Major role of adipocyte prostaglandin E₂ in lipolysis-induced macrophage recruitment

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Abbreviations: AA, arachidonic acid; adPLA₂, adipocyte phospholipase A₂; ATMs, adipose tissue macrophages; CaMK, calmodulin-dependent kinase; CCL2, CC-chemokine ligand 2; COX, cyclooxygenase; cPLA₂, cytosolic phospholipase A₂; EP, prostaglandin E receptor; IBMX, isobutylmethylxanthine; LA, linoleic acid; OA, oleic acid; PA, palmitic acid; PGE₂, PGD₂, prostaglandin E₂ and D₂; SVF, stromal-vascular fraction; TAG, triacylglycerol; WAT, white adipose tissue.
ABSTRACT

Obesity induces accumulation of adipose tissue macrophages (ATMs), which contribute to both local and systemic inflammation and modulate insulin sensitivity. Adipocyte lipolysis during fasting and weight loss also leads to ATM accumulation, but without proinflammatory activation suggesting distinct mechanisms of ATM recruitment. We examined the possibility that specific lipid mediators with anti-inflammatory properties are released from adipocytes undergoing lipolysis to induce macrophage migration. In the present study, we showed that conditioned medium from adipocytes treated with forskolin to stimulate lipolysis, can induce migration of RAW 264.7 macrophages. In addition to FFAs, lipolytic stimulation increased release of prostaglandin E2 (PGE\(_2\)) and PGD\(_2\), reflecting cytosolic phospholipase A\(_2\)\(\alpha\) (cPLA\(_2\)\(\alpha\)) activation and enhanced cyclooxygenase 2 (COX2) expression. Reconstituted medium with the anti-inflammatory PGE\(_2\) potently induced macrophage migration while different FFAs and PGD\(_2\) had modest effects. The ability of conditioned medium to induce macrophage migration was abolished by treating adipocytes with the COX2 inhibitor sc236 or by treating macrophages with the EP4 antagonist AH23848. In fasted mice, macrophage accumulation in adipose tissue coincided with increases of PGE\(_2\) levels and COX1 expression. Collectively, our data show that adipocyte originated PGE\(_2\) with inflammation suppressive properties plays a significant role in mediating ATM accumulation during lipolysis.

Supplementary keywords: Adipose tissue, Cyclooxygenase, Eicosanoids, ERK, Fatty acid, Inflammation, Lipase
Adipose tissue is the major repository of excess energy stored in the form of triacylglycerol (TAG). During energy need, adipocyte lipolysis involving the hydrolysis of TAG releases FFAs for energy production by different tissues (1, 2). Adipose tissue also secretes various adipokines, which influence energy homeostasis and insulin sensitivity of distant tissues (3, 4). In addition to its important role in metabolic regulation, adipose tissue modulates the immune system by recruiting and activating lymphoid and myeloid cells when adipocyte fat storage is exaggerated as occurs in obesity (5). In obese individuals, there is a strong positive correlation between adipocyte size and the accumulation of proinflammatory adipose tissue macrophages (ATMs) (6, 7).

However, ATM recruitment also occurs in mice after fasting or with calorie-restriction (8) and in obese patients maintained on low calorie diets during early weight loss (9), but under these conditions it does not associate with inflammation. The above findings suggest that the mechanisms that operate in ATM recruitment in obesity or calorie restriction are not identical. Both fasting or pharmaceutically induced lipolysis increase macrophage content in adipose tissue, and lipolysis measures correlate with increased ATM content independent of adiposity (10). This suggests that lipid turnover and not lipid accumulation per se is important for ATM recruitment. Moreover, the recruited ATMs play an adaptive role during lipid flux conditions such as with weight loss and fasting by buffering local concentration of FFAs through their ability to accumulate TAG in lipid droplets (8). Whether adipocyte release of FFA or FA-derived molecules might signal for ATM recruitment in addition to driving ATM lipid accumulation is not known.

Free FAs, especially the saturated type, usually activate classical inflammatory responses in macrophages through engagement of pattern recognition receptors, including TLRs (11). However, they are unlikely to explain the calorie restriction associated ATM recruitment, which normally does not associate with inflammation. Although multiple lines of evidence support the concept that FFAs released upon lipolytic stimulation play an important role in ATM recruitment and accumulation (12), the differential effects of individual FFA and potential involvement of FA metabolites are not well known. In the present study, we compared the effects of a series of lipid species released by adipocytes, aiming to
determine the major molecules that trigger macrophage recruitment. Our results suggest that adipocyte
originated prostaglandin E$_2$ (PGE$_2$) plays a significant role in mediating the lipolysis-induced macrophage
recruitment and influences the inflammatory response.

