et al., 2012). To clarify the cellular targets affected by LXs in this model, we designed a similar in vitro system

(C) WAT TNF-α and IL-6 expression were analyzed by qPCR; n = 4.

(D) WAT adiponectin was analyzed by ELISA; n = 4.

(E) WAT Annexin-A1 protein was determined by western blot; a representative blot is shown and densitometry quantification of n = 3 experiments. Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ANOVA with Bonferroni correction.

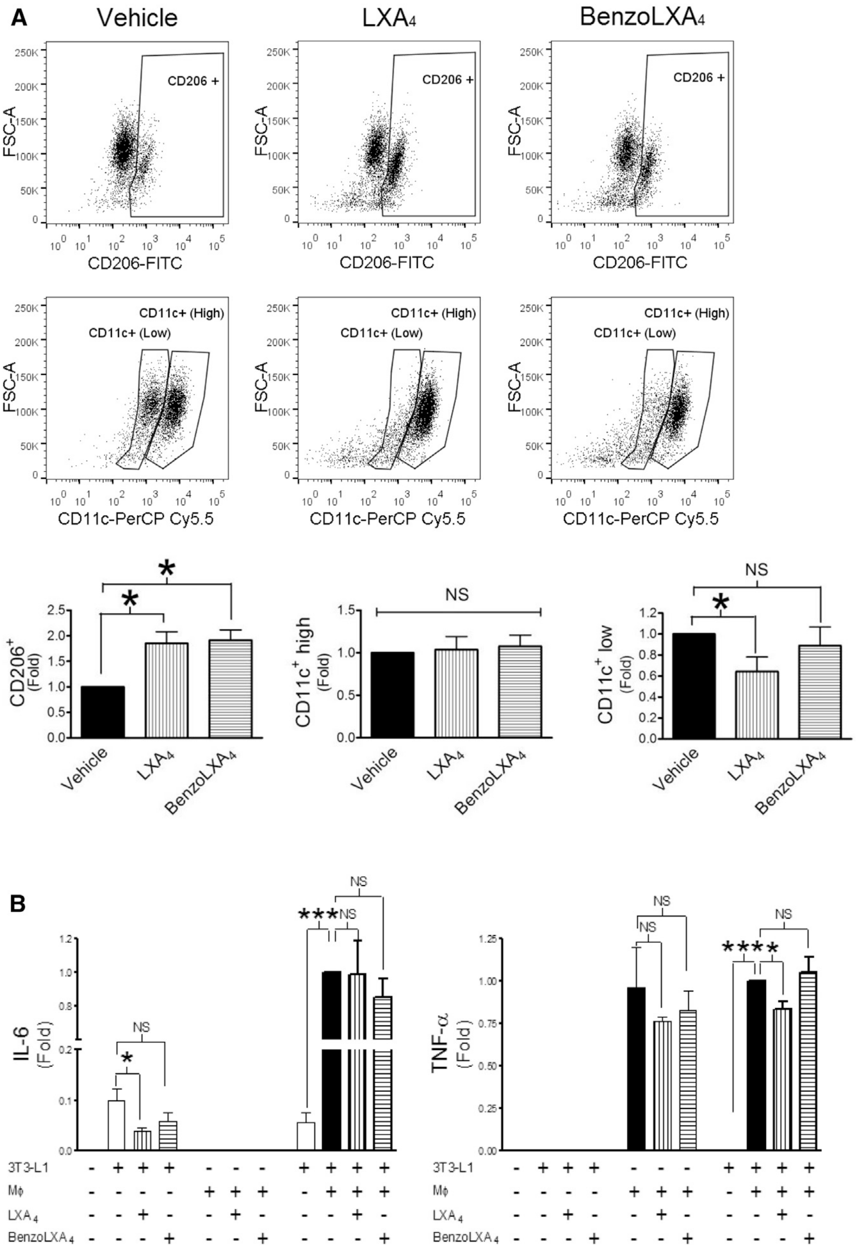

Figure 2. Lipoxins Attenuated Adipose Inflammation and Shift Adipose Macrophage Phenotype toward Resolution in Vitro

J774 macrophages (MΦs), constitutively expressing an M1 phenotype, were incubated with vehicle, 1 nM LXA_4, or 10 pM $BenzoLXA_4$ for 16 hr. MΦ phenotype was analyzed by flow cytometry, and supernatants were collected. Serum-starved hypertrophic 3T3-L1 adipocytes were treated with vehicle, 1 nM LXA_4, or 10 pM $BenzoLXA_4$, or alternatively with MΦ-conditioned supernatants, as indicated. Following 24-hr incubation, adipocyte supernatants were collected and analyzed by ELISA.

(A) J774 MΦ phenotype was analyzed by flow cytometry, and representative dot plots are shown of anti-inflammatory M2 ($CD206^+$) MΦs and pro-inflammatory M1 ($CD11c^+$) MΦs, with respective quantification below; n = 3.

(B) Adipocyte cytokine production was analyzed by ELISA; n = 3. Data are presented as mean ± SEM; n = 3. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ANOVA with Bonferroni correction.

comprised of inflammatory MΦs and hypertrophic adipocytes, where the latter resemble the adipocyte biology seen in obesity (Yoshizaki et al., 2012). The aim was to investigate whether LXs exert their protective effects via altering of MΦ phenotype and/or adipocyte cellular function. First, we characterized the ability of LXs to shift MΦ phenotype in vitro, using the constitutively M1-activated J774 cell line, which presents with high $CD11c^+$ and low $CD206^+$ expression (Figure 2). Both LXA_4 and $BenzoLXA_4$ significantly increased $CD206^+$ expression in vitro ($p < 0.05$) (Figure 2A). Interestingly, J774 expressed two distinct $CD11c^+$ populations: $CD11c^{low}$ and $CD11c^{high}$. Similarly to the in vivo scenario, LXA_4 attenuated $CD11c^+$ expression, specifically targeting the $CD11c^{low}$ population ($p < 0.05$) (Figure 2A).

We subsequently stimulated hypertrophic adipocytes either directly with vehicle, LXA_4, or $BenzoLXA_4$, or alternatively with MΦ-conditioned media from J774 cells treated with either vehicle or LXs, as illustrated in Figure 2B. In accordance with previous research (Borgeson et al., 2012), LXA_4 significantly attenuated adipocyte IL-6 secretion (Figure 2B). Of note, the hypertrophic adipocytes did not produce detectable levels of TNF-α or IL-10 (data not shown). In contrast, the J774 MΦs secreted high levels of TNF-α (Figure 2B), but not IL-6 or IL-10 (data not shown). When co-culturing adipocytes with MΦ-conditioned media, LXA_4 treatment attenuated MΦ-induced TNF-α production ($p < 0.05$), although IL-6 remained unaltered (Figure 2B), similar to findings in our in vivo study (Figure 1C). Importantly, the MΦ-conditioned media appeared to increase adipose IL-6 production, which appears to have masked the basal LXA_4-mediated attenuation of IL-6 secretion (Figure 2B).

