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Characterization of Eicosanoids Produced by Adipocyte Lipolysis: IMPLICATION OF CYCLOOXYGENASE-2 IN ADIPOSE INFLAMMATION

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Running Title: COX-2 activation in lipolysis-triggered adipose inflammation

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ABSTRACT

Excessive adipocyte lipolysis generates lipid mediators and triggers inflammation in adipose tissues. However, the specific roles of lipolysis-generated mediators in adipose inflammation remain to be elucidated. In present study, cultured 3T3-L1 adipocytes were treated with isoproterenol to activate lipolysis and the fatty acyl lipidome of released lipids was determined by using LC-MS/MS. We observed that β-adrenergic activation elevated levels of approximately fifty lipid species, including metabolites of cyclooxygenases, lipoxygenases, epoxygenases, and other sources. Moreover, we found that β-adrenergic activation induced cyclooxygenase 2 (COX-2), not COX-1, expression in a manner that depended on activation of hormone-sensitive lipase (HSL) in cultured adipocytes and in the epididymal white adipose tissue (EWAT) of C57BL/6 mice. We found that lipolysis activates the JNK/ NFκB signaling pathway and inhibition of the JNK/ NFκB axis abrogated the lipolysis-stimulated COX-2 expression. In addition, pharmacological inhibition of COX-2 activity diminished levels of COX-2 metabolites during lipolytic activation. Inhibition of COX-2 abrogated the induction of CCL2/ MCP-1 expression by β-adrenergic activation and prevented recruitment of macrophage/ monocyte to adipose tissues. Collectively, our data indicate that excessive adipocyte lipolysis activates the JNK/ NFκB pathway leading to upregulation of COX-2 expression and recruitment of inflammatory macrophages.

Obesity is a global epidemic that is associated with numerous morbidities, such as type 2 diabetes, cardiovascular diseases, hypertension, and certain types of cancers (1-5). There is a growing appreciation that for certain individuals, obesity results in low grade chronic inflammation that largely emanates from the adipose tissue (6,7) and results in systemic insulin resistance (8-10). Adipose tissue inflammation consists of hypertrophied adipocytes that secrete adipokines and free fatty acids into the circulation. An excess of these free fatty acids increases the likelihood of lipotoxicity and the formation of atherosclerotic plaques. The hypertrophied adipocytes also have
impaired functions, including their ability to respond to insulin, which often leads to systemic insulin resistance and eventually type 2 diabetes.

Adipose tissue is known to produce inflammatory cytokines/chemokines that regulate local and systemic pro-inflammatory responses (11). For example, adipose-derived pro-inflammatory chemokines (C-C motif) ligand 2/monocyte chemotactic protein 1 (CCL2/MCP-1) plays a critical role in inflammatory cell recruitment to adipose tissues (12,13) which is important in the pathology of metabolic syndrome (13). Also, interleukin-6 (IL-6) produced by inflamed adipose tissue can contribute to hepatic insulin resistance (14). However, the signaling pathways involved in the production of inflammatory cytokines/chemokines remain elusive. We recently reported that acute activation of \( \beta_3 \)-adrenergic receptors (ADRB3) triggers expression of pro-inflammatory genes including MCP-1, IL-6, PAI-1, among others (15). ADRB3-mediated inflammation mimics inflammation produced by chronic treatments, like high fat feeding, and thus offers a tractable model for investigating molecular mechanisms of adipose tissue inflammation. Importantly, inflammation induced by ADRB3 agonists depends on activation of Hormone Sensitive Lipase (HSL), suggesting involvement of lipolytic products as pro-inflammatory mediators. Using this adipose lipolysis model, we recently showed that adipose ADRB3/HSL signaling pathway activates sphingosine kinase 1 which leads to the production of pro-inflammatory cytokine IL-6 (16).

Adipocyte lipolysis is known to produce lipid mediators, but it is poorly understood how specific mediators regulate pro-inflammatory signaling in adipose tissue. We have established LC-MS/MS methods to quantitatively measure more than 600 species of fatty acyl lipids in a single chromatographic run (17-19). In this study, we characterized the fatty acyl lipidome produced by adipocyte lipolysis using LC-MS/MS lipidomic methods. We observed that adipose lipolysis increases the production of approximately 50 lipid species which are metabolites of cyclooxygenase (COX), lipoxygenases, epoxygenases, and other sources. Furthermore, our data indicate that adipocyte lipolysis up-regulates cyclooxygenases-2 (COX-2), which contributes to adipose pro-inflammatory signaling. Thus, targeting COX-2 may provide a novel means for modulating obesity-induced inflammation.

RESULTS
Lipidomic characterization of lipid mediators generated by the adipocyte ADBR3/HSL-mediated lipolytic process.