MATERIALS AND METHODS

Materials

Calf serum, FBS and DMEM were purchased from Invitrogen (Grand Island, NY, USA). Antibodies for phosphor-ERK1/2, total ERK1/2, phospho-cPLA$_2$$\alpha$, and tubulin were ordered from Cell Signaling Technology (Beverly, MA, USA). SuperScript VILO cDNA synthesis kit, TRIzol$^\text{®}$ and SYBRGreen reagents were ordered from Life Technologies (Foster City, CA, USA). Antibody for calnexin was from Enzo Life Sciences (Farmingdale, NY, USA) and antibody for Ran from Santa Cruz Biotechnology (Santa Cruz, CA, USA). COX2 antibody was purchased from R&D Systems (Minneapolis, MN, USA). CD11b, CD64, F4/80, NOS2, CD301 and CD206 antibodies were purchased from eBioscience (San Diego, CA, USA). Sc236 and AH23848 were ordered from Cayman Chemical (Ann Arbor, MI, USA). Other reagents were from Sigma (St. Louis, MO, USA).

Cell culture

3T3-L1 cells (from American Type Culture Collection) were cultured to confluence in DMEM with 4.5 mg/ml glucose containing 20% calf serum, as described previously (13). Two days after confluence, differentiation was initiated by incubation with DMEM containing 10% FBS, 500 $\mu$M isobutylmethylxanthine (IBMX), 0.25 $\mu$M dexamethasone, and 4 $\mu$g/mL insulin for 2 days. IBMX and dexamethasone were then removed, and insulin maintained for another 2 days. Thereafter, cells were grown in DMEM containing 10% FBS. RAW 264.7 macrophages (from American Type Culture Collection) were cultured in DMEM containing 10% FBS.
siRNA transfection of adipocytes

Adipocytes were seeded at ~70% confluence and transfection of siRNA (10 nM final concentration) in adipocytes was performed using Lipofectamine RNAiMAX, as we recently described (14). A scrambled siRNA (Invitrogen) was used as a negative control. Most of the experiments were performed 3 days after siRNA transfection.

Treatment of adipocytes and collection of conditioned medium

Adipocytes were serum starved for four hours prior to different treatments in 0.4% BSA/DMEM. Cells were then washed with PBS and incubated in fresh BSA/DMEM overnight. The culture media were centrifuged at 10,000 g for 30 min and the supernatants collected as conditioned media (CM) for the assays.

Measurement of glycerol, FFA and prostaglandins

Glycerol content in the culture media was determined by using free glycerol reagent (Sigma, St Louis, MO). For the FFA measurement, lipids from culture media were extracted in presence of an internal control (C19:0 FA), and separated on silica gel 60-Å plates as previously described (13). The FFA fraction was scrapped from the TLC plates and collected in glass tubes after visualization with 0.01% rhodamine 6G. Fatty acid methyl esters were prepared by reaction with methanol:acetyl chloride 4:1 at 70°C for 1 h and quantitative GC-MS analysis conducted (Hewlett-Packard 5890GC, Palo Alto, CA, USA) as described (14). Release of PGE$_2$ and prostaglandin D$_2$ (PGD$_2$) was measured using EIA kits (Cayman Chemical Co.).

Migration assay of RAW 264.7 cells

Migration of RAW 264.7 cells was measured by a modified Boyden chamber migration assay using transwell inserts with an 8.0 μm polycarbonate membrane (Corning, NY), as previously described (15). The RAW 264.7 cells suspended in 0.4% BSA/DMEM were placed in the upper chamber, whereas, adipocyte CM or media containing different lipids was placed in the lower chamber and 0.4%
BSA/DMEM was used as a control. After allowing cell migration for 4-5 hours, cells were fixed in formalin and stained with hematoxylin. Cells that had not migrated and remained in the upper chamber were removed by gently swiping the filters with wet cotton tips. Raw264.7 cells that migrated onto the filter were counted with a microscope (Nikon E800) and quantified (Image J) from four fields per condition.

Isolation of membrane and cytosol fractions

Membrane fraction was prepared as described previously (16), with minor modifications. Briefly, cells were lysed in 0.25 M sucrose buffer, containing 1 mM deoxycholate with both protease and phosphatase inhibitors, and then homogenized. Cell nuclei were removed through centrifugation (1,000 x g, 4 °C, 10 min). The supernatant was separated from the pellet and fat layers, and centrifuged at 100,000g (4 °C, 1 hr) using a TLS-55 rotor. The resulting membrane-enriched pellet and cytosol-containing supernatant were then analyzed using Western blotting.

Gel electrophoresis and Western blotting

Whole cell lysates of mouse adipose tissue or 3T3-L1 adipocytes were prepared with lysis buffer containing a protease inhibitor cocktail and a phosphatase inhibitor cocktail (Roche Pharmaceuticals). Proteins were separated on 4-12% gradient SDS-PAGE and transferred to nitrocellulose membranes. The membranes after blocking were probed with primary antibodies. After incubating with infrared fluorophore-coupled secondary antibodies, the proteins were visualized on a direct infrared fluorescence detection system (Odyssey Imaging System, LICOR, Lincoln, NE).