Lipoxins Modulate Obesity-Induced Adipose Autophagy

Obesity causes excessive upregulation of WAT autophagy, which is linked to adipose stress and inflammation (Stienstra et al., 2014). We analyzed two of the main markers of autophagy and autophagic flux: LC3 and p62. LC3 is an ubiquitin-like protein involved in the biogenesis of autophagosome. It is cleaved from the microtubular network to form LC3-I, which is lipidated by the Atg12-Atg5-Atg16L complex to generate LC3-II. Lipidated LC3-II is incorporated into the phagophore during its formation and guides the autophagosome. Conversion of LC3-I to LC3-II is a hallmark of autophagy induction, and the LC3-II:LC3-I ratio is

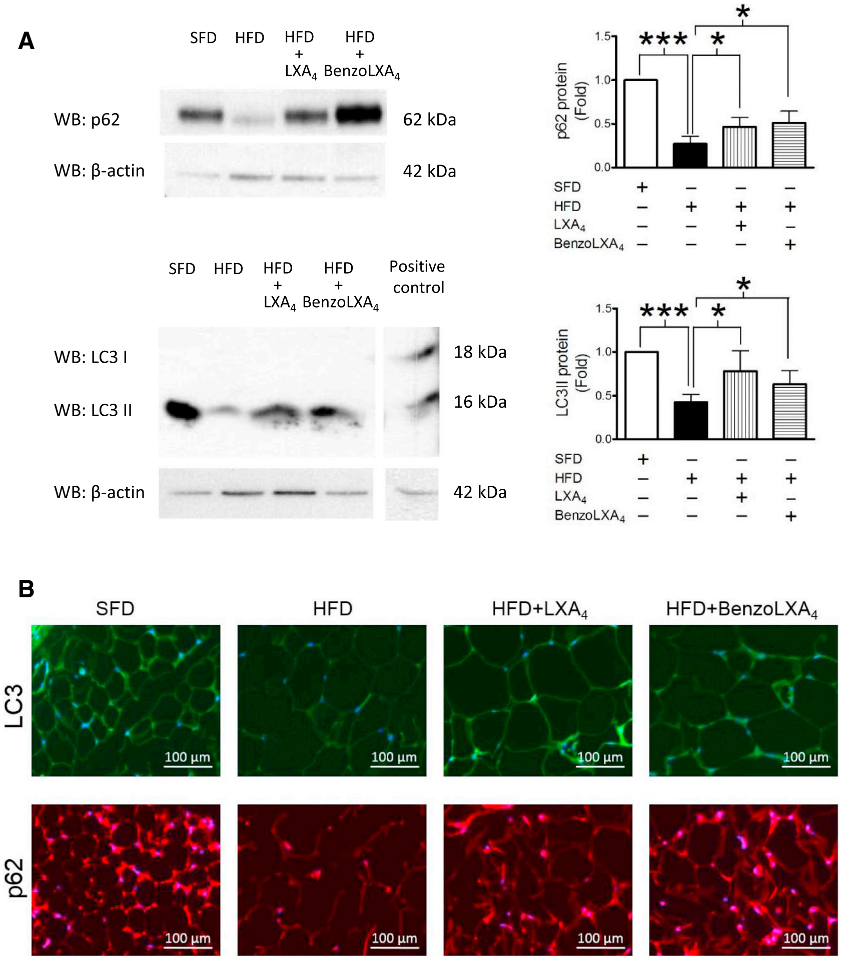

Figure 3. Lipoxins Modulate Obesity-Induced Adipose Autophagy

C57BL/6 mice, fed a standard-fat diet (SFD: 10% fat) or a high-fat diet (HFD: 60% fat) for 12 weeks, were treated with vehicle, LXA_4 (5 ng/g), and $BenzoLXA_4$ analog (1.7 ng/g) three times per week between weeks 5 and 12.

(A) Representative western blots are shown of adipose autophagy markers (p62 and LC3-II protein, normalized to β-Actin). Quantification is expressed as a fold ratio to control (right panels; n = 4).

(B) Representative immunofluorescence staining of WAT LC3-II (green) and p62 (red) is shown (n = 3). Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ANOVA with Bonferroni correction.

used to assess autophagy. We observed a reduction in the WAT LC3-II levels in obese mice, indicating reduced expression or enhanced degradation of LC3-II (Figure 3A). In agreement with previous reports of obesity-induced enhancement of autophagy in WAT, we envisage that the reduced levels of LC3-II are a consequence of enhanced lysosomal degradation due to higher autophagic flux. Similarly, we observed reduced WAT p62 levels in HFD mice (Figure 3A). p62/sequestosome1 (SQSTM1) is a ubiquitin-binding scaffold protein serving as an adaptor for the recruitment of ubiquitinated protein aggregates to the autophagosome. Similar to LC3-II, p62 accumulates as autophagy is inhibited and decreases when autophagy is induced. Interestingly, LXs restored the obesity-induced reduction in the levels of LC3-II ($p < 0.05$) and p62 ($p < 0.05$) (Figure 3A). These findings are confirmed by immunofluorescence staining of autophagy markers p62 and LC3-II (Figure 3B), indicating that LXs downregulated obesity-induced autophagy. To gain mechanistic insights into the mechanism for LX's regulation of autophagy, we performed WB analysis to determine mTOR and AMPK activity and expression. AMPK promotes autophagy by promoting Ulk1 phosphorylation at Ser317 and Ser777, and, conversely, mTOR inhibits autophagy by inhibiting Ulk1 by phosphorylation at Ser 757. Surprisingly, LXA_4 or $BenzoLXA_4$ did not affect mTOR and AMPK significantly (data not shown), suggesting that LXs regulate autophagy independent of mTOR and AMPK. It is noteworthy that p62/SQSTM1 has been identified as a key protein promoting lipid metabolism and limiting inflammation in the recently identified "metabolically activated" MΦs (mMe-MΦs) (Kratz et al., 2014). Thus, LX-mediated restoration of p62 may correlate with a restoration not only of M1/M2 phenotype, but also of the mMe-MΦ population.

