Macrophage phenotype and bioenergetics are controlled by oxidized phospholipids identified in lean and obese adipose tissue

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Adipose tissue macrophages (ATMs) adapt their metabolic phenotype either to maintain lean tissue homeostasis or drive inflammation and insulin resistance in obesity. However, the factors in the adipose tissue microenvironment that control ATM phenotypic polarization and bioenergetics remain unknown. We have recently shown that oxidized phospholipids (OxPL) uniquely regulate gene expression and cellular metabolism in Mox macrophages, but the presence of the Mox phenotype in adipose tissue has not been reported. Here we show, using extracellular flux analysis, that ATMs isolated from lean mice are metabolically inhibited. We identify a unique population of C3CR1γ/CD44/CD80low ATMs that resemble the Mox (Tnfr1⁺/HO1⁺) phenotype to be the predominant ATM phenotype in lean adipose tissue. In contrast, ATMs isolated from obese mice had characteristics typical of the M1/M2 (CD11c⁺/CD206⁺) phenotype with highly activated bioenergetics. Quantifying individual OxPL species in the stromal vascular fraction of murine adipose tissue, using targeted liquid chromatography-mass spectrometry, revealed that high fat diet-induced adipose tissue expansion led to a disproportional increase in full-length over truncated OxPL species. In vitro studies showed that macrophages respond to truncated OxPL species by suppressing bioenergetics and up-regulating antioxidant programs, mimicking the Mox phenotype of ATMs isolated from lean mice. Conversely, full-length OxPL species induce proinflammatory gene expression and an activated bioenergetic profile that mimics ATMs isolated from obese mice. Together, these data identify a redox-regulatory Mox macrophage phenotype to be predominant in lean adipose tissue and demonstrate that individual OxPL species that accumulate in adipose tissue instruct ATMs to adapt their phenotype and bioenergetic profile to either maintain redox homeostasis or to promote inflammation.

Significance

Adipose tissue macrophages (ATMs) maintain adipose tissue homeostasis. However, during obesity ATMs become inflammatory, resulting in impaired adipose tissue function. Oxidative stress increases during obesity, which is thought to contribute to adipose tissue inflammation. To date, the connection between oxidative stress and adipose tissue inflammation remains unclear. In this study, we identify two classes of phospholipid oxidation products in lean and obese adipose tissue, which polarize macrophages to an antioxidant or proinflammatory state, respectively. Furthermore, we show that these phospholipids differently affect macrophage cellular metabolism, reflecting the metabolisms of ATMs found in lean and obese adipose tissue. Identification of pathways controlling ATM metabolism will lead to novel therapies for insulin resistance.

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Here we characterize the bioenergetic profile of ATMs from lean and obese mice. We used flow cytometry to link the ATM bioenergetic profile to established in vitro macrophage polarization states (i.e., proinflammatory M1, antiinflammatory M2, or antioxidant M0x). Furthermore, quantification of individual OxPL species in whole blood and the ATM-containing stromal vascular fraction (SVF) of adipose tissue allowed us to define the unique OxPL compositions present in physiological and pathological states of obesity. Finally, we tested the different OxPL compositions that we found in vivo on their ability to differentially reprogram macrophage bioenergetics and phenotypic polarization states in vitro.