RNA isolation and real-time PCR

Total RNA was isolated from 3T3-L1 adipocytes and adipose tissues using TRIzol® reagent and concentration measured (Nanodrop). Reverse transcription was performed with a SuperScript VILO cDNA synthesis kit and gene expression determined with real time qPCR using SYBRGreen reagent as previously described (14). Primer sequences are listed in supplemental Table S1.
**Fluorescent microscopy**

3T3-L1 adipocytes were electroporated with enhanced green fluorescent protein tagged cPLA$_2$$\alpha$ (EGFP-cPLA$_2$$\alpha$) plasmid and seeded on cover slides. Forty-eight hours later, the cells were treated with 10 μM forskolin for 0, 2 and 5 min, fixed and counterstained with DAPI (nuclei, blue). Images were acquired on a Nikon Eclipse TE2000-U microscope operated by MetaMorph software.

**Flow cytometry (FC) of macrophages in adipose tissues**

Age-matched C57BL/6 WT mice were fed ad libitum or fasted overnight. After CO$_2$ asphyxiation, the epididymal fat pads were excised, rinsed in PBS, minced into fine pieces, and digested with collagenase. The digested samples were passed through mesh and subjected to a brief centrifugation. The pellets or stromal-vascular fraction (SVF) was collected for flow cytometry. Cells in the SVF were incubated in RBC lysis buffer (BD Biosciences) for five min at 4°C to remove red cells and then with FC blocking buffer for 10 min before staining with fluorescent-labeled antibodies for 30 min at 4°C. Cells were washed and suspended before analysis. M1 and M2 macrophages were identified as CD64+/CD11b+/NOS2+ or CD64+/CD11b+/CD206+/CD301+ cells, respectively. Macrophage number was calculated by multiplying the number of total cells after digestion by the percentage of cells gated as DAPI-CD45$^-$/CD45$^+$CD64$^+$CD11b$^+$ for each treatment conditions. All animal experiments were approved by the Animal Studies Committee of Washington University School of Medicine.

**Statistical analyses**

The data are presented as means ± SE. Statistical significance was analyzed with GraphPad Prism 6.0 software. One-way ANOVA or student’s t-test was performed with Tukey’s post hoc analysis depending on the experimental design. The correlation between COX1 and F4/80 expression was analyzed using Pearson’s correlation analysis. $P < 0.05$ was considered to be significant.
RESULTS

Adipocyte lipolysis induces RAW 264.7 macrophage chemotaxis

3T3-L1 adipocytes with or without pretreatment with 10 μM forskolin were washed and then switched to fresh medium without the stimulant for an overnight incubation to procure CM (Fig. 1A). Levels of FFA and glycerol in CM from cells pre-treated with forskolin were markedly increased as expected (Fig. 1B). The total concentration of all major FFA together in CM from forskolin-treated (FSK-CM) adipocytes is 91 μM with the concentration of palmitic acid (PA) being the highest at around 31 μM (Supplemental Table S2). To begin to examine the effect of adipocyte lipolysis on macrophage migration, we first compared the effect of FSK-CM to that of control medium not incubated with adipocytes (CTL) or CM from untreated adipocytes with only vehicle included in the preincubation medium (DMSO-CM). More migrated cells were counted in FSK-CM as compared with DMSO-CM (5.6-fold; *P* < 0.001) or control medium (12.8-fold; *P* < 0.001) (Fig. 1C, D) consistent with the effect of lipolysis to induce macrophage migration. We then examined whether the enhanced macrophage migration is due to the FFA released from the lipolytically active adipocytes. Macrophage chemotaxis was examined in response to media containing 100 μM individual FFA; PA, oleic acid (OA), linoleic acid (LA) or 100 μM of a FFA mixture (PA, OA and LA) all added complexed to BSA at a ratio of 4:1 (FA:BSA). As shown in Fig. 1E, PA, OA and LA enhanced macrophage migration by 2.7, 1.7 and 2.1-fold, respectively, and the FA mixture was more effective, increasing migration by 4.0-fold, suggesting a potentially small cooperative regulation. The FA:BSA ratio in the FSK-CM is much lower than that in the reconstituted media since we had to include high BSA as a FA acceptor to drive lipolysis. Thus, unbound FFA in the reconstituted media are at concentrations higher than those in FSK-CM, but had only modest effects as compared to FSK-CM. Collectively, these results indicate that FFA release during lipolysis is one of the factors mediating macrophage accumulation but that FFA alone are unlikely to account for the whole effect.