Lipoxins Attenuate Obesity-Induced Liver Disease

Obese mice presented with increased absolute liver weight ($p < 0.05$) compared to SFD mice. Interestingly, liver expansion was attenuated by LXA_4 ($p < 0.01$) (Figure 4A), even though the total body weight remained unaltered with the LX treatment (Figure S1A). In light of this finding, we further investigated serum alanine aminotransferase (ALT) levels, a cardinal sign of liver injury. Obesity increased ALT by 3.15-fold ($p < 0.05$), which was attenuated by both LXA_4 and $BenzoLXA_4$ to basal levels ($p < 0.05$) (Figure 4B). Circulating and hepatic triglyceride (TG) levels were significantly increased during obesity. Interestingly, LXA_4 ($p < 0.05$) and $BenzoLXA_4$ ($p < 0.01$) attenuated liver TG deposition, although serum TGs remained unaltered (Figure 4C). HFD increased serum cholesterol E, but no significant effects were seen with LXA_4 (data not shown). Obesity did not increase hepatic MΦ infiltration, assessed through F4/80^{+} staining, and no obvious changes were observed with LX treatment (Figure 4D). Furthermore, hepatic gene expression of inflammatory cytokines, including TNF-α, IL-6, and anti-inflammatory cytokine IL-10, was assessed, but no significant differences were observed with HFD (data not shown). Finally, we assessed liver morphology using Ki67 and H&E staining; no differences in proliferation were detected (data not shown), but obesity induced some mild vacuolization, which appeared attenuated by LX treatment (Figure 4E).

Lipoxins Attenuate Obesity-Induced CKD

Obese mice presented with CKD, as evidenced by increased albuminuria, confirming that the model reflects obesity-induced CKD (Figure 5A). LXA_4 significantly attenuated obesity-induced

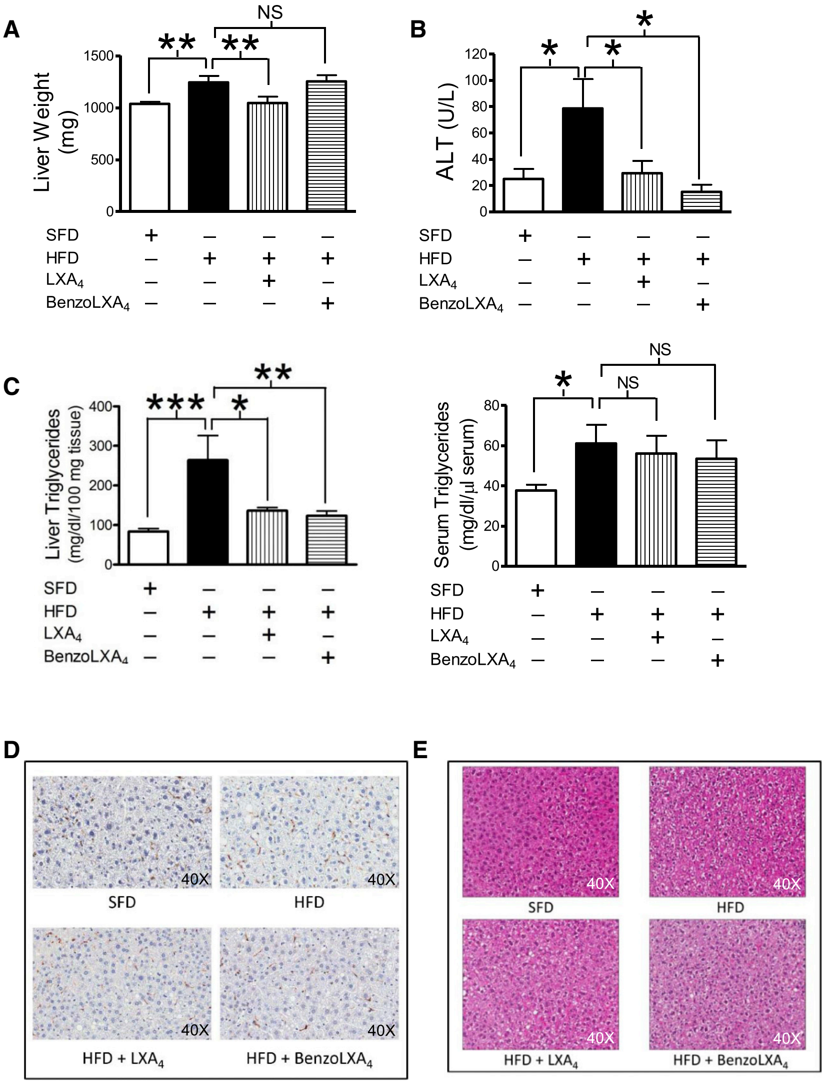

Figure 4. Lipoxins Attenuate Obesity-Induced Liver Injury

C57BL/6 mice, fed a standard-fat diet (SFD: 10% fat) or a high-fat diet (HFD: 60% fat) for 12 weeks, were treated with vehicle, LXA_4 (5 ng/g), or $BenzoLXA_4$ (1.7 ng/g) three times per week between weeks 5 and 12.

(A–C) (A) Liver weight was analyzed at harvest; n = 10. To further assess liver injury we analyzed (B) serum alanine aminotransferase (ALT) and (C) hepatic and serum TG content; n = 4.

(D and E) Representative images of hepatic $F4/80^+$ macrophages and H&E staining are shown; n = 3. Data are presented as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ANOVA with Bonferroni correction.

albuminuria, demonstrating protection against disease (Figure 5A). LXA_4 attenuated obesity-induced urinary H_2O_2, demonstrating protection against renal injury, possibly by attenuation of free radical production (Figure 5B). HFD-induced glomerular expansion and mesangial matrix expansion were significantly attenuated by LXA_4 and $BenzoLXA_4$ (Figure 5D). To further analyze tubulointerstitial injury and fibrosis, collagen deposition was assessed using Sirius Red staining, specifically detecting newly deposited collagen. Interestingly, HFD-induced tubulointerstitial collagen deposition was significantly attenuated by LXA_4 and $BenzoLXA_4$ (Figure 5E). Renal MΦ infiltration was investigated by flow cytometry. HFD increased the ratio of $CD11c^+$ M1 MΦs, but $CD206^+$ M2 MΦs were only detected at minimal levels. In addition, LXs did not affect HFD-induced renal MΦ infiltration in this model, as assessed by flow cytometry analysis (data not shown).

