Lipidomic Profiling of High-Fat Diet-Induced Obesity in Mice: Importance of Cytochrome P450-Derived Fatty Acid Epoxides

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Objective: Enzymatic metabolism of polyunsaturated fatty acids leads to formation of bioactive lipid metabolites (LMs). Previous studies have shown that obesity leads to deregulation of LMs in adipose tissues. However, most previous studies have focused on a single or limited number of LMs, and few systematical analyses have been carried out.

Methods: A LC-MS/MS-based lipidomics approach was used, which can analyze >100 LMs produced by cyclooxygenase, lipoxygenase, and cytochrome P450 (CYP) enzymes, to analyze the profile of LMs in high-fat diet-induced obesity in mice.

Results: LC-MS/MS showed that high-fat feeding significantly modulated profiles of LMs in adipose tissues. Among the three major polyunsaturated fatty acid metabolizing pathways (cyclooxygenase, lipoxygenase, and CYP), CYP-derived fatty acid epoxides were the most dramatically altered LMs. Almost all types of fatty acid epoxides were reduced by 70% to 90% in adipose tissues of high-fat diet-fed mice. Consistent with the reduced levels of fatty acid epoxides, the gene expression of several CYP epoxygenases, including Cyp2j5, Cyp2j6, and Cyp2c44, was significantly reduced in adipose tissues of high-fat diet-fed mice.

Conclusions: Results show that CYP-derived fatty acid epoxides are the most responsive LMs in high-fat diet-induced obesity, suggesting that these LMs could play critical roles in obesity.

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Introduction

Obesity is a major health concern in the U.S.: more than one-third of U.S. adults (34.9% or 78.6 million) have obesity (1). Individuals with obesity have significantly increased risks of developing many diseases, including cardiovascular diseases, hypertension, type II diabetes, and certain types of cancer (2). The annual medical care cost to treat obesity and obesity-associated diseases is estimated to be around $190 billion in the U.S. (3). Therefore, it is of critical importance to better understand the mechanism by which obesity increases the risks of various human diseases, which could facilitate the development of effective therapeutic strategies.

The enzymatic metabolism of polyunsaturated fatty acids (PUFAs), such as arachidonic acid (ARA, 20:4ω-6), leads to formation of bioactive lipid metabolites (LMs), which are important lipid signaling molecules involved in the regulation of many fundamental physiological and pathological processes (4-6) (see a simplified scheme in Supporting Information Figure S1a; the abbreviations of LMs are in Supporting Information Table S1). There are three major pathways involved in enzymatic metabolism of PUFAs: cyclooxygenase (COX-1 and COX-2), lipoxygenase (5-LOX and 12/15-LOX), and cytochrome P450 (CYP). The COX pathway leads to formation of prostaglandins, which are important mediators to induce inflammation and pain; and COX-2 is the therapeutic target of many anti-inflammatory drugs on the market (4). The LOX pathway leads to formation of leukotrienes and hydroxyl fatty acids, which are predominately proinflammatory and play critical roles in inflammatory diseases such as asthma (4). The CYP pathway converts PUFAs to fatty acid epoxides, which have a variety of beneficial effects such as anti-
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Inflammatory, cardioprotective, vasodilative, and analgesic actions (5,6). Besides ARA, other PUFAs, including linoleic acid (LA, 18:2ω-6), α-linolenic acid (α-LA, 18:3ω-3), γ-linolenic acid (γ-LA, 18:3ω-6), dihomoga-Linolenic acid (DGLA, 20:3ω-6), eicosapentaenoic acid (EPA, 20:5ω-3), and docosahexaenoic acid (DHA, 22:6ω-3), are also efficient alternative substrates of these enzymes and are converted to the corresponding LMs with unique biological activities (6-8) (Supporting Information Figure S1b-f). Together, this leads to formation of a large array of LMs with diverse chemical structures, many of which have potent biological activities.