Lipolytic stimulation increased arachidonic acid (AA) release by activation of cPLA₂α
Since FSK-CM has a greater effect in inducing macrophage migration than what is observed with FFA (Fig. 1D vs E), we explored potential involvement of additional lipid mediators. Arachidonic acid (AA) is the primary source of prostaglandins that mediate inflammatory responses and play a role in macrophage migration. Cellular arachidonic acid is primarily esterified into phospholipids and free AA is kept very low. Arachidonic acid release is controlled by activation of cytosolic phospholipase A\textsubscript{2} (cPLA\textsubscript{2}) and adipocyte phospholipase A\textsubscript{2} (adPLA) (17, 18). Interestingly, forskolin-treated adipocytes were found to produce more AA than vehicle-treated cells (Fig. 2A). We explored the potential involvement of different PLA\textsubscript{2} in adipocyte AA release following forskolin. We first examined the expression levels of cPLA\textsubscript{2}\textalpha, cPLA\textsubscript{2}\textgamma and adPLA\textsubscript{2} and observed a dramatic increase of cPLA\textsubscript{2}\textalpha expression after exposure to forskolin (Fig. 2B). However, forskolin treatment did not alter cPLA\textsubscript{2}\textgamma expression and significantly decreased adPLA\textsubscript{2} level (supplemental Fig. S1). It has been reported that cPLA\textsubscript{2}\textalpha is activated by phosphorylation at serine-505 by extracellular signal-regulated kinase (ERK) (19, 20). We determined that phosphorylation of cPLA\textsubscript{2}\textalpha and ERK occurred rapidly at 15 min, and further increased at 30 min following forskolin treatment of 3T3-L1 adipocytes (Fig. 2C). Similarly, adrenergic activation by isoproterenol also increased phosphorylation of both cPLA\textsubscript{2}\textalpha and ERK (supplemental Fig. S2).

In addition to phosphorylation, cPLA\textsubscript{2} activity is also enhanced by its translocation from cytosol to membrane to access AA-containing phospholipid substrates. Forskolin decreased p-cPLA\textsubscript{2}\textalpha in the cytosolic fraction, while increasing in the membrane fraction (Fig. 2D). We further examined subcellular localization of EGFP-cPLA\textsubscript{2}\textalpha in adipocytes by fluorescent microscopy. Under basal condition, cPLA\textsubscript{2}\textalpha displayed diffused cytoplasmic distribution in adipocytes. However, forskolin treatment triggered the formation of numerous fluorescent puncta (Fig. 2E), confirming its translocation from cytosol to membrane.

\textbf{PGE\textsubscript{2} released from adipocytes mediated macrophage migration}
In addition to increase of AA release (Fig. 2A), foskolin pretreatment also resulted in higher levels of both PGE$_2$ and PGD$_2$ in the FSK-CM (Fig. 3A). Accordingly, we compared the effects of AA, PGE$_2$, and PGD$_2$ on macrophage chemotaxis. AA did not show stimulatory effect on RAW macrophage, while macrophage chemotaxis was strongly activated by PGE$_2$ (17.8-fold) while PGD$_2$ had a much weaker impact (3.5-fold) (Fig. 3B, C). The effect of PGE$_2$ on macrophage migration was also more potent than that of FFA (Fig. 1E and 3B), suggesting that PGE$_2$ could be the most important mediator linking adipocyte lipolysis and macrophage migration. We further examined cyclooxygenase (COX) gene expression and found that COX2 expression was strongly stimulated by forskolin treatment while COX1 level was not significantly changed (Fig. 3D).

To evaluate the relative contribution of COX-generated eicosanoids in the FSK-CM to the induction of macrophage recruitment, we examined effect of a specific COX2 inhibitor, sc236 (21). Adipocytes were incubated with forskolin in presence or absence of sc236 prior to collection of CM. Sc236 did not affect forskolin-induced release of glycerol and FFA (Fig. 4A), but significantly inhibited PGE$_2$ and PGD$_2$ production as would be expected (Fig. 4B). In the presence of the COX2 inhibitor, CM derived from forskolin-treated adipocytes lost most of its ability to stimulate macrophage migration (Fig. 4C, D). These results indicate that prostaglandins (mostly likely PGE$_2$) and not FFAs play a major role in inducing macrophage recruitment during adipocyte lipolysis.

PGE$_2$ regulates various pathways by binding to multiple prostaglandin E receptors (EPs), including EP1, EP2, EP3 and EP4. Recently the EP4 receptor was shown most important for PGE$_2$-mediated macrophage migration (22). Pretreatment of macrophages with the EP4 antagonist, AH23848, significantly inhibited FSK-CM-induced macrophage migration (Fig. 4E), suggesting that CM PGE$_2$ is acting via the macrophage EP4 receptor. Interestingly, virtual elimination of PGE2 in adipocytes or inhibition of EP4 on the macrophages blunts most but not all of the FSK effect on cell migration, suggesting contribution from FFAs or other unidentified factors in FSK-CM.
Increased macrophage infiltration in adipose tissue during fasting