**Results**

**ATMs from Lean Mice Are Metabolically Inhibited, While Those from Obese Mice Are Highly Energetic.** ATMs were shown to regulate obesity-associated adipose tissue dysfunction and insulin resistance (9). Metabolic activation of macrophages in obesity has been implied based on in vitro stimulation (13), yet measurement of the bioenergetic profile of resident or infiltrating ATMs ex vivo has not been reported. Previous studies suggest that ATMs from lean mice are of the antiinflammatory M2 phenotype (31, 32), which would indicate heavy reliance on oxidative phosphorylation and mitochondrial function (33), while in the context of obesity, a shift in ATM polarization to proinflammatory M1 macrophages was proposed (31, 32), which would rely heavily on aerobic glycolysis (34). We used extracellular flux assays, which allow for direct evaluation of cellular bioenergetic profiles ex vivo by measuring oxygen consumption rate (OCR, measure of oxidative phosphorylation) and extracellular acidification rate (ECAR, measure of aerobic glycolysis), to test the hypothesis that ATMs from lean mice exhibit a high OCR and low ECAR (M2 metabolism), while ATMs from obese mice show a low OCR and high ECAR (M1 metabolism). To analyze ATM metabolism in lean and obese adipose tissue, we fed C57BL/6 mice chow diet or high-fat diet (HFD) for 12 wk. Next, we isolated the SVF from the epididymal fat pads for bioenergetic profiling (Fig. 1A) and assessed OCR and ECAR in SVF cells using extracellular flux analysis (35, 36). Unexpectedly, we found that both the respiratory capacity (OCR) and aerobic glycolysis (ECAR) of SVF isolated from lean mice were low, while they were both significantly increased in SVF cells isolated from obese mice (Fig. 1B). This result indicates that SVF cells isolated from lean mice exist in a relatively low bioenergetic state, while SVF cells in obesity adopt an energetic (high respiratory capacity, high glycolysis) metabolic phenotype. Because the SVF isolated from adipose tissue contains a variety of cell types, we enriched the SVF for CD11b+ cells (which are predominantly macrophages) using magnetic beads coupled to an anti-CD11b antibody (SI Appendix, Fig. S1A). Surprisingly, CD11b+ cells isolated from adipose tissue of lean mice exhibit a strikingly suppressed OCR and ECAR, resulting in a bioenergetic profile reminiscent of a quiescent state (Fig. 1C). Remarkably, the respiratory capacity (OCR) of CD11b+ enriched SVF from obese mice was almost three times greater than lean mice, where glycolysis (ECAR) was four times greater than CD11b+ SVF from lean mice. Taken together, these data show that CD11b+ ATMs maintain a quiescent metabolic phenotype (low respiratory capacity and glycolysis) in lean adipose tissue, but in obesity, CD11b+ cells exert a highly energetic metabolic phenotype. Moreover, this is in striking difference from what would be expected for M2 polarized macrophages (high OCR) or M1 macrophages (high ECAR). This unexpected finding led us to further investigate the polarization state of resident ATMs in lean mice and to monitor changes during obesity.

**A Population of CX3CR1+/F4/80+ Cells Is Predominant in Lean Adipose, While Obesity Induces Accumulation of CX3CR1+/F4/80+ Cells in Adipose Tissue.** To examine the relationship between ATM phenotypic polarization and the respective cellular bioenergetic profiles in obesity, we placed mice on a HFD for 8 and 16 wk to induce adipose tissue expansion and immune cell infiltration (SI Appendix, Fig. S1B). We isolated the SVF from the epididymal fat pads of each mouse and analyzed the macrophage content by flow cytometry using CD45, F4/80, and CD11b as markers for ATM (37–39) (see SI Appendix, Fig. S1C for gating strategy). Within the F4/80+CD11b+ ATM population, we identified two distinct populations that were distinguished by CX3CR1 expression (Fig. 2A). In lean mice, ~6% of ATMs were CX3CR1+; however, this percentage increased to over 60% and

![Fig. 1.](image-url)
over 80% after 8 and 16 wk of HFD feeding, respectively (Fig. 2B). We observed that CX3CR1 expression coincided with high expression of F4/80 and accordingly identified two ATM populations characterized as F4/80hi and F4/80lo (Fig. 2C and D). Similarly, two populations of macrophages have been previously characterized based on their F4/80 and CD11b expression in other tissues, and have been referred to as F4/80hiCD11blo and F4/80loCD11blo (also F4/80hiCD11blose or F4/80loCD11blose) (37–40). Like the CX3CR1+ population (Fig. 2C), the F4/80hi population was predominant in lean adipose tissue (83%), and doubled in number after 8 wk of HFD (from 1.1 × 10^5 to 2.5 × 10^5 cells per fat pad), but decreased in percentage (83% in lean, 55% after 8 wk and 12% after 16 wk HFD) (Fig. 2D). In contrast, F4/80lo macrophages drastically increased in number during the HFD (from 0.2 × 10^5 to 8.4 × 10^5 cells per fat pad; 17% in lean, 53% after 8 wk and 84% after 16 wk HFD) (Fig. 2D). These results indicate that resident ATMs are predominantly CX3CR1+F4/80hi and that ATMs infiltrating during obesity are primarily CX3CR1+F4/80lo.