Differentiated 3T3-L1 mouse adipocytes were treated with and without the isoproterenol (ISO), a nonselective beta adrenergic agonist, for three hours to induce maximal lipolysis (15). Culture medium was collected and analyzed for the levels of fatty acyl lipids by the LC-MS/MS. We have established LC-MS/MS methods to quantitatively measure more than 600 species of fatty acyl lipids in a single chromatographic run (17-19). We found that approximately sixty lipid species were significantly increased following ISO stimulation (Supplemental Tables 1 and 2), including metabolites of cyclooxygenase (COX), lipoxygenase, and epoxygenase.

Activation of hormone sensitive lipase (HSL) is responsible for approximately 2/3 of the total fatty acids released during beta-adrenergic receptor activation (15,20,21). We then determine which ISO-increased lipid mediators are regulated by the activation of HSL. Differentiated 3T3-L1 mouse adipocytes were pretreated for 1 hour in the presence or absence of BAY 59-9435, a selective HSL inhibitor (15,16), followed by treatment with and without ISO for an additional three hours (15,16). As shown in Figure 1 and Supplemental Table 1, pretreatment with BAY 59-9435 largely eliminated the ISO-induced production of cyclooxygenase, lipoxygenase and epoxygenase metabolites (Figure 1, Supplemental Table 1).

HSL-mediated lipolysis stimulates COX-2 expression.

We next investigated mechanisms underlying increased production of cyclooxygenase metabolites during adipocyte lipolysis. As shown in Figure 2A, isoproterenol upregulated COX-2 mRNA levels. As expected from the lipidomic data, inhibition of HSL with BAY 59-9435 eliminated induction of COX-2 gene expression. This result indicates that induction of COX-2 mRNA by ISO involves HSL-mediated lipolysis. In contrast, expression of COX-1 was unaffected by adrenergic activation or HSL inhibition (Figure 2B). Up-regulation of COX-2 gene expression was
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accompanied by selective induction of COX-2 protein (Figure 2C). Furthermore, selective inhibition of COX-2 activity with celecoxib eliminated production of cyclooxygenase metabolites induced by ISO treatment (Figure 2D, Supplemental Table 2). Our lipidomic analysis identified PGE2 is the most abundance cyclooxygenase metabolite induced by ADRB3/HSL activation (Supplemental Table 1 & 2). Our previous study showed that culture media with respect to incubation time, temperature, and composition of the media greatly affect the stability of lipid species identified using multiple reaction monitoring (MRM) LC-MS/MS method (17). This may be a reason for the difference in minor lipid species identified in different experiments (e.g. 11dh-TXB3 and TXB2 in Figure 1 and 2, respectively).

Lastly, we tested whether activation of adipocyte lipolysis in vivo affected COX-2 expression by injecting mice with the selective β3-adrenergic receptor (ADRB3) agonist CL 316-243 in the presence and absence of HSL inhibition. As shown in Figure 2E, administration of CL 316-243 upregulated levels of COX-2 mRNA, and this effect was abolished by inhibition of HSL with BAY 59-9435. Collectively, our data suggest that adrenergic activation of lipolysis up-regulates COX-2, not COX-1, in vitro and in vivo, leading to the increased production of lipid metabolites of cyclooxygenase pathway.

Adipose lipolysis activates JNK/ NFκB pro-inflammatory signaling axis, leading to COX-2 up-regulation.

We next characterized the mechanism by which lipolysis regulates COX-2 activity and expression. We previously reported that adipose ADRB3-triggered lipolysis activates stress kinases such as c-Jun N-terminal kinase (JNK) (15,16). As shown in Figure 3A, pharmacological inhibition of JNK significantly abolished up-regulation of COX-2 by isoproterenol. NFκB is a known downstream molecular target that is activated in response to JNK activation (22-24), and NFκB has been found to induce COX-2 gene expression (25-28). Therefore, we examined whether adipocyte lipolysis activates NFκB. Treatment of 3T3-L1 adipocytes with isoproterenol led to the progressive translocation of NFκB into the cell nucleus (arrows, Figure 3B), indicating NFκB activation. Moreover, the ISO-induced COX-2 up-regulation was significantly attenuated by pharmacological inhibition of NFκB with BAY 11-7082 (Figure 3C). These data suggest that induction of COX-2 gene expression depends on activation of the JNK/ NFκB signaling pathway.