Previous research has shown that LMs play critical roles in the pathology of obesity. In obese Zucker rats, there is attenuated production of prostacyclin (PGI2), which is a LM produced by COX enzymes with potent anti-inflammatory and vasodilative effects (9). Reduced levels of this beneficial LM could contribute to increased adipose inflammation and impaired adipose tissue blood flow (ATBF) in obesity. Dietary feeding of a high-fat diet (HFD) increased tissue levels of LOX-derived leukotriene B4 (LTB4); and inhibition of LTB4 receptor protected mice from HFD-induced insulin resistance and hepatic steatosis (10), suggesting that the LOX pathway contributes to increased risks of obesity-associated diseases. HFD also reduced levels of CYP-derived epoxyeicosatrienoic acids (EETs), which have potent anti-inflammatory, vasodilative, and cardioprotective effects (11-13). Pharmacological inhibition or transgenic deletion of soluble epoxide hydrolase (sEH, the dominant enzyme in degrading EETs) has been shown to protect mice from various adverse effects induced by obesity (14-21). Together, these results support that LMs play critical roles in regulating the pathology of obesity.

Most previous studies of LMs in obesity have only studied single or limited numbers of LMs (9,10,14-21). However, the enzymatic metabolism of PUFAs produces hundreds of LMs, which could have different or even opposite biological activities, it would be difficult to understand the biological processes by only studying single or limited number of LMs (22). Therefore, it is important to conduct comprehensive profiling of a variety of LMs in tissues, which could help us to better understand their roles in the pathology of obesity, in order to develop novel biomarkers or therapeutic targets for obesity and obesity-associated diseases. To this end, here we used a LC-MS/MS-based lipidomics approach, which can simultaneously measure the concentrations of >100 LMs produced by COX (COX-1, COX-2), LOX (5-LOX, 12/15-LOX), and CYP enzymes from ARA, LA, α-LA, DGLA, EPA, and DHA (23,24) (see Supporting Information Table S1), to systematically analyze how lipid signaling is deregulated in obesity.

**Methods**

**Obesity experiment**

The animal experiment was conducted in accordance with the protocols approved by the Institutional Animal Care and Use Committee of UMass-Amherst. C57BL/6 male mice (6 weeks of age) were maintained on HFD (60% kcal% fat, purchased from Research Diet Inc., catalog number D12492) and a control diet (10 kcal% fat, D12450f from Research Diet Inc.) for 8 weeks. Diet information can be found at [http://www.researchdiets.com/opendisse-diets/stock-diets/dio-series-diets](http://www.researchdiets.com/opendisse-diets/stock-diets/dio-series-diets).

**LC-MS/MS-based lipidomics analysis**

To extract LMs from adipose tissues, ~100 mg tissues were mixed with an antioxidant solution (0.2 mg/mL butylated hydroxytoluene and 0.2 mg/mL triphenylphosphine in methanol), 10 μL of deuterated internal standards (500 nM of d4-6-keto PGL6, d4-TXB2, d4-PGE2, d4-LTB4, d11-14,15-DHET, d11-9-HODE, d8-5-HETE, d11-11,12-EET), and 400 μL extract solution (0.1% acetic acid with 0.2 mg/mL butylated hydroxytoluene in methanol) and then homogenized; the resulting homogenates were kept in −80°C overnight. After centrifugation of the homogenates, the pellets were washed with methanol (containing 0.1% butylated hydroxytoluene and 0.1% acetic acid) and then combined with the supernatant. The LMs in the combined solutions were loaded onto pre-washed Waters Oasis® solid phase extraction (SPE) cartridges and washed with 95:5 v/v water/methanol with 0.1% acetic acid, and the eluates were eluted with methanol and ethyl acetate and dried using a centrifugal vacuum evaporator, then reconstituted in methanol for LC-MS/MS analysis. The LC-MS/MS analysis was carried out on an Agilent 1200SL HPLC system (Agilent, Santa Clara, CA) coupled to a 4000 QTRAP® MS/MS (AB Sciex, Foster City, CA) as described in our previous report (24). The lipid mediators whose levels were above the detection limit of LC-MS/MS were reported.