ATM recruitment occurs in mice after fasting, which does not associate with inflammation (8). Based on the results that macrophage migration was stimulated by adipocyte lipolysis in vitro, we aimed to examine whether PGE2 could play a role in ATM recruitment in fasting mice. We first confirmed ATM accumulation in white adipose tissue (WAT) from fed and fasted mice by measuring expression levels of macrophage/myeloid cell-specific markers F4/80 and CD11b. Significant increases in the expression of these markers were observed after overnight fasting and immunohistochemistry of adipose tissue with the sF4/80 antibody also showed that ATM numbers were markedly increased (data not shown). ATMs were further analyzed by flow cytometry with a broad mononuclear gate consisting of CD64+ /CD11b+ cells (Fig. 5A), and this confirmed the effect of overnight fasting to enhance macrophage accumulation in adipose tissue (Fig. 5B). ATMs are broadly divided into the pro-inflammatory M1 and the anti-inflammatory M2 subclasses. We analyzed the different populations by flow cytometry. As shown in Fig. 5C, under both fed and fasted conditions, expression of the CD206 and CD301, markers of M2 macrophages, were much higher than those of the M1 marker NOS2. Fasting did not significantly alter the M1/M2 ratio and the anti-inflammatory M2 phenotype remained dominant over the pro-inflammatory M1. This confirmed that adipocyte lipolysis induces ATM recruitment without inducing inflammation in WAT.

ATM content correlates with COX expression in WAT

We examined if the increased ATM accumulation in fasted WAT in mice might also associate with PGE2 production. Indeed, overnight fasting increased PGE2 level in WAT almost threefold (Fig. 6A). In vitro PGE2 levels in explant of WAT were also increased by forskolin treatment (Fig. 6B). We examined COX1 and COX2 expression in WAT and found that COX1 expression in WAT was increased threefold after an overnight fast while COX2 expression was not stimulated (Fig. 6C). Notably, COX1 mRNA expression highly correlated (r² = 0.84, P < 0.0001) with tissue f4/80 expression levels (Fig. 6D). In agreement with the fasting-induced upregulation of COX1 mRNA level in WAT, COX1 protein level
also increased after fasting (Fig. 6E). Collectively, our results suggest that adipocyte lipolysis enhances AA release from membrane phospholipids and PGE\(_2\) production by activating cPLA\(_2\)\(\alpha\) and upregulating COX level. The net result is promotion of macrophage recruitment in the absence of inflammation.

**DISCUSSION**

Recent studies suggested that macrophage recruitment to adipose tissue is a normal response that associates with activation of adipocyte lipolysis during the early phase of weight loss and fasting (8, 23). However, the metabolic factors that induce adipose tissue macrophage infiltration during lipolysis are still undefined. The present study using *in vitro* and *in vivo* models identified production by adipocytes upon lipolytic stimulation of the lipid mediator PGE\(_2\) which strongly promotes macrophage migration. Our data provided multiple lines of evidence to support PGE\(_2\) as the most credible mediator of ATM recruitment and showed that its production is due to lipolysis-induced activation of cPLA\(_2\)\(\alpha\) and to upregulation of COX level (Fig. 6F).