Next, we investigated the HSL-mediated signaling pathways which contribute to the activation of JNK/NFκB pathway, leading to COX-2 up-regulation. Activation of HSL generates free fatty acids including palmitic and oleic acids from triglyceride. Treatment of 3T3-L1 adipocytes with palmitic acid rapidly activated JNK kinases, as indicated by the phosphorylation status of p54\(^{JNK}\) and p46\(^{JNK}\) (Figure 3D). In addition, palmitate treatment resulted in parallel phosphorylation of IκB\(α\) and elevation of COX-2 protein levels (Figure 3D). The rapid phosphorylation of IκB\(α\) and expression of COX-2 protein suggests substantial amplification of signals generated by JNK activation (i.e. pp56\(^{JNK}\) and pp46\(^{JNK}\)). Collectively, these data suggest that free fatty acids produced by HSL activation stimulate the JNK/NFκB/COX-2 signaling axis in adipocytes.

COX-2 inhibition significantly suppressed the ISO-stimulated NFκB activation (Figure 3E), indicating that COX-2 activity is required for NFκB activation. In contrast, celecoxib did not affect ISO-stimulated phosphorylation of IκB\(α\) and elevation of COX-2 protein levels (Figure 3D). COX-2 inhibition significantly suppressed the ISO-stimulated NFκB signaling axis in adipocytes.

Lipolysis-stimulated CCL2/ MCP-1 expression is dependent on JNK/ NFκB/ COX-2 pathway.

We previously showed that stimulation of lipolysis activates JNK and p38 kinases, leading to the up-regulation of pro-inflammatory cytokines including CCL2/MCP-1 and IL-6 in adipocytes (15). Therefore, we examined whether the lipolysis regulates the expression of pro-inflammatory cytokines via the JNK/ NFκB/ COX-2 signaling pathway. Inhibition of JNK significantly diminished the lipolysis-increased expression of CCL2/ MCP-1 and IL-6 (Figure 4A). In contrast, p38 inhibition had no significant effect on CCL2/ MCP-1 and IL-6 up-regulation (Figure 4B). Strikingly, we observed that NFκB inhibition completely abrogated the lipolysis-increased CCL2/ MCP-1 expression, but had no effect on induction of IL-6 expression (Figure 4C).
Similarly, COX-2 inhibition suppressed induction of CCL2/ MCP-1, but had no effect on IL-6 (Figure 4D).

To determine whether expression of COX2 is sufficient to induce expression of CCL2 in the absence of isoproterenol, we acutely transduced 3T3-L1-CAR cells with COX2 expressing adenovirus (16). As shown in Figure 4E, expression of COX-2 rapidly stimulated the production of CCL-2 without affecting expression of IL-6. These results suggest that CCL2/ MCP-1, but not IL-6, is a downstream target of the lipolysis-activated JNK/ NFκB/ COX-2 signaling pathway.

**COX-2 inhibition abrogates ADRB3-stimulated CCL2/ MCP-1 up-regulation in mice.**

Next, we injected C57BL/6 male mice with and without CL 316-243 to induce the adipose tissue lipolysis (15,16). Mice were pre-treated with or without celecoxib to evaluate the role of COX-2 in acute lipolysis-triggered adipose inflammation. As shown in Figure 5A, 5B, and 5C, CL 316-243 treatment markedly increased mRNA and protein levels of COX-2 in epididymal white adipose tissue (EWAT), whereas CL 316-243 treatment had no effect on COX-1 levels. Furthermore, administration of CL 316-243 significantly induced CCL2/ MCP-1 expression when mice were pre-treated with control vehicle (n=5; p < 0.01, CL(-)/ Cel(-) vs. CL(+)/ Cel(-), Two-way ANOVA) (Figure 5D). Also, celecoxib treatment significantly diminished CL 316-243-stimulated up-regulation of CCL2/ MCP-1 (n=5; p < 0.01, Two-way ANOVA) (Figure 5D). In contrast, celecoxib treatment did not diminish the CL 316-243-induced IL-6 increase (Figure 5E). These results support our *in vitro* observations and suggest that the adipose lipolysis activates COX-2, which ultimately leads to the up-regulation of CCL2/ MCP-1 in adipose tissues.

**COX-2 regulates adipose macrophage/ monocyte infiltration induced by lipolysis.**

CCL2/ MCP-1 is known to be a critical pro-inflammatory chemokine which promotes the recruitment of macrophages/ monocytes to the site of inflammation (29-31). Therefore, we examined whether the lipolysis stimulates the recruitment of macrophages/ monocytes into adipose tissues, and whether the macrophage/ monocyte recruitment is mediated by COX-2 activity. Hematoxylin and eosin staining of gonadal WAT suggested that ADRB3 activation leads to tissue extravasation and immune cell infiltration (data not shown). As shown in Figure 6, administration of CL 316-243 significantly increased the infiltration of macrophages/ monocytes into adipose tissues, as determined by immunohistochemistry for F4/80. Moreover, the increased macrophage/ monocyte infiltration was completed abrogated by COX-2 inhibition. The immunohistochemical observation of COX-2-dependent macrophage recruitment was further supported by qPCR analysis for levels of Mac-2 (Figure 6C), a macrophage cell surface protein (32).