**Real-time PCR analysis**

Total RNA was isolated from gonadal adipose tissues using TRIzol Reagent (Life Technologies, Carlsbad, CA) according to manufacturer’s instruction. Conversion of up to 2 μg of total RNA to single stranded cDNA was preformed using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) according to manufacturer’s instruction. Quantitative real-time PCR (RT-PCR) was conducted using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Agawam, MA) on a DNA Engine Opticon® 2 System (Bio-Rad Laboratories, Hercules, CA) with specific mouse primers. The primers used in this research were: Cyp2β (sense) 5′-CTCTGGAAGACTCCATCTCA-3′ and (antisense) 5′-CCTCCTTTGTTAGTTTGG-3′, Cyp2β (sense) 5′-TTCAGGCACAGTCC-3′ and (antisense) 5′-TCGGGGGATAGTTCTTGG-3′, Cyp2c44 (sense) 5′-GCTGCTTACTACAGATGCCG-3′ and (antisense) 5′-TTCAGGCACAGTCC-3′, Ehh2 (sense) 5′-GGGTCTGAGCGAGTGG-3′ and (antisense) 5′-TGTAGCTTCCATCATGTTG-3′, Alox15 (sense) 5′-GGTCCCAACAGCGAAGTGTCATAC-3′ and (antisense) 5′-AGTTATTTGCACTACATCACCCTTT-3′, Alox3 (sense) 5′-ACTACATCTACCTCATCACTATCAT-3′ and (antisense) 5′-GTTGACATCGTGGAGTTCCAC-3′, Cox2 (sense) 5′-TTCAACACACTCTACACTCCG-3′ and (antisense) 5′-AGAAAGCGTTGTGGTACTCAT-3′, Ptges (sense) 5′-GGATGCGCTGAAGTACGTGGA-3′ and (antisense) 5′-CAGGAATGACTACAGGAAACGC-3′, Ptges (sense) 5′-GGATGCGCTGAAGTACGTGGA-3′ and (antisense) 5′-CAGGAATGACTACAGGAAACGC-3′.
Fatty acid composition analysis

Total lipids from gonadal adipose tissue were extracted as previously described (25), then treated with 3-N-methanolic HCl at 55°C for 40 min to prepare the fatty acid methyl esters (FAMEs) (26). The resulted FAMEs dissolved in hexane were used for GC-MS analysis, using Shimadzu GC-MS-QP2010 SE (Tokyo, Japan). Oven conditions: initial temperature 50°C; temperature increase: 20°C/min to 200°C, then increase 2°C/min to 220°C and held for 142.5 min. Other conditions: injector temperature 250°C; detector temperature 250°C; carrier gas helium, split ratio: 10:1. Column: SUPELCOWAX 10 (fused silica), 100 m × 0.25 mm × 0.25 μm.

The FAMEs were identified by comparing with the standards (Sigma-Aldrich, St. Louis, MO, or Nu-Chek Prep, Elysian, MN) or by their mass spectra, which were further compared with the NIST Mass Spectral Library.

Data analysis

All data are expressed as the mean ± standard error of the mean (SEM). For the comparison between the control group and the HFD group, Shapiro-Wilk test was used to verify the normality of data. When data were normally distributed,
statistical significance was determined using two-sided \( t \)-test; otherwise, significance was determined by Mann-Whitney \( U \) test. All of these data analyses were performed by using SigmaPlot software (San Jose, CA). The principal component analysis (PCA) was implemented using MetaboAnalyst (http://www.metaboanalyst.ca/). The data were scaled using auto-scaling before the analysis. \( P \) values less than 0.05 are reported as statistically significant.

**Results**

**CYP-derived LMs in adipose tissues**

After 8 weeks of dietary feeding, HFD significantly increased body weight and adipose tissue weight in C57BL/6 mice (Supporting Information Figure S2). These results are consistent with previous studies of HFD on obesity (27). We used LC-MS/MS-based lipidomics to compare the profiles of LMs in inguinal, gonadal, and interscapular adipose tissues of mice fed on HFD or control diet. Among the three major PUFA metabolizing pathways (COX, LOX, and CYP), CYP-derived fatty acid epoxides were the most dramatically altered LMs in adipose tissues (Figure 1 and Supporting Information Figure S3). PCA analysis of the lipidomics data showed the difference between the HFD group and the control group, and fatty acid epoxides were the most important lipid mediators contributing to the differentiation (Supporting Information Figure S4). There was no difference in terms of recovery of deuterated LM standards between the control and HFD groups (see Supporting Information Figure S5).