Lipoxin-Mediated Protection Is Independent of Adiponectin

Obesity-induced impairment of the adiponectin/AMPK pathway causes both liver and kidney disease (Polyzos et al., 2010; Sharma, 2009; Sharma et al., 2008). We noted a trend toward increased WAT adiponectin levels in LX-treated mice (Figure 1D), and thus we evaluated whether the LX-mediated hepatic and reno-protective effects were mediated via the adiponectin axis by comparing the HFD-induced pathology observed in the C57BL/6J wild-type (WT) (Figure 5) versus adiponectin$^{-/-}$ knockout (KO) mice (Figure 6). The adiponectin$^{-/-}$ mice were more susceptible to obesity-induced kidney and liver disease, as indicated by increased albuminuria ($p < 0.01$, ##), urine H_2O_2 ($p < 0.05$, #), and serum ALT ($p < 0.05$, #) (Figures 6A–6C). LXs also displayed reno-protective actions in adiponectin$^{-/-}$ mice, as demonstrated by a reduction in albuminuria ($p < 0.05$, *), and both LXA_4 ($p < 0.001$, ***) and $BenzoLXA_4$ ($p < 0.01$, **) attenuated HFD-induced urine H_2O_2 in the mice lacking the adiponectin gene (Figures 6A and 6B). Similarly, LXs significantly reversed the obesity-induced increase of serum ALT in the adiponectin$^{-/-}$ mice (Figure 6C). These findings demonstrate unique properties of LXA_4 compared to other ω3-derived SPMs, which regulate obesity via direct action on adiponectin (Clària et al., 2012; Rius et al., 2014).

As anticipated, obese animals displayed decreased sensitivity to insulin-stimulated glucose uptake. LXA_4 and $BenzoLXA_4$ did not rescue obesity-induced impairment of glucose tolerance in C57BL/6J mice, suggesting that protection against liver disease and CKD occurs independent of rescued insulin sensitivity (Figure 6D, WT data). Adiponectin$^{-/-}$ mice displayed exaggerated impairment of glucose tolerance compared to respective WT control ($p < 0.001$, ###) (Figure 6D). LXs restored HFD-induced impairment of glucose uptake, and LXA_4 attenuated fasting blood glucose in the KO strain (Figure 6D). To investigate our findings further in vitro, hypertrophic adipocytes were used as an experimental model of obesity (Yoshizaki et al., 2012). As described, adipocytes were incubated with vehicle, LXA_4, $BenzoLXA_4$, or alternatively with MΦ-conditioned media from J774 cells treated with either vehicle or LXs. The LXs did not attenuate basal adiponectin production in hypertrophic adipocytes (Figure 6E), although LXA_4 attenuates other cellular

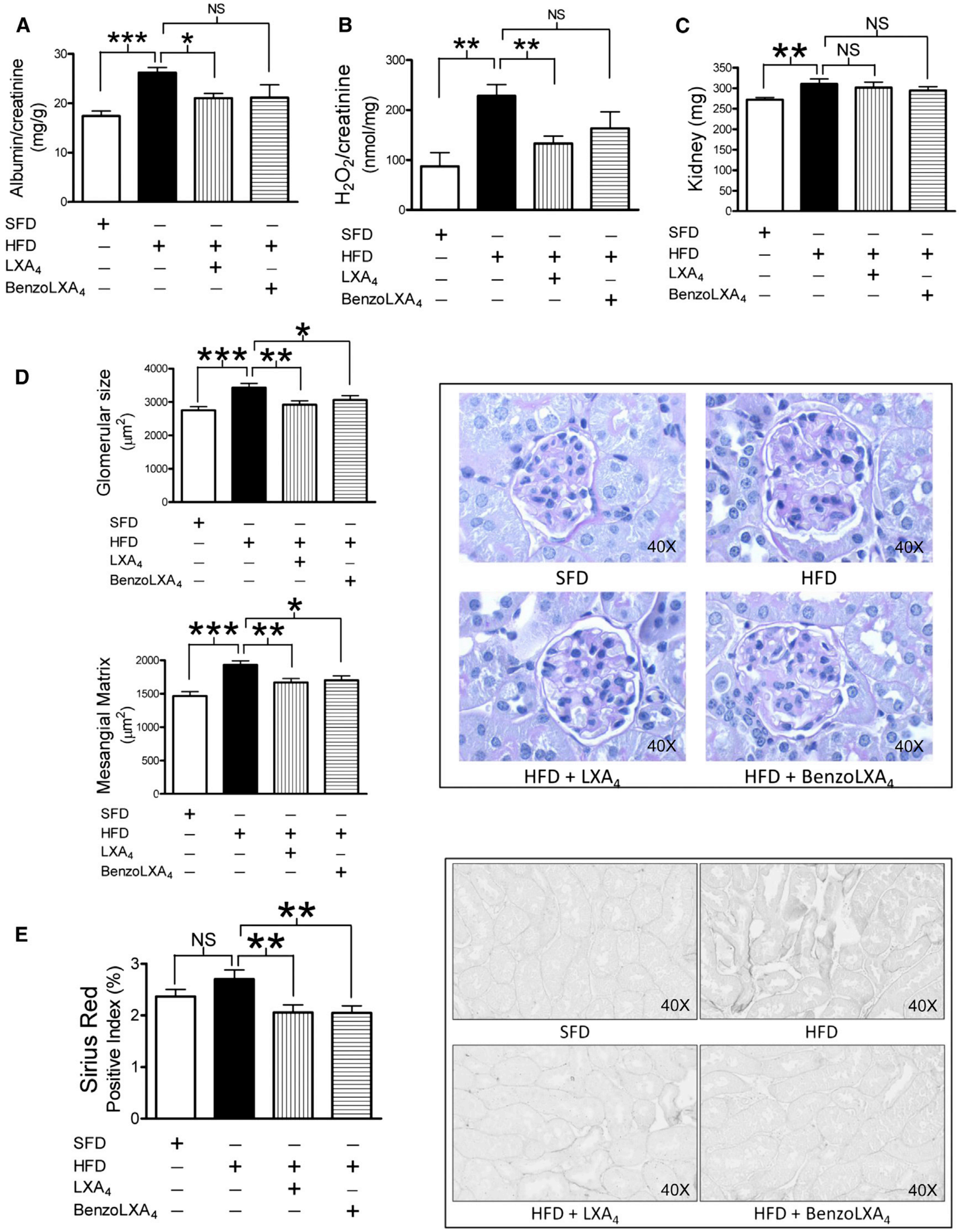

Figure 5. Lipoxins Attenuate Obesity-Induced CKD

C57BL/6 mice, fed a standard-fat diet (SFD: 10% fat) or a high-fat diet (HFD: 60% fat) for 12 weeks, were treated with vehicle, LXA$_4$ (5 ng/g), or BenzoLXA$_4$ (1.7 ng/g) three times per week between weeks 5 and 12. At 1 week prior to harvest, 24-hr urine samples were collected. Parameters of renal injury, including (A) albuminuria and (B) urine hydrogen peroxide (H_2O_2)/creatinine and (C) renal hypertrophy were assessed; n = 10.