Collectively, our results indicate that free fatty acids generated from the HSL-mediated lipolysis activate the JNK/ NFκB/ COX-2 signaling axis in adipose tissues (Figure 6D). Subsequently, COX-2 activation stimulates CCL2 expression, leading to the infiltration of immune cells in adipose tissue.

**DISCUSSION**

Previous work has shown that excessive lipolysis is associated with adipose tissue inflammation and immune cell infiltration (15,16,33). However, pro-inflammatory lipid mediators produced by adipocyte lipolysis remain to be defined. The present study utilized the LC-MS/MS to profile the fatty acid lipidome generated during adipocyte lipolysis. Our data suggest that COX-2 is responsible for elevating levels of prostaglandins, prostacyclins, and thromboxanes from arachidonic acid in response to adrenergic activation of lipolysis. COX-2 was shown to be a critical inflammatory molecule which is induced in various tissues and in obese individuals (34-36). Therefore, we decided to focus on characterizing the involvement of COX-2 in lipolysis-triggered adipose inflammation in present study. Our results show that COX-2 expression is significantly induced and activated in cultured adipocytes and adipose tissue upon beta-adrenergic activation. The up-regulation of COX-2 was inhibited by selective pharmacological inhibition of HSL, indicating that lipolysis-increased COX-2 expression is dependent on HSL activity. Furthermore, our study suggests that COX-2 upregulation of CCL2 production may play an important role in immune cell infiltration of adipose tissue.
Previously, we reported that adipose lipolysis activates JNK and p38 stress kinases, which play important roles in lipolysis-stimulated production of pro-inflammatory cytokines/chemokines (15,16). In present study, we observed that beta adrenergic activation induced nuclear translocation of NFκB, a key inflammatory regulator. Moreover, pharmacological inhibition of either JNK or NFκB suppressed the lipolysis-induced COX-2 up-regulation. Lipolysis is known to generate free fatty acids by hydrolyzing triglycerides. Direct treatment of adipocytes with palmitate, a free fatty acid, activates the JNK/NFκB/COX-2 signaling pathway. These results together suggest that free fatty acids produced by adipose lipolysis activate the JNK/NFκB pathway, leading to COX-2 up-regulation. Also, we showed that lipolysis-induced expression of IL-6 is mediated by JNK activation (15). However, unlike CCL2, pharmacological inhibition of NFκB or COX-2 had no effect on the lipolysis-stimulated IL-6 expression. In this regard, we recently reported that lipolysis-induced upregulation of IL-6 is mediated by production of sphingosine-1-phosphate, and this pathway requires upregulation of sphingosine kinase 1 (SphK1) via the JNK/AP-1 pathway (16). Collectively, these results indicate that the regulation of CCL2 and IL-6 both involve the generation of lipid mediators, but the specific pathways (COX2 and SphK1) diverge following JNK activation.

In the present study, we found that lipolysis triggers an acute infiltration of macrophages/monocytes into adipose tissues which is mediated by the JNK/NFκB/COX-2 signaling axis. The physiological or patho-physiological significance of the lipolysis-driven macrophages/monocytes infiltration awaits future investigation. Our previous studies suggest that lipolysis-driven infiltration of macrophages/monocytes regulates inflammation, apoptosis, and remodeling of adipose tissues (15,16,21,37). In addition, it has been suggested that adipose macrophages can buffer local fatty acid concentrations by uptake of fatty acids and suppression of adipocyte lipolysis (33).

How COX-2 could potentially regulate the expression of CCL2/MCP-1 in adipocytes is not yet known. Our lipidomic analysis showed that PGE2, a pro-inflammatory prostaglandin involved in numerous inflammatory processes (38-41), is one of the most abundant lipidomic metabolites generated from the COX-2 enzyme during lipolysis. Also, it has been reported that PGE2 treatment upregulates MCP-1/CCL2 expression in mesangial cells (42). However, we were unable to demonstrate that exposure of 3T3-L1 adipocytes to PGE2 alone (up to 50 µM for up to 24 hr) could upregulate CCL2 (not shown). Furthermore, various combinations of other prostaglandins (e.g. PGD2, PGJ2, d12-PGJ2, 0-25 µM) were also ineffective. It is possible that exogenous PGE2 dampens COX-2-dependent pro-inflammatory signaling by activating the EP4 receptor (43-46). Thus, we speculate that either a combination of prostaglandins and/or other lipid mediator(s) could be responsible for the COX-2-mediated up-regulation of CCL2/MCP-1. Alternatively, it is possible that upregulation of CCL2/MCP-1 is mediated by intracellular effects of eicosanoids generated by COX-2 pathway. Future studies are needed to reveal the molecular link between specific lipolysis-stimulated COX-2 products and CCL2/MCP-1 expression in adipocytes.