It is likely that acute ATM recruitment induced by lipolytic stimulation of adipocytes is under distinct regulation and has different effects as compared to chronic ATM accumulation during the development of obesity. Adipose tissue during the development of obesity produces a variety of bioactive molecules that initiate infiltration of M1 macrophages and produces pro-inflammatory cytokines such as TNF-\(\alpha\) and CC chemokine ligand 2 (CCL2) (24, 25). Macrophages are functionally and numerically the most important type of immune cells recruited in obese WAT (26, 27). The role of WAT macrophage infiltration during the development of obesity in the etiology of local and systemic chronic inflammation and in insulin resistance has been intensively investigated (25). However, activation of adipocyte lipolysis although strongly inducing macrophage recruitment does not induce inflammation. The expression of CCL2 and TNF\(\alpha\) was not enhanced in adipocytes pretreated with forskolin (data not shown).
and while fasting of mice increased ATM accumulation, this did not associate with a change in macrophage polarization as the M2 anti-inflammatory phenotype remained the predominant macrophage type present. It has been proposed that lipolysis and the associated increase in FFA concentration regulate macrophage accumulation without activation of the proinflammatory M1-polarized state (8). In addition to pro-inflammatory cytokines, some FA metabolites have been proposed as potential triggers of macrophage recruitment, especially arachidonic acid and eicosanoids (28, 29). Our results show that macrophage chemotaxis was only mildly promoted by FFAs including arachidonic acid but that it was strongly activated by the fatty acid metabolite PGE\(_2\), which dramatically increased in the culture media from forskolin-treated adipocytes (Fig. 3A) or WAT explants (Fig. 6B), and in the WAT from fasted mice (Fig. 6A). Furthermore, inhibition of COX2 in adipocytes (Fig. 4C, D) or of EP4 in macrophages (Fig. 4E) diminished the ability of FSK-CM to induce macrophage migration. These findings demonstrate that PGE\(_2\) release from adipocytes directly mediates macrophage infiltration during lipolysis. Interestingly, PGE\(_2\) acting via its EP3 receptor is a documented anti-lipolytic action as it suppresses hormone-stimulated lipolysis in adipocytes (30). This suggests that PGE\(_2\) production by adipocytes undergoing lipolysis serves to buffer the local increase in fatty acid concentration by recruiting M2 macrophages that can store FA in cytosolic lipid droplets and by exerting negative feedback to prevent excess lipolysis (Fig. 6F). In line with this interpretation, rapid appearance of lipid droplets in ATMs during a fast has been described (8). In addition, PGE\(_2\) can suppress production of acute inflammatory mediators and inhibit attraction of proinflammatory cells (31). Suppressing PGE\(_2\) production by deletion of microsomal prostaglandin E synthase-1 exacerbates neutrophil-mediated inflammation (11) while activation of the PGE\(_2\) receptor EP4 inhibits inflammasome activation through EP4 receptor and intracellular cAMP in human macrophages (32). These results suggest that PGE\(_2\) normally plays a key role in regulating anti-inflammation and immune suppression during lipolysis. Synthesis of PGE\(_2\) is impaired in adipocytes isolated from obese Zucker rats (33) and high fat feeding leads to suppression of microsomal prostaglandin E synthase-1 in WAT (34). Accordingly, it is possible that a more limited PGE\(_2\) production during the development of obesity might play a role in the activation of inflammatory immune cells.
Release of AA from phospholipids is essential for the initiation of eicosanoid generation and the release process is dependent on PLA$_2$ activation (35). In the present study, forskolin treatment dramatically increased expression level of cPLA$_2$$\alpha$, but not that of cPLA$_2$$\gamma$ or adPLA$_2$ in adipocytes (Fig. 2B). The importance of cPLA$_2$$\alpha$ in adipocyte AA release and PGE$_2$ production following forskolin treatment was further confirmed by the increase in its phosphorylation and membrane localization. Both CaMKII and ERK can phosphorylate cPLA$_2$$\alpha$, respectively at Ser515 and Ser505 (19). Our results show that activation of lipolysis induces cPLA$_2$$\alpha$ phosphorylation at Ser505 in adipocytes (Fig. 2). Thus the increase in cAMP that initiates lipolysis also leads to downstream activation of ERK which phosphorylates and activates cPLA$_2$$\alpha$ (36).

COX exists in two isoforms and COX1 is generally considered to be constitutively expressed and responsible for the production of prostaglandins important for homeostatic functions. In contrast, COX2 is highly inducible in response to specific stimuli and growth factors and its expression is usually restricted under basal conditions (37). The response of adipocyte COX2 to forskolin in our study is consistent with the established role of cAMP-dependent signaling in regulating COX2 expression (38). Regulation of the COX isoforms under physiological conditions in animal tissues is more complicated. Fasting induces increases in levels of several hormones notably glucocorticoids, in addition to norepinephrine and epinephrine, and glucocorticoids enhances COX1 gene expression (39) and selectively inhibit COX2 expression (40). Thus, it is likely that COX1 was induced after fasting in WAT by glucocorticoid, while COX2 induction by cAMP is neutralized (Fig. 6F). The selective response of adipose tissue COX1 and not COX2 during fasting may explain why WAT recruits macrophages but does not induce inflammation.

The identified ERK/cPLA$_2$$\alpha$/PGE$_2$ pathway may also provide insight into the pathology of diseases influenced by altered cell migration. For example, cPLA$_2$$\alpha$ is highly expressed in many tumors, and the lipid metabolites formed by cPLA$_2$ and COX might modulate migration of tumor cells and cancer
metastasis (41, 42). Depletion of cPLA$_2$ levels inhibited VEGF-induced migration and tube formation of human retinal microvascular endothelial cells (43) and cPLA$_2$ inhibition suppressed pulmonary metastasis in a mouse model for pulmonary tumors (42). These studies suggest that the metabolites produced consequent to cPLA$_2$ activation including PGE$_2$ might play an important role in regulation of cell migration involving many cell types.