(D and E) (D) Glomerular expansion and matrix deposition were assessed by periodic acid-Schiff and (E) tubulointerstitial collagen by Sirius Red; n = 5. Data are presented as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; ANOVA with Bonferroni correction.

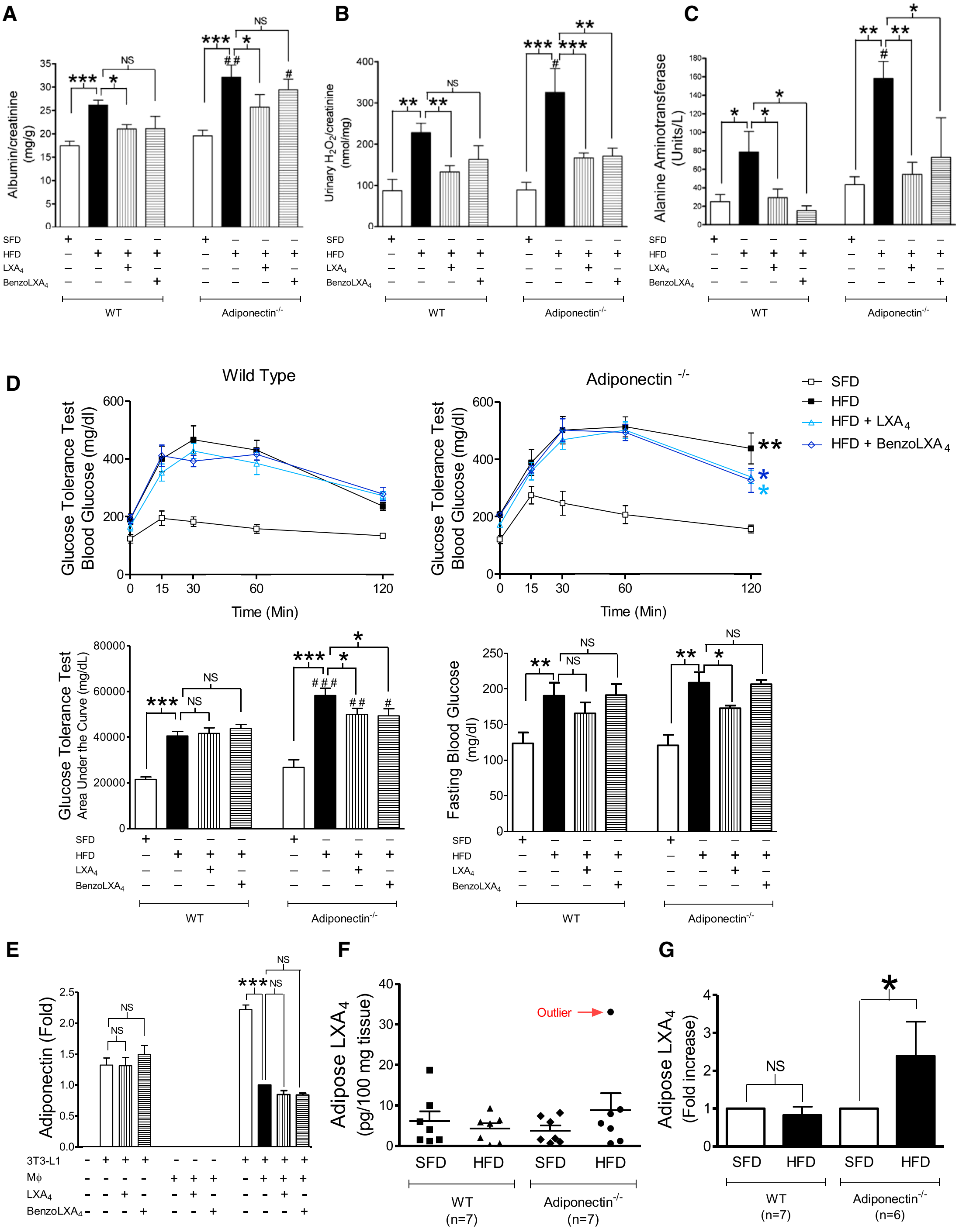

Figure 6. Lipoxin-Mediated Protection of Obesity-Induced Pathology Is Independent of Adiponectin

C57BL/6 or adiponectin$^{-/-}$ mice, fed a standard-fat diet (SFD: 10% fat) or a high-fat diet (HFD: 60% fat) for 12 weeks, were treated with vehicle, LXA_4 (5 ng/g), or BenzoLXA_4 (1.7 ng/g) three times per week between weeks 5 and 12. Parameters of renal injury, including (A) albuminuria and (B) urine hydrogen peroxide (H_2O_2)/creatinine were analyzed; n = 10.

(C) Serum alanine aminotransferase (ALT) was analyzed; n = 4.

(D) Glucose tolerance was assessed over 120 min 1 week prior to harvest. The area under curve (AUC) was calculated for respective groups and used for statistical analysis, as well as basal fasting glucose; n = 10.

(E) Hypertrophic adipocytes were incubated with vehicle, LXA_4 (1 nM), or BenzoLXA_4 (10 pM) for 24 hr. Alternatively, adipocytes were incubated for 24 hr with supernatants from MΦs pretreated with vehicle, LXA_4 (1 nM), or BenzoLXA_4 (10 pM). Following respective treatment, the adipocyte supernatants were collected, and cytokine production was analyzed by ELISA; n = 3.

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responses in this in vitro system, e.g., basal IL-6 production (Figure 2B). LXA_4 did not rescue MΦ-induced attenuation of adipose adiponectin production (Figure 6E), although LXA_4 attenuated MΦ-induced TNF-α in this experimental setup (Figure 2B).

Finally, we investigated endogenous LXA_4 production in SFD- and HFD-treated WT and adiponectin$^{-/-}$ mice (Figures 6F and 6G). Using LM-metabololipidomics (Figure S4), endogenous LXA_4 was identified and quantified at biologically relevant amounts (5.7 ± 1.1 pg/100 mg tissue) (Krishnamoorthy et al., 2010), which are comparable to levels of eicosanoids reported by others, e.g., LTB4 (0.7 pg/100 mg tissue) (Li et al., 2015). We also identified mediators from the DHA, EPA, and AA bioactive metabolomes including RvD1 (4.2 ± 1.0 pg/100 mg tissues), RvD5 (1.6 ± 0.4 pg/100 mg tissues), and maresin 1 (1.9 ± 0.3 pg/100 mg tissues). These were not significantly altered in either condition, although a trend toward reduced LXB_4 levels was observed in obese animals, which will be interesting to investigate further in future studies. Of note, HFD administration reduced LXA_4 levels in adipose tissue (WT, SFD 6.1 ± 2.4; versus HFD, 4.3 ± 1.3 pg/100 mg tissue) (Figure 6F). We also found a reduction in LXA_4 levels between lean WT and lean adiponectin$^{-/-}$ mice, although in both cases the difference did not reach statistical significance. However, the adiponectin$^{-/-}$ mice displayed a trend of increased endogenous LXA_4 production following HFD treatment (KO, SFD 3.8 ± 1.2; versus HFD, 8.8 ± 4.2 pg/100 mg tissue) (Figure 6F). When comparing the fold changes between lean and obese animals in respective mouse strains, it became apparent that obesity significantly increased endogenous LXA_4 production in the adiponectin$^{-/-}$ group (Figure 6G; $p < 0.05$).