We observed that celecoxib treatment alone slightly, but significantly, increased levels of COX-2, CCL2, an IL-6 in animal gonadal WATs (Figure 5). The mechanism for elevated expression of those adipose inflammatory markers by COX-2 inhibition alone is currently unknown. We found that noticeable quantity of PGE2 is secreted by cultured adipocytes, and levels of PGE2 was significantly reduced by celecoxib treatment (Figure 2D and Supplemental Table 2). As discussed earlier, PGE2 was shown to suppress lipolysis (43-46). Thus, it is possible that the basal level of PGE2 secreted by adipocytes functions to suppress adipose lipolysis and inflammation, and this process would be reversed somewhat by COX-2 inhibition.

In summary, our data reveal that lipolysis induces large changes in the fatty acyl lipidome of adipocytes, including the production of pro-inflammatory mediators. Furthermore, our study delineates a pro-inflammatory pathway involving COX-2 activation, induction of CCL2 expression, and infiltration of immune cells in adipose tissue. Our results indicate that lipid mediators derived from lipolysis activate divergent pathways, and that an understanding of these pathways could define new approaches to
controlling adipose tissue inflammation and associated metabolic dysfunction.

EXPERIMENTAL PROCEDURES

Reagents

Isoproterenol (ISO) (Sigma) was dissolved in H₂O. BAY 59-9435 (BAY), chemically synthesized as described (47), was dissolved in 0.5% methylcellulose. CL 316-243 (CL) (Sigma) was dissolved in H₂O. Cyclooxygenase-2 inhibitor, celecoxib (Sigma), was dissolved in DMSO. JNK inhibitor (SP-600125, Calbiochem) and NFκB inhibitor (BAY 11-7082, Calbiochem) were dissolved in DMSO. Antibodies against COX-1, COX-2, phospho-HSL, HSL, phospho-JNK, JNK, phospho-IκBα were from Cell Signaling. Polyclonal rabbit anti-F4/80 and anti-GAPDH was purchased from Abcam and Santa Cruz, respectively. Other reagents, unless specified, were from Sigma.

Cell culture

3T3-L1 and 3T3-L1-CAR cells were cultured and differentiated as previously described (15,16). Two days post-differentiation, cells were cultured overnight in serum-free DMEM. Subsequently, media were replaced with phenol red free plain DMEM. Cells were treated with 10 µM of ISO or PBS control for 3 hours at 37°C. Alternatively, cells were pretreated with a selective HSL inhibitor BAY59-9435 (10 µM), JNK inhibitor (SP-600125, 10 µM), NFκB inhibitor (BAY 11-7082, 10µM), or cyclooxygenase-2 inhibitor (celecoxib, 5 µM) for 1 hour, followed by stimulating with or without isoproterenol (ISO, 10 µM) for an additional 3 hours. Cell pellets and culture media were collected and processed for biochemical analysis and lipids quantification by LC-MS/MS methods, respectively, as described below. Transduction of 3T3-L1-CAR cells with adenoviral particles was performed essentially as we previously described (16).

Fatty acyl extraction from cell culture media

Culture media were added with Internal Standard mixture (5 ng each of 15(S)-HETE-d8, LTB4-d4, and PGE1-d4, delivered in 5 µl methanol) (48), followed by the addition of methanol to a final concentration of 15%. The samples were mixed thoroughly and stood at room temperature for 30-60 minutes. The samples were then applied to Strata-X cartridges that were preconditioned with methanol. The sample tube and cartridge were each rinsed twice with 1 mL of 15% methanol and dried briefly. Then, the cartridge was washed with 2 mL hexane and dried. The cartridge was eluted with 0.5 mL methanol containing 0.1% formic acid into a 1 mL glass vial. The eluate was evaporated to dryness with nitrogen gas. The lipid extracts were reconstituted with 30 µL methanol and 30 µL 25 mM ammonium acetate in MilliQ water, and used for LC-MS/MS fatty acyl analysis as described below.