Our results support a model by which lipolytic stimulation in adipocytes induces macrophage migration via cPLA$_2$$\alpha$ activation and PGE$_2$ production. Moreover, the ATMs acutely recruited by PGE$_2$ are mostly in the anti-inflammatory M2 state. This recruitment would help to limit the increase of local lipid concentration and lipotoxicity, unlike the chronic stimulation of ATM recruitment in obese WAT which leads to inflammation. Dissection of the distinct cellular pathways that lead to differential ATM recruitment and activation should help identify attractive targets to limit obesity-associated inflammation and insulin resistance.
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Figure Legends:

**Fig. 1.** Adipocyte lipolysis induces macrophage chemotaxis. A: Schematic diagram of protocol used for preparing conditioned medium (CM): 3T3-L1 adipocytes (Ad) were first serum-starved, then incubated in 0.4% BSA/DMEM plus DMSO or 10 μM forskolin (FSK) for four hours, and finally switched to fresh medium (0.4% BSA/DMEM) for an overnight incubation. The following day the medium was collected as conditioned medium (CM) and kept frozen at -80°C until use. B: Glycerol and FFA levels in the CM collected from adipocytes with or without 10 μM forskolin (FSK) pretreatment. Data are means of triplicates from three separate experiments. **P < 0.01. C, D: Macrophage (RAW264.7) chemotaxis: Macrophages suspended in DMEM with 0.4% BSA were placed in the upper chamber of a transwell. The lower chamber contained either DMEM with 0.4% BSA (CTL) or CM from vehicle treated (DMSO-CM) or forskolin treated (FSK-CM) adipocytes. After five hours the macrophages that adhered to the transwell were fixed, stained with hematoxylin, imaged using software Olympus DP controller (C), and quantified using image J (D). Data are means of duplicates from three separate assays. **P < 0.01, ***P < 0.001.

**E, F: Effect of different fatty acids (100 μM, FA:BSA = 4:1) on macrophage (RAW264.7) chemotaxis:** The lower chamber contained DMEM with 0.4% BSA (CTL) or supplemented with palmitic acid (PA, 100 μM), oleic acid (OA, 100 μM), linoleic acid (LA, 100 μM) or a FA mixture containing equal amounts of PA, OA and LA (FA mix, total concentration of 100 μM). Data shown are means of duplicates from two experiments. *P < 0.05, **P < 0.01, compared with CTL group.

**Fig. 2.** Adipocyte lipolysis releases arachidonic acid (AA) by activating cPLA₂α. A: Arachidonic acid levels in the CM collected from vehicle treated (DMSO) or forskolin treated (FSK) adipocytes. Data are means of triplicates from three experiments. **P < 0.01. B: mRNA levels of cPLA₂α in vehicle treated (DMSO) or forskolin treated (FSK) adipocytes. Data shown are means of triplicates from two separate experiments. ***P < 0.001. C: Whole-cell lysates of adipocytes treated with forskolin (FSK) for 0, 15 and 30 min were prepared and analyzed by Western blot for phospho-cPLA₂α (S505) (p-cPLA₂α),
phospho-ERK1/2 (T202, Y204) (p-ERK), total ERK1/2 (t-ERK) and Ran. Protein levels were quantified using Li-Cor technology. Data are from two experiments conducted in triplicates. p-cPLA2 adjusted to the control Ran and p-ERK to t-ERK are presented relative to vehicle treated cells and are plotted as means ± SE. *P < 0.05, **P < 0.01, compared with 0 min. D: cPLA2 translocation in response to forskolin (FSK) treatment in adipocytes. 3T3-L1 adipocytes were starved and treated with forskolin for 15 min. Cells were lysed, separated into cytosol and membrane fractions and probed for p-cPLA2 and calnexin. Signal intensity was analyzed by Li-Cor technology and is plotted as means ± SE. Data in the graph are from two separate experiments conducted in duplicates. *P < 0.05, **P < 0.01. E: 3T3-L1 adipocytes transfected with enhanced green fluorescent protein tagged cPLA2α (EGFP-cPLA2α) plasmid were treated with 10 μM forskolin (FSK) for 0, 2 or 5 min, and then fixed and counterstained with DAPI (nuclei, blue) before imaging with MetaMorph. The data are representative of three experiments.

Fig. 3. PGE2 produced by adipocytes during lipolysis induces macrophage migration. A: PGE2 and PGD2 levels in CM from vehicle treated (DMSO) or forskolin treated (FSK) adipocytes. Data are from duplicates of two experiments, **P < 0.01. B,C: Macrophage (RAW264.7) chemotaxis: Macrophages suspended in DMEM with 0.4% BSA were placed in the upper chamber of the transwell and the lower chamber was filled with DMEM plus 0.4% BSA (CTL), 100 μM arachidonic acid (AA) complexed to BSA (FA:BSA=0.8), 5 nM PGE2 or PGD2. Migrated cells were quantified with image J (C). Data are means of duplicates from three independent assays. **P < 0.01, ***P < 0.001, compared with CTL group. D: mRNA levels of COX enzymes in adipocytes treated with 10 μM forskolin (FSK). All data are means of triplicates from two separate experiments. * P < 0.05, *** P < 0.001.