DISCUSSION

Obesity is an independent risk factor for serious pathological conditions, including diabetes, liver disease, and CKD. Adipose inflammation appears to be the common denominator of obesity-related pathologies (Donath and Shoelson, 2011; McNelis and Olefsky, 2014). Promoting the resolution of adipose inflammation is therefore a potential therapeutic approach that could alleviate obesity-associated organ dysfunction (Börgeson and Godson, 2012; Clària et al., 2012; Donath, 2014; Donath et al., 2013; Spite et al., 2014; Tabas and Glass, 2013). The results presented here demonstrate that LXA_4 attenuates obesity-associated adipose inflammation and thereby alleviates liver and kidney diseases associated with obesity.

LXs rescued HFD-induced kidney and liver disease, and the key mechanism of action appeared to be the attenuation of visceral WAT inflammation. In contrast to other studies (Oh et al., 2012) we do not report an increase in total WAT F4/80$^+$ MΦs in HFD versus SFD. Importantly, we observed an obesity-induced shift of the MΦ phenotype from M1 (CD11c$^+$) to M2 (CD206$^+$). The LXA_4-induced shift in WAT MΦ phenotype correlated with other attributes of resolution, such as attenuation of the inflammatory cytokine TNF-α. These data may be compared with other in vivo models, where LXA_4 promotes an M1-to-M2 MΦ polarization in a mouse air-pouch model of inflammation (Vasconcelos et al., 2015). Indeed, LXA_4 may attenuate the pro-inflammatory M1 MΦ phenotype by modulating IκBα degradation, NF-κB translocation, and IKK expression, resulting in suppressed NF-κB activation (Huang et al., 2014; Kure et al., 2010). In contrast, LXA_4 may promote an M2a and M2c MΦ phenotype by modulating STAT3 (Li et al., 2011) and prolonging the MΦ lifespan by inhibiting LPS-induced apoptosis via PI3K/Akt and ERK/Nrf-2 pathways (Prieto et al., 2010). Furthermore, our finding that LXA_4 promotes a WAT M1-to-M2 MΦ polarization correlates with earlier reports that depletion of CD11c$^+$ cells results in rapid normalization of obesity-induced insulin sensitivity, paralleled by a decrease in adipose and systemic inflammation (Patsouris et al., 2008). Similarly, ω3-derived SPMs (e.g., Rvs, protectins, and maresins) reduce insulin resistance, increase adiponectin secretion, and modulate adipose MΦ functions toward a "pro-resolution phenotype" in genetic models of obesity (González-Périz and Clària, 2010; Gonzalez-Periz et al., 2009; Hellmann et al., 2011; Neuhofer et al., 2013; Ost et al., 2010).

It is important to recognize that in addition to their well-established actions on leukocytes (Serhan, 2007), LXs affect numerous cell types, including adipocytes (Borgeson et al., 2012). It is therefore reasonable to ask whether LXA_4-mediated attenuation of WAT inflammation occurred through modulation of the MΦ phenotype and/or via direct manipulation of the adipocyte cell function. LXA_4 restores TNF-α-induced impairment of insulin signaling in normal 3T3-L1 adipocytes (Borgeson et al., 2012). In this study, we used a similar experimental setup, composed of murine MΦs and hypertrophic adipocytes, the latter in order to better mimic obesity (Yoshizaki et al., 2012). The results confirmed that LXA_4 may reduce inflammation by affecting both MΦs and adipocytes. We confirmed in vitro that LXA_4 shifts the MΦ phenotype from M1 to M2, and that this translates to an attenuation of MΦ TNF-α production. LXA_4 has a direct effect on hypertrophic adipocyte IL-6 secretion; although this effect may be overwhelmed by the strong inflammatory stimuli from M1 MΦs. Interestingly, this in vitro system correlated with our in vivo data, where the LXA_4 primarily affected adipose TNF-α gene expression.

The LXA_4 receptor is expressed in the adipose "target tissue." The murine homologs of ALX/FPR2 are Fpr2/Fpr-rs1 and Fpr3/Fpr-rs2, and we have previously demonstrated that Fpr-rs2/FPR3 is expressed in both the murine adipocytes and macrophages (Borgeson et al., 2012). Furthermore, Clària et al. report that expression of the LXA_4 receptor is present in mouse WAT, and that this expression is sustained in obese mice (Clària et al., 2012). Interestingly, they also show that the human LXA_4 receptors ALX/FPR2 and GPR32 are identified in human adipose tissue (Clària et al., 2012).

In addition to attenuating WAT inflammation, LXs modulated HFD-induced adipose autophagy. The general consensus is that chronic obesity causes excessive activation of autophagy in the adipose tissue, which correlates with increased cell death

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(F) The endogenous adipose LXA_4 production was assessed by LC-MS/MS in vehicle-treated SFD and HFD wild-type (WT) and adiponectin$^{-/-}$ mice (n = 7).
(G) The HFD-induced fold increase of endogenous LXA_4 production was calculated, excluding the outlier identified in (F). Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Statistically significant differences between respective condition in the WT versus adiponectin$^{-/-}$ strain are indicated as #$p < 0.05$, ##$p < 0.01$, ### $p < 0.001$; ANOVA with Bonferroni correction.

and adipose inflammation (Martinez et al., 2013; Stienstra et al., 2014). In accordance with previous studies (Cummins et al., 2014; Stienstra et al., 2014), our data show that HFD is associated with enhanced levels of autophagy in WAT, as evident from decreased detectable p62 and LC3-II, suggesting high autophagy flux and enhanced degradation of the proteins. Interestingly, LXs restored the HFD-induced decrease in p62 and LC3-II protein to the levels observed in lean mice. Importantly, the LX-mediated attenuation of autophagy was independent of mTOR and AMPK activity, and we hypothesize that LXs may regulate autophagy by modulating the autophagic flux via regulation of autophagosome maturation (fusion of autophagosomes with lysosome). However, future studies are needed to investigate the detailed molecular mechanisms involved in LX-mediated regulation of autophagy and its significance in LX-mediated attenuation of adipose inflammation. Interestingly, p62/SQSTM1 has been identified as one of the key proteins that promote lipid metabolism and limit inflammation in the recently identified mMe-MΦs (Kratz et al., 2014). Activation of p62 results in inhibition of NF-κB and thus limits inflammation. It is therefore particularly noteworthy that LXs restore obesity-induced attenuation of p62, as it may correlate with a restoration not only of M1/M2 phenotype, but also an anti-inflammatory mMe-MΦ population. This will be the focus of future work.