LC-MS/MS quantification

LC-MS/MS quantification was performed as described (17,48). For LC-MS/MS analysis, reverse phase HPLC was performed using C18 column (Luna, C18, 3 µm, 2 mm × 150 mm, Phenomenex, CA) using a gradient elution on Waters Alliance 2695 system (Waters Corporation, MA). The mobile phase consisted of methanol, water, acetonitrile, and ammonium acetate. Solvent A: methanol:10 mM aqueous ammonium acetate:acetonitrile (85:10:5, v/v); Solvent B: methanol:10 mM aqueous ammonium acetate:acetonitrile (10:85:5, v/v). The column was eluted isocratically from 0 to 10 min at 55% A followed by a linear gradient to 100% A from 10 to 20 min. Samples were injected using the autosampler (an integral part of the Waters Alliance 2695 system) maintained at 10 ± 2 °C and the injection volumes were 10 µl for each sample. Total injection cycle for each sample was 25 min including column equilibration to initial conditions. The flow rate was 0.2 ml/min. The HPLC eluent was directly introduced to Quattro LC mass spectrometer (Micromass-Waters, MA). The mass spectrometric detector settings were as follows: ESI needle voltage, 2.8 kV; source block temperature, 120 °C; desolvation temperature, 350 °C; desolvation gas flow, 540 l/h; nebulizer gas flow, 80 l/h; and the collision gas pressure was 3.2 × 10⁻⁴ bar. Cone voltage and collision energy for each MRM transition were optimized. Chromatographic data were analyzed by Quanlynx module of the Masslynx software (Waters Corporation, MA) to integrate the chromatograms for each MRM transition.

Animal studies

All animal procedures were performed according to the NIH and institutional guidelines, and were approved by the Wayne State University Animal Use and Care Committee. C57BL/6 mice (8 weeks old male, Jackson Laboratory) were used in this
study. To examine the role of ADRB3/HSL signaling in the regulation of COX-2 expression, mice were intraperitoneally (i.p.) injected with the selective HSL inhibitor, BAY59-9435 (30 mg/kg), celecoxib (100 mg/kg body weight), or vehicle control as previously described (20,21,49). One hour later, mice were i.p. injected with 10 nmol of CL 316-243 or saline for additional 3 hours (20,21). Mice were euthanized, and the epididymal white adipose tissue (EWAT) pads were collected and processed for biochemical and immunohistochemical analysis as described below.

**Real-time PCR**

Total RNA was isolated from cultured cells using Trizol and was reversely transcribed with an oligo-dT primer (Promega) by M-MLV Reverse Transcriptase (Promega) for first strand cDNA synthesis. Total RNA was isolated from the EWAT using liquid nitrogen and a mortel and pestel to grind the tissue in to a powder and then Trizol was added. Then, the RNA was reversely transcribed with an oligo-dT primer (Promega) by M-MLV Reverse Transcriptase (Promega) for first strand cDNA synthesis. For real-time PCR quantitation, 50 ng of reversely transcribed cDNAs were amplified with the ABI 7500 system (Applied Biosystems) in the presence of SYBR Green master mix. PCR primer pairs used were: mouse PTGS1 (COX-1): sense, 5’-- ACA AAA GAA CCC AGT GTC CA - 3’, anti-sense, 5’ -AGA ACT GTG GTG GTT TTC AA - 3’; mouse PTGS2 (COX-2): sense, 5’ -TGA TCG AAG ACT ACG TGC AA - 3’, anti-sense, 5’ -GTG AGT CCA TGT TCC AGG AG - 3’; mouse GAPDH: sense, 5’ – CAC CTT CGA TGC CGG GGC TG - 3’, anti-sense, 5’ – GGC CAT GAG GTC CAC CAC CC - 3’; mouse CCL2: sense, 5’ - CAC AGT TGC CGG CTG GAG CAT – 3’; anti-sense, 5’ - GCT TCT TTG GGA CAC CTG C - 3’; and mouse Mac-2: sense, 5’ - AGG AGA GGG AAT GAT GTT GCC – 3’, anti-sense, 5’ - GGT TTG CCA CTC TCA AAG GG - 3’.

Protein extraction procedure and western blot analysis were performed as described (50). Cells were collected in ice-cold PBS using cell scrapers followed by centrifugation (250 x g, 5 min). Cell extracts were prepared in RIPA buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (Calbiochem) with constant agitation at 4 °C for 30 min. After centrifugation at 15,000 x g for 20 min, supernatant was collected and protein concentration was measured using a bicinchoninic acid protein assay kit with BSA as standard. 50 μg of protein extracts were dissolved in 2× Laemmli sample buffer, heated at 95 °C for 5 min, and resolved on a 10% SDS-PAGE gel. After electrophoresis, gels were transferred to nitrocellulose membranes. Subsequently, membranes were blocked in 5% non-fat dry milk (Lab Scientific) in TBST buffer (20 mM Tris–HCl, pH 7.4, 500 mM NaCl and 0.05% Tween-20). Membranes were washed and incubated with the indicated primary antibodies (1:1000 dilution) on a rotary shaker at 4 °C overnight. The blots were then incubated with peroxidase-conjugated goat anti-rabbit secondary antibody for 1 h at room temperature, and developed with enhanced chemiluminescent reagent (Thermo Scientific).