The fatty acid epoxides were further metabolized by sEH to generate the corresponding fatty acid diols (10). Consistent with reduced adipose levels of fatty acid epoxides, the levels of fatty acid diols were also significantly reduced (Figure 2a–c). For example, the levels of 11,12-diHET, which is a sEH metabolite of 11,12-EET, were reduced by 84.5% (mean ± SEM) in gonadal adipose tissues of HFD-fed mice (Figure 2b). We need to point out in the adipose tissues, the concentrations of fatty acid epoxides were much higher than those of the corresponding fatty acid diols. For example, the concentration of 11,12-EET was 73-fold higher than its sEH metabolite 11,12-DHET in gonadal adipose tissues of control mice (Figure 2b).

We further analyzed whether there was a correlation of adipose tissue weight with adipose concentration of CYP-derived LMs. In gonadal fat tissues, the adipose tissue weight inversely correlated with adipose concentration of fatty acid epoxides such as 11,12-EET and 19,13-EET in adipose tissues of HFD-fed mice (Figure 2d).
COX-derived LMs in adipose tissues

The metabolism of PUFAs by COX enzymes leads to formation of prostaglandin H$_2$ (PGH$_2$), which is further enzymatically metabolized to generate various prostaglandins (Figure 3a). Compared with CYP-derived LMs, the profiles of COX-derived LMs showed more complicated patterns. In inguinal adipose tissues, the levels of COX-derived LMs were not changed (Figure 3b), suggesting that the COX pathway is not likely to be involved in the biology of inguinal adipose tissues. In gonadal adipose tissues, the concentrations of PGI2 (as measured by its stable metabolite 6-keto-PGF1a) and PGE2 were significantly reduced in HFD-fed mice (Figure 3c). In interscapular adipose tissues, the concentrations of PGF2a and PGE2 were significantly reduced in HFD-fed mice ($P < 0.05$, Figure 3d).

5-LOX-derived LMs in adipose tissues

The metabolism of ARA by 5-LOX leads to formation of 5-hydroperoxyeicosatetraenoic acid (5-HpETE), which is then converted to at least two classes of LMs: (1) 5-hydroxyeicosatetraenoic acid (5-HETE), or similar LMs such as $\alpha$-LA-derived 9-HOTrE and EPA-derived 5-HEPE, and (2) LTB$_4$, or similar LMs such as EPA-derived LTB$_4$ (Figure 4a). HFD feeding reduced levels of 5-HETE-series LMs (such as 5-HETE, 9-HOTrE, and 5-HEPE) while increasing levels of LTB$_4$ in adipose tissues (Figure 4b–d). The tissue levels of LTB$_4$ were significantly increased in inguinal, gonadal, and interscapular adipose tissues of HFD-fed mice (Figure 4b–d), which is in agreement with recent studies which showed that HFD increased tissue levels of LTB$_4$ (10).

12/15-LOX-derived LMs in adipose tissues

The metabolism of PUFAs by 12/15-LOX leads to formation of a series of LMs (Figure 5a). LC-MS/MS showed that many of these LMs were reduced in adipose tissues of HFD-fed mice (Figure 5b–d). For example, the tissue levels of 15-HETE were significantly reduced in inguinal, gonadal, and interscapular adipose tissues of HFD-fed mice (Figure 5b–d). 12-HETE, which is among the most abundant LOX-derived LMs in adipose tissues, was reduced by 71.7 ± 6.78% (mean ± SEM) in interscapular adipose tissue of HFD-fed mice (Figure 5d).
Fatty acid composition and expression of COX, LOX, and CYP in adipose tissues

The tissue profiles of LMs are in part mediated by fatty acid composition and expression of PUFA metabolizing enzymes in the tissues (6). To understand the mechanisms by which HFD modulated LMs in adipose tissues, we analyzed fatty acid composition and gene expression of COX, LOX, and CYP in adipose tissues. We focused on gonadal adipose tissues, which are the largest adipose tissues, since we have observed significant changes of most LMs in gonadal fat (Figures 1–5).