Obesity is an independent risk factor for kidney disease, even when excluding variables such as diabetes and hypertension (Börgeson and Sharma, 2013). Additionally, obesity accelerates the progression of pre-existing kidney disease (Mathew et al., 2011). We have previously shown that LXA_4 and $BenzoLXA_4$ attenuate experimental tubulointerstitial fibrosis induced by unilateral ureteric obstruction (Borgeson et al., 2011; Brennan et al., 2013). LXs have also been shown to be protective in acute renal injury, attenuating the inflammatory response to ischemia reperfusion injury (Börgeson and Godson, 2012; Leonard et al., 2002). However, the potential of using LXA_4 in models of obesity-induced CKD has not previously been investigated. The present study showed that both LXA_4 and $BenzoLXA_4$ attenuated obesity-induced CKD, as evidenced by reduced glomerular expansion and mesangial matrix deposition. The LXs also attenuated the mild tubulointerstitial fibrosis observed in this experimental system. LXA_4 rescued albuminuria, a cardinal sign of kidney disease, and significantly lowered HFD-induced urine H_2O_2 levels, which were used as a marker of reactive oxygen species (ROS) and renal injury. Interestingly, renal leukocyte infiltration was not significantly affected by LXs in this disease model, suggesting that the protection was not due to a direct effect on the renal inflammatory milieu.

Obesity-induced non-alcoholic fatty liver disease (NAFLD) is also becoming a major health problem. NAFLD ranges from steatosis (accumulation of hepatic TGs) to non-alcoholic steatohepatitis (NASH or steatohepatitis, steatosis with an inflammatory component). Hepatic steatosis is associated with enhanced hepatic susceptibility to progression into irreversible forms of liver disease (Spite et al., 2014). Other SPMs (RvE1, Protectin D1, and D-series Rvs) attenuate obesity-induced liver disease (Clària et al., 2012; Gonzalez-Periz et al., 2009). LXA_4 enhances organ function in murine liver transplantation, attenuating serum ALT and inducing a pro-resolving shift in cytokine production, decreasing IFN-γ while increasing IL-10 (Levy et al., 2011; Liao et al., 2013). Recent data highlight a protective effect of LXs and other arachidonate-derived mediators in cardiovascular disease associated with increased reverse cholesterol transfer and lower plasma LDL (Demetz et al., 2014). In our model, obesity caused mild liver injury, as apparent by increased serum ALT, liver weight, and TG accumulation. Interestingly, LXs attenuated HFD-induced liver injury, as both LXA_4 and $BenzoLXA_4$ attenuated serum ALT and hepatic TG deposition to baseline levels. LXA_4 significantly attenuated liver weight, which is a particularly noteworthy finding because the total body weight was not affected. The attenuation of hepatic TG deposition may correlate with the restoration of hepatic expansion. However, this may not be the sole explanation, as $BenzoLXA_4$-induced attenuation of hepatic TGs did not translate to altered organ weight. LXA_4-induced reduction of hepatic edema may be an additional explanation for the significant effect mediated by LXA_4 on attenuation of liver weight. Collectively, our findings suggest that LX-mediated reduction of hepatic steatosis may make the liver more resistant against additional insults. Previous research demonstrates that an accumulation of WAT MΦs and resulting adipose inflammation correlates with liver pathology (Cancello et al., 2006; Ix and Sharma, 2010; Tordjman et al., 2009). We thus propose that the LX-mediated restoration of the WAT M1/M2 ratio is a key mechanism of action in the reduction of liver pathology. In line with this argument, our data reveal that LXA_4 increases WAT levels of the pro-resolving AnxA1, which is protective of NASH in mice (Locatelli et al., 2014). Specifically, this interesting study demonstrates that WAT MΦ-derived AnxA1 plays a functional role in modulating hepatic inflammation and fibrogenesis during NASH progression. The LXA_4-mediated increase in AnxA1 is thus likely a key mechanism of action in the observed attenuation of liver injury.

In addition to LXA_4, we evaluated the therapeutic potential of a (1*R*)-stereoisomer analog (referred to as $BenzoLXA_4$) in these experiments. This analog differs structurally from the benzo-analog o-(9,12)-benzo-ω6-epi-LXA_4 described by Sun et al. (Sun et al., 2009) and is protective in acute inflammation and tubulointerstial fibrosis (Borgeson et al., 2011; O'Sullivan et al., 2007). It should be noted that the $BenzoLXA_4$ analog exerted similar actions as LXA_4, although the native compound proved more effective in the present study. Thus, the analog provides us with valuable insights into the effect on biological activity of modifying the triene unit of native LXA_4 to a metabolically more stable benzene ring as well as the importance of (*R*)-stereochemistry at the benzylic carbinol center, which will aid future analog design.

The adiponectin/AMPK axis is implicated in obesity-induced liver and kidney pathology (Mathew et al., 2011; Sharma et al., 2008). Previous work demonstrates that LXA_4 increases adiponectin in adipose explants (Clària et al., 2012) and is present in human adipose tissue (Clària et al., 2013) and urine (Sasaki et al., 2015), and herein we observed a trend toward increased WAT adiponectin production in LX-treated mice (Figure 1D). Therefore, we hypothesized that LXs may mediate protection in our obesity model via induction of adiponectin in WAT. To explore this possible mechanism of action, we carried out the experimental design in both WT and adiponectin$^{-/-}$ mice. The assumption was that if our hypothesis was correct, the LX-mediated protection would not be observed in the KO strain. The

LX-mediated restoration of albuminuria was evident in both WT and adiponectin$^{-/-}$ mice, although LXs displayed increased ability to attenuate HFD-induced urine H_2O_2 production in the KO strain. Furthermore, the in vitro data show that neither LXA_4 nor $BenzoLXA_4$ induced adiponectin production in hypertrophic 3T3-L1 adipocytes. In addition, the LXs did not rescue MΦ-induced attenuation of adiponectin production in the adipocytes. Collectively, the data thus show that LX-mediated attenuation of obesity-induced disease is adiponectin independent in this model of HFD-induced renal and liver injury. In relation to our initial observation that LXs caused a trend toward increased WAT adiponectin production, this may simply be due to an improved general health of these mice, rather than being a direct mediator of LX's beneficial effects.