**Immunohistochemical staining**

The immunohistochemical staining procedure followed the protocol from the Vector Laboratories Vectastain Universal Elite ABC Kit (Anti-mouse IgG/ Rabbit IgG, Cat. No. PK-6200). Briefly, mouse EWAT tissues were fixed in 10% formalin followed by paraffin embedding. Paraffin sections (5 μm) were performed antigen retrieval in citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) at 90°C for 10 min, and then deparaffinized by incubating the slides in xylene followed by a graded series of ethanol and then water. Endogenous peroxidase activity was quenched with 0.3% H2O2 for 5 minutes. After washes, sections were incubated with blocking serum (normal horse serum) for 20 minutes. Subsequently, samples were incubated with anti-F4/80 (1:200 dilution in PBS) at 4°C for overnight. The slides were then washed with PBS and incubated with the diluted biotinylated secondary antibody for 30 minutes. After washing with PBS, Vectastain ABC Reagent was applied to the slides for 30 minutes. After washing with PBS, DAB substrate reagent was
added to the slides for 10 minutes and then washed several times with water. Slides were examined and analyzed using the Leica inverted microscope and the image acquisition was from the SPOT Pursuit monochrome digital camera.

Statistical analysis

Results are shown as mean ± SD. Differences between various treatments were analyzed by ANOVA. Statistical significance was measured by student’s t – test. p value < 0.01 is considered highly significant and p < 0.05 is considered statistically significant.

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Author contribution: AG, JZ, and SC designed the study and conducted experiments. EM designed study and performed animal experiments. GCV and YHA chemically synthesized HSL inhibitor. KRM lipidomic analysis of lipid mediators. AS provided research reagent. AG, JG, and MJL designed experiments and prepared the manuscript.

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Abbreviations: COX, cyclooxygenase; ADRB3, β3-adrenergic receptor; BAY, BAY 59-9435, selective HSL inhibitor; CL, CL-316243, specific agonist of β3-adrenergic receptor; EWAT, epididymal white adipose tissues; HSL, hormone sensitive lipase; IL-6, interleukin 6; ISO, isoproterenol, specific agonist of β-adrenergic receptor.

FIGURE LEGENDS

FIGURE 1: The generation of lipid mediators by ADRB3 activation is dependent on HSL activity. Differentiated 3T3-L1 mouse adipocytes were treated with ISO (10 µM) or PBS for 3 hours in the presence and absence of BAY 59-9435 (BAY) (10 µM), a specific HSL inhibitor. The media was collected and analyzed by LC-MS/MS lipidomic methods: lipid metabolites of the (A) cyclooxygenase, (B) lipoxygenase, (C) epoxygenase, and (D) other metabolites following treatments. (D) LA, metabolites of linoleic acid; EPA, metabolites of eicosapentaenoic acid; DHA, metabolites of docosahexaenoic acid. Data are from a representative experiment which was repeated three times with similar results. Statistical analysis shows that all listed lipids are less than 0.05 (t-test) in comparing control vs. ISO and ISO vs. BAY + ISO (Supplemental Table 1). Note that BAY treatment significantly inhibits the ISO-increased lipid species.

FIGURE 2: Adipose ADRB3/ HSL signaling pathway up-regulates cyclooxygenase-2 (COX-2), not COX-1 expression. Differentiated 3T3-L1 cells were treated with or without ISO (10 µM) for 3 hours in the presence and absence of BAY 59-9435 (10 µM). mRNA levels of COX-2 (A) and COX-1 (B) were measured by qPCR analysis and protein levels (C) were assessed by Western-blotting analysis. (D) Differentiated 3T3-L1 cells were pretreated with celecoxib (5 µM) or control vehicle for 1 hour, followed by stimulation with or without ISO for 3 hours. Lipid metabolites of cyclooxygenase pathway were quantitated by LC-MS/MS lipidomic method. Right panel, Heat map analysis. *P values of all shown lipid species are less than 0.05 (t-test) in comparing control vs. ISO and ISO vs. celecoxib + ISO (Supplemental Table 2). (E) C57BL/6 mice were intraperitoneally (i.p.) injected with BAY 59-9435 (30 mg/kg) for 1 hour, followed by i.p. injection with CL 316-243 (10 nmol). 3 hours later, the epididymal white adipose tissue (EWAT) was analyzed for COX-2 mRNA levels by qPCR analysis. Data represent mean ± SD of triplicate determinations. Each panel was repeated at least two times with similar result. *, p < 0.05, t – test