For fatty acid composition, as expected, triglycerides were major lipids in the gonadal adipose tissue, with a minimum amount of phospholipids (determined by TLC method based on Ref. 28; data not shown), which is consistent with previous studies (29). Therefore, we analyzed fatty acid composition from the total lipid of adipose tissues. GC-MS analysis showed that HFD feeding did not change the tissue levels of ARA and α-LA and slightly increased tissue levels of LA (22.50 ± 0.18% in the HFD group vs. 17.78 ± 0.44% in the control group, \( P < 0.001 \)) (Supporting Information Table S2). These results support that HFD feeding did not reduce levels of PUFAs in adipose tissues, suggesting that the reduced levels of many LMs in adipose tissues were not due to lack of PUFA substrates.

For gene expression of PUFA metabolizing enzymes, RT-PCR showed that the expression of several CYP epoxygenases, such as Cyp2j5, Cyp2j6, and Cyp2e44, was significantly reduced in the gonadal adipose tissues of HFD-fed mice (Figure 6a), which is well consistent with the reduced levels of CYP-derived fatty acid epoxides in adipose tissues (see Figure 2b). The expression of Ephx2 (encoding sEH) was not changed (Figure 6a). For the COX pathway, the gene expression of Cox2 was not changed, while the expression of Ptges (encoding microsomal prostaglandin E synthase) and Ptgis (encoding PGI2 synthase) was significantly reduced in HFD-fed mice (Figure 6b). This is consistent with the LC-MS/MS analysis which showed that only PGE2 and PGI2, but not other COX-derived LMs, were reduced in adipose tissues of HFD-fed mice (Figure 6c). For LOX pathways, the gene expression of Alox15 (encoding 12/15-LOX) and Alox5 (encoding 5-LOX) was not significantly changed (Figure 6c), suggesting that the effects of HFD on LOX-derived LMs may be though modulations of downstream enzymes. Finally, we analyzed the expression of Pla2 (including cytosolic calcium-dependent Pla2g4a and secretory Pla2g12a (4)) and found little change of this gene (Figure 6c). Together, these results support that HFD changed tissue profiles of LMs mainly through modulation of the expression of PUFA metabolizing enzymes in adipose tissues.

Discussion

In this study, we conducted a LC-MS/MS-based lipidomics analysis of HFD-induced obesity in mice. Our central finding is that HFD significantly modulated the profiles of LMs in adipose tissues of...
mice. Among the three major PUFA metabolizing pathways (COX, LOX, and CYP), CYP-derived fatty acid epoxides were the most dramatically changed LMs in adipose tissues of HFD-induced obesity. Almost all types of fatty acid epoxides, including EpOMEs derived from LA, EETs from ARA, EpDPEs from DHA, and EpODEs from α-LA, were reduced by 70% to 90% in different types of adipose tissues. Based on our GC-MS analysis of fatty acid composition and RT-PCR analysis of PUFA metabolizing enzymes, these changes were most likely caused by reduced expression of CYP epoxygenases, not because the PUFA substrates were reduced in adipose tissues of HFD-fed mice. The ratios of fatty acid epoxides to the corresponding diols were reduced in the tissues of HFD-fed mice, while the gene expression of Ephx2 (encoding sEH) was not changed. This may be because the fatty acid diols could be further metabolized, such as by phase II enzymes through conjugations of the hydroxyl groups, leading to removal of fatty acid diols (6). Our results are consistent with previous studies, which showed that EETs are reduced in adipose tissues of HFD-fed mice (11). Many of these fatty acid epoxides have beneficial effects on health. ARA-derived EETs have been shown to have potent anti-inflammatory, vasodilatory, antihypertensive, cardioprotective, renal-protective, and analgesic actions (30). DHA-derived EpDPEs were shown to be the most potent fatty acid epoxides in dilation of blood vessels, with EC_{50} values of 0.5 to 24 pM for dilation of porcine coronary arterioles (31). Our own study showed that EDPs have potent antiangiogenic, anticancer, and antimetastatic effects in vitro and in vivo (32). Therefore, reduced levels of these beneficial LMs, in particular EETs and EDPs, may contribute to the adverse effects of obesity. This is supported by recent studies, which have shown that pharmacological inhibition or transgenic deletion of sEH, which is the dominant enzyme in degrading fatty acid epoxides, protected mice from various adverse consequences of obesity, such as endoplasmic reticulum stress, metabolic syndrome, hepatic steatosis, inflammation, and endothelial dysfunction (11,14-21). Together, these results strongly support that CYP-derived fatty acid epoxides play important roles in regulating the pathology of obesity.