LXs did not mediate protection via enhancing glucose tolerance in the WT animals, indicating that the mechanism of action did not involve improved pancreatic insulin function. However, both LXA_4 and $BenzoLXA_4$ reduced GTT 120 min post-injection ($p < 0.05$) in the KO mice, although the animals still remained significantly more insulin resistant compared to lean mice. LXA_4 also reduced fasting glucose in the adiponectin$^{-/-}$ strain, suggesting that LXA_4 may exert some modulation of insulin resistance.

As the LXA_4-mediated protection appeared enhanced in the KO strain, we measured endogenous LXA_4 production in the adipose tissue of the SFD- and HFD-treated groups. Obese adiponectin$^{-/-}$ mice expressed significantly more endogenous LXA_4 when challenged with HFD. Thus, it is plausible that in conditions of adiponectin deficiency there is a compensatory response to further increase LX production due to enhanced inflammation and the other arachidonic acid (AA)-derived products. Further exogenous administration of LXs may be necessary to restore insulin sensitivity. The regulation of LXs by adiponectin is worthy of future studies. Whether LXs may have an effect to improve insulin sensitivity and lower blood glucose in other models of diabetes remains to be tested.

In conclusion, these results indicate that LXs have therapeutic potential in obesity-induced pathologies, such as liver disease and CKD. LXA_4 likely mediates protection via reducing adipose inflammation and modulation of WAT MΦ phenotype. Interestingly, LXs reversed HFD-induced adipose autophagy, which has been linked to obesity-induced disease. LX-mediated protection was independent of systemic adiposity and glucose tolerance. Moreover, the LX-mediated actions were adiponectin independent in this system. Collectively, these results demonstrate the potential of using SPMs, such as LXA_4 and LX-stable analogs, to protect from obesity-induced pathologies.

EXPERIMENTAL PROCEDURES

Detailed protocols are described in Supplemental Experimental Procedures.

In Vivo Obesity Study

C57BL/6J (n = 10) and C57BL/6J adiponectin$^{-/-}$ mice (n = 7) (Jackson Laboratory) were fed a SFD (10% fat) or a HFD (60% fat) for 12 weeks. Vehicle (1% ethanol), LXA_4 (5 ng/g), or $BenzoLXA_4$ (1.7 ng/g) were given as 100 μl intraperitoneal (i.p.) injections three times per week between weeks 5 and 12. Vehicle does not increase baseline WAT inflammation (Qin et al., 2014). LXA_4 (5(*S*)-6(*R*)-15(*S*)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid) was bought from EMD Millipore, and (1*R*)-$BenzoLXA_4$ was synthesized by P.J.G. (O'Sullivan et al., 2007). Insulin-stimulated glucose tolerance, micro-albuminuria, and urinary H_2O_2 were assessed 1 week prior to sacrifice, and organs were harvested under isoflurane sedation. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

Flow Cytometry Analyses

Leukocytes isolated from homogenized WAT and kidneys were stained and characterized by flow cytometry as pan-MΦs (F4/80PE^{hi}CD11bAPC^{hi}), M1 MΦs (CD11c^{+}PerCPcy5.5 of CD45^{+}F480^{hi}CD11b^{hi} cells), or M2 MΦs (CD206^{+} FITC of CD45^{+}F480^{hi}CD11b^{hi} cells) (Figure S2). B cells (CD19^{+}) and T cells (CD4^{+} versus CD8^{+}) were also identified.

Lipid Mediator Lipidomics

Endogenous LXA_4 production was determined in snap-frozen WAT from SFD and HFD animals (n = 7), using targeted LC-MS/MS-based lipidomics (Colas et al., 2014). One outlier was identified by the ROUT method in the adiponectin$^{-/-}$ HFD group (Figure 6F) and excluded in the paired "Fold increase" analyses (Figure 6G).

Immunohistochemistry

Renal glomerular expansion and matrix deposition were assessed by Periodic Acid-Schiff and tubulointerstitial fibrosis by Sirius Red staining. Livers and WAT were stained for H&E, ki67, and F4/80^{+} MΦs. WAT p62 and LC3-II proteins were detected by immunofluorescence. Staining was quantified by color deconvolution algorithms (Aperio Software).

Protein Analyses

WAT protein (40 μg) was immunoblotted on a 16% SDS-PAGE gel and transferred onto 0.2 μm PVDF membranes. Proteins identified as LC3, p62, pmTOR, mTOR, pAMPK, AMPK, AnxA1, and β-Actin were quantified using Adobe Photoshop.

Adipose and Liver mRNA Expression

RNA was isolated from tissues homogenized in TRIzol. Relative TNF-α, IL-6, and IL-10 mRNA expression was analyzed by the $\Delta\Delta CT$ method using TaqMan primer/probe (Life Technologies) and normalized to 18S.

Liver Function Analyses

Liver tissue (100 mg) suspended in 3 M KOH (in 65% EtOH) was incubated at 70°C for 1 hr, to activate digestion, and diluted 1:3 with 2 M Tris-HCl (pH 7.5). Subsequently, ALT, TGs, and cholesterol E were analyzed in both liver extracts and serum.

In Vitro Experiments

J774 MΦs were incubated with vehicle (0.1% ethanol), LXA_4 (1 nM), or $BenzoLXA_4$ (10 pM) for 16 hr. Supernatants were collected, and MΦ phenotype was determined by flow cytometry as M1 (CD11^{+}) versus M2 (CD206^{+}). Serum-starved hypertrophic 3T3-L1 adipocytes were treated with vehicle, LXA_4 (1 nM), or $BenzoLXA_4$ (10 pM) for 24 hr. Alternatively, adipocytes were treated with the MΦ-conditioned supernatants for 24 hr. Adipocyte supernatant TNF-α, IL-6, and adiponectin levels were assessed by ELISA.

Statistical Analyses

The in vivo study was calculated to an experimental power of 80%. Assuming Gaussian distribution, ANOVA with Bonferroni correction was used to assess statistical significance ($p < 0.05$). Data are presented as mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.05.003.

AUTHOR CONTRIBUTIONS

E.B., K.S., and C.G. designed the experiments and wrote the manuscript. E.B. executed all experiments presented, except the LC-MS/MS-based lipidomics. A.M.F.J. and Y.S.L. assisted in optimization of flow cytometry analyses. A.T.

and G.H.S. assisted in optimization of autophagy analyses. S.T.A.-S. and P.J.G. designed and synthesized the BenzoLXA$_4$ analog. R.A.C., J.D., and C.N.S. carried out LC-MS/MS profiling. All authors actively reviewed and edited the manuscript.

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