FIGURE 3: ADRB3 activation-induced COX-2 up-regulation is mediated by JNK/ NFκB signaling pathway. (A) Differentiated 3T3-L1 cells were treated with ISO (10 µM) in the presence and absence of JNK inhibitor (SP-600125, 10 µM). mRNA levels of COX-2 were quantitated by qPCR analysis. Data are mean ± SD (n=3; **, p < 0.01, t-test). (B) Differentiated 3T3-L1 cells were treated with ISO for 0, 15, and 30 minutes followed by immunostaining with anti-NFκB. Note that ISO treatment progressively increases nuclear localization of NFκB (arrow in middle and lower panels). Red = NFκB, Blue = DAPI nuclear staining. (C) 3T3-L1 cells were treated with ISO in the presence and absence of an NFκB inhibitor (BAY
mRNA levels of COX-2 were quantitated by qPCR analysis. Data are mean ± SD (n=3; **, *p < 0.01, t-test). (D) Differentiated 3T3-L1 cells were treated with palmitic acid (PAL, 0.5 mM) for various times. Cellular extracts were blotted with indicated antibodies. Lower panel, intensity of western-blotting were quantitated with NIH Image J software (normalized to GAPDH). n =3; * and **, p < 0.05 and 0.01, respectively; t-test. (E) Differentiated 3T3-L1 cells were treated with ISO (10 µM) in the presence and absence of COX-2 inhibitor (Cel, celecoxib, 5 µM) for 30 min. Cellular extracts were blotted with indicated antibodies. Lower panel, data represent mean ± SD (n=3; **, p < 0.01; NS, non-statistical significance; t-test).

FIGURE 4: Lipolysis-increased CCL2/ MCP-1 expression is dependent on JNK/ NFκB/ COX-2 signaling pathway. Differentiated 3T3-L1 cells were pretreated with or without an inhibitor of JNK (SP 600125, 10 µM) (A), p38 kinase (SB 203580, 10 µM) (B), NFκB (BAY 11-7082, 10 µM) (C), and COX-2 (celecoxib, 5 µM) (D) for 1 hour. Subsequently, cells were stimulated with or without ISO (10 µM) for an additional 3 hours. Levels of CCL2/ MCP-1 (left panels) and IL-6 (right panels) were quantitated by qPCR analysis. Data represent mean ± SD of a representative experiment (n = 3), which was repeated at least two times with similar results. **, *p < 0.01; *, p < 0.05; NS, not statistically significant; t-test. (E) 3T3-L1-CAR cells were transduced with a multiplicity of 200 of adenoviral particles carrying rat COX-2 (Ad-COX-2) for indicated times. Expression levels of CCL2, IL-6 (Left panel), or rat COX-2 (right panel) were measured by qPCR analysis. Note that ectopic expression of COX-2 significantly increased CCL2 expression. Data are mean ± S.D. of triplicate determinations (**, p < 0.01; t-test).

FIGURE 5: COX-2 activity is required for the ADRB3-stimulated CCL2 up-regulation in animal adipose tissues. C57BL/6 mice (male, 8 weeks old) were i.p. injected with celecoxib (100 mg/kg) or control vehicle for 1 hour. Subsequently, mice were i.p. injected with or without CL 316-243 (10 nmol). Three hours later, epididymal white adipose tissues (EWAT) were collected, and measured for levels of COX-2 (A), COX-1 (B), CCL2/ MCP-1 (D), and IL-6 (E) by qPCR analysis. **, *p < 0.01; NS, not statistically significant (n =5, Two-way ANOVA). (C) Protein levels of COX-2 and COX-1 were measured by Western-blotting analysis.

FIGURE 6: COX-2 activity is required for the ADRB3-stimulated macrophage/monocyte infiltration in adipose tissues. C57BL/6 mice (male, 8 weeks old) were i.p. injected with celecoxib (100 mg/kg) or control vehicle for 1 hour. Subsequently, mice were i.p. injected with or without CL 316-243 (10 nmol). Three hours later, epididymal white adipose tissues (EWAT) were collected. (A) Paraffin sections (5 µm) of EWAT were immunohistochemically stained with anti-F4/80. a, vehicle; b, CL 316-243; c, celecoxib alone; d, celecoxib + CL 316-243; e and f, enlarged image of boxed area in a and b, respectively. Arrows, infiltrated macrophages/monocytes. (B) Macrophages/ monocytes present in each treatment (4-5 microscopic fields) were scored. *, p < 0.05, t-test. (C) Levels of Mac-2, a macrophage maker, were quantitated by qPCR. *, p < 0.05, t-test. (D) A model of our findings, revealing a COX-2-mediated mechanism through which the HSL-driven lipolysis stimulates macrophages/ monocytes infiltration into adipose tissues. Various ways to inhibit this signaling pathway may reduce adipose inflammation triggered by acute lipolytic process.
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