The profiles of COX- and LOX-derived LMs showed more complicated patterns in adipose tissues of HFD-induced obesity. For the COX pathway, the relative balance of vasodilative PGI_{2} (as measured by its stable metabolite 6-keto-PGF_{1α}) and vasoconstrictive TXA_{2} (as measured by its stable metabolite TXB_{2}) plays a critical role in regulating vascular tone and cardiovascular functions (4,33). Our study shows that HFD feeding reduced adipose levels of vasodilative PGI_{2}, while had little effect on vasoconstrictive TXA_{2}. These results support that the PGI_{2} pathway, but not the TXA_{2} pathway, may contribute to some adverse effects of obesity. Our results are consistent with previous studies which showed that biosynthesis of PGI_{2}, but not TXA_{2}, was attenuated in obese Zucker rats (9). We also found that the tissue levels of PGE_{2} were significantly reduced in obese mice. This is consistent with previous studies of HFD on adipose tissue levels of PGE_{2} (34-37). The biological significance of PGE_{2} remains to be determined. On one hand, PGE_{2} is a potent vasodilator, and reduced levels of PGE_{2} could contribute to reduced ATBF of obesity (4); on the other hand, PGE_{2} is a potent inducer of inflammation (4), and reduced levels of PGE_{2} are not consistent with the enhanced adipose inflammation in obesity. Previous studies have shown that HFD induced a dynamic change of adipose level of

![Figure 5](https://www.obesityjournal.org)
PGE₂: in the early stage of HFD feeding (day 4 post-HFD feeding), PGE₂ was increased in adipose tissues, while at a later stage (day 14), its concentration was reduced in adipose tissues (35). These results support that there may be a highly time-dependent change of tissue levels of LMs in order to respond to varied cellular stimulations at different stages of obesity development.

For the LOX pathway, only the concentration of 5-LOX-derived LTB₄ was significantly increased in adipose tissues. This is consistent with recent studies which showed that HFD increased tissue levels of LTB₄; in addition, inhibition of the LTB₄ receptor protected mice from HFD-induced insulin resistance and hepatic steatosis (10), supporting a critical role of LTB₄ in the pathology of obesity. For the 12/15-LOX pathway, our results show that the many 12/15-LOX-derived LMs (12-HETE, 15-HETE, 15(s)-HETrE, and 13-HOTrE) were reduced in adipose tissues of HFD-fed mice. Our results are consistent with previous studies which showed that HFD reduced adipose concentrations of 12-HETE and 15-HETE (37). Some previous studies have shown that the 12/15-LOX pathway is activated in obesity (38); and these different results could be because different animal models of obesity were used. Many of the LOX-derived hydroxy fatty acids and leukotrienes have potent...
effects to regulate inflammation and vascular tone (4). It remains to be determined whether reduced levels of 12/15-LOX-derived LMs contribute to the adverse effects of obesity.

In conclusion, our lipidomics analysis shows that HFD significantly modulates the profiles of LMs in adipose tissues of mice. In particular, CYP-derived fatty acid epoxides are the most dramatically altered LMs in HFD-induced obesity, suggesting that these novel LMs could play critical roles in the pathology of obesity. This lipidomics study lays the foundation to further investigate the functional roles of LMs in obesity, which could facilitate the development of novel biomarkers or therapeutic targets for obesity and obesity-associated diseases.

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