Fig. 4. Inhibition of PGE2 production suppresses macrophage migration induced by adipocytes lipolysis. A: Levels of glycerol and FFA in CM collected from vehicle treated (DMSO) or forskolin treated (FSK) adipocytes or cells pretreated with forskolin and sc236 (FSK+ SC). Data are means of triplicates from
two independent assays. ** $P < 0.01$. NS, not significant. B: PGD$_2$ and PGE$_2$ content in CM. Data are means of triplicates from one assay. C, D: Macrophage (RAW264.7) chemotaxis: Macrophage suspended in DMEM with 0.4% BSA were placed in the upper chamber of a transwell. The lower chamber contained either DMEM with 0.4% BSA (CTL) or CM from vehicle treated (DMSO), forskolin treated (FSK) adipocytes or cells pretreated with forskolin and sc236 (FSK+SC). After five hours the macrophages that adhered to the transwell were fixed, stained with hematoxillin, imaged using software Olympus DP controller (C), and quantified using image J (D). Data are means of duplicates from three separate assays. ** $P < 0.01$. E, F: Macrophage (RAW264.7) chemotaxis: Cells were pre-incubated with 3 μM of AH23848, an EP4 receptor antagonist before the migration assay using FSK-CM (FSK+AH). Data are means of duplicates from two independent experiments. ** $P < 0.01$, *** $P < 0.001$.

**Fig. 5.** Macrophage content in adipose tissues of mice in fed or fasted states. A: Representative flow cytometry plots of CD11b$^+$/CD64$^+$ cells in adipose tissue; n=2, 3 mice pooled each experiment. B: Adipose tissue macrophage numbers are shown as CD11b$^+$/CD64$^+$ cells; Data are means ± SE. n=2, 3 mice pooled each experiment. ** $P < 0.01$. C: Mean fluorescence intensity (MFI) of markers for M1 (NOS2, gray fill) and M2 (CD301 and CD206, no fill) macrophages in adipose tissue; Data are means ± SE. n=2, 3 mice pooled in each experiment.

**Fig. 6.** Macrophage markers correlate with COX1 expression. A: PGE$_2$ levels in adipose tissue of 6 mice/group. B: PGE$_2$ levels in medium from adipose tissue explants treated with vehicle (DMSO) or 10 μM of forskolin (FSK); n=3 explants. C: mRNA levels of COX enzymes of adipose tissues from fed and fasted mice. Fed, n=6 mice/group; fasted, n=6 mice/group. D: Correlation between COX1 mRNA and F4/80 mRNA in adipose tissues. Pearson’s correlation coefficient is shown. The points represent data from individual fed (black diamond) and fasted (white diamond) mice. E: Total lysates of epididymal
adipose tissue from fasted mice were probed for COX1 and β-actin. Signal intensity was analyzed by Li-Cor technology and is plotted as means ± SE. n=3 mice/group. *P < 0.05, **P < 0.01. F: Model of how adipocyte lipolysis induces macrophage recruitment in adipose tissue. Fasting or FSK treatment increases cAMP levels in adipocytes which induces lipolysis of stored triglycerides. At the same time cAMP activates the ERK1/2 which phosphorylates cPLA2α enhancing its activity. Translocation of cPLA2α to membranes releases AA from phospholipids, a step that is essential for production of the prostaglandin PGE2. COX2 expression in adipocytes is upregulated by FSK via cAMP-dependent signaling. In WAT, it is likely that COX1 was induced after fasting by GR activation, while COX2 induction by cAMP is neutralized. The PGE2 produced by adipocytes acting via its receptor EP4 promotes recruitment of M2 macrophages. In addition PGE2 feedbacks to prevent excess lipolysis by adipocytes. PGE2 release during lipolysis would help to limit the increase of local lipid concentration and lipotoxicity, unlike the chronic stimulation of ATM recruitment in obese WAT which leads to inflammation. FSK, forskolin; β-AR, β adrenergic receptor; GR, glucocorticoid receptor.
**FIGURE 1**

A. Ad

- Starve cells
- 4hr

- Change to fresh media
- DMSO
- FSK
- 4hr
- Overnight

- Collect CM

B. Graph showing relative cell numbers against different conditions.

C. Images showing cell morphology under various treatments:

- CTL
- DMSO
- FSK

D. Bar graph showing relative cell numbers.

E. Images showing cell morphology under different fatty acid treatments:

- CTL
- PA
- OA

- LA
- FA mix

F. Bar graph showing relative cell numbers for different fatty acid treatments.
FIGURE 3

A

B

C

D

** Relative cell numbers

*** Relative mRNA levels

A

DMSO 

FSK

B

PGE2 PGD2

PGD2

PGE2

AA

CTL

COX1 COX2

** PGE2 PGD2

0

1

2

3

4

5

ng/mg protein

CTL AA PGE2 PGD2
FIGURE 4

A

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FIGURE 5

A

fed

fasted

B

Relative CD11b+/CD64+ cell number/mg WAT

fed

fasted

C

fed

fasted

CD11b C

CD64

ATM

CD301 CD206 NOS2

MFI

CD301 CD206 NOS2

MFI

0

4000

8000

12000

16000

20000

0

3000

6000

9000

12000

0

6000

9000

0

3000