The Majority of Resident ATMs Are Positive for Mox Markers and Are Out-Populated by M1/M2 ATMs During Obesity. To further investigate phenotypic polarization of ATMs, we interrogated the F4/80hi and F4/80lo ATM populations for macrophage phenotypic polarization. We used the established markers CD11c for M1 (31) and CD206 for M2 (41, 42) ATMs. Additionally, we used redox homeostatic enzymes Tnrx1 and HO1 (whose expression is driven by the transcription factor Nrf2) for Mox, a macrophage polarization state previously identified in atherosclerotic lesions (14). We found that expression of the Mox markers Tnrx1 and HO1 was exclusively associated with the resident F4/80hi (also CX3CR1+) population, while expression of CD11c and CD206 appears almost exclusively in the F4/80lo (also CX3CR1–) population (Fig. 3A), reminiscent of the inflammatory macrophage phenotype found in human obesity (43). The total number of Mox remained constant (1.2 × 10^5 in lean, 1.4 × 10^5 after 8-wk, and 1.0 × 10^5 after 16-wk HFD-fed mice) and consistent with the numbers of F4/80lo macrophages, while CD11c+, CD206+, and CD11cCD206+ cells significantly increased upon HFD feeding (Fig. 3B). Back-gating of the CD11c+, CD206+, and CD11cCD206+ macrophage populations confirmed that these populations were negative for HO1 and Tnrx1 (SI Appendix, Fig. S1D). Remarkably, while in lean mice 84% of F4/80+CD11b+ cells expressed the Mox markers HO1 and Tnrx1, Mox still comprised 47% and 13% of all macrophages after 8 and 16 wk of HFD (Fig. 3C). Together, over the course of HFD-induced cell infiltration into the SVF of adipose tissue, the dynamics of macrophage phenotypic polarization reflect a relative decrease of the Mox macrophage population, with concomitant increases of the M1/M2 hybrid and M2 populations (Fig. 3D). However, these results also support the notion that sufficient numbers of Mox macrophages are present in adipose tissue of not only lean but also obese mice. Indeed, we find that crown-like structures contain Mox macrophages, characterized by expression of HO1+ and Tnrx1+, as well as Nrf2 that has translocated to the nucleus (SI Appendix, Fig. S1E).

Analysis of Full-Length and Truncated OxPAPC Species in Blood from Lean and Obese Mice. To analyze individual OxPL species, we developed a targeted liquid chromatography-mass spectrometry (LC-MS) approach (modified from refs. 44 and 45), aimed at quantifying the levels of oxidation products derived from 1-palmitoyl-2-araachidonoyl-3-sn-glycero-phosphatidylcholine (PAPC) and 1-palmitoyl-2-linoleoyl-3-sn-glycero-phosphatidylcholine (PLPC), collectively referred to as OxPAPC and OxPLPC (46, 47). To better characterize the relative abundance of individual classes of phospholipid oxidation products, we assigned 15 of the previously identified species of OxPAPC (and OxPLPC) to one of four groups based on the structure of the oxidized moiety and previously characterized biological activity (29, 30, 48–50). Full-length species were divided into the HETE/HPETE and the isoprostane classes, and truncated species into the carboxyl (including PLPC oxidation products PONPC and PAzPC) and the γ-keto/hydroxyl classes, based on criteria described in Materials and Methods (Fig. 4A and SI Appendix, Fig. S2A and C). Establishing an LC method with more polar lipids eluting earlier and fewer polar lipids eluting later in the run allowed us to reliably monitor the air-oxidation of PAPC over time (SI Appendix, Fig. S2C and H), quantitatively representing relative amounts of each OxPAPC class present at different time points (SI Appendix, Fig. S2D).

To quantify the levels of OxPAPC species in whole blood from lean and obese mice, we used mice that had been fed chow or a HFD for 9 or 17 wk, resulting in a significant increase in body weight (Fig. 2E). In contrast, PAPC levels were significantly lower in obese mice compared to lean mice after 16 wk of HFD (Fig. 4B), and the levels of OxPAPC species were also significantly lower in obese compared to lean mice (Fig. 4, C–E). The levels of OxPAPC species in the blood of lean mice were significantly lower after 16 wk compared to 8 wk of HFD feeding, while the levels of OxPAPC species in the blood of obese mice remained relatively constant over the same time period (Fig. 4C). This was consistent with a significant decrease in body weight over time in the obese group (Fig. 2E).

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weight over time (SI Appendix, Fig. S4A). In whole blood, LysoPC (specifically 16:0), which can be derived from both phospholipid oxidation (52) and phospholipase A2 (PLA2) activity (Fig. 4A), was highly abundant and significantly increased with HFD, and remarkably, significantly correlated with body weight (Fig. 4B and SI Appendix, Fig. S3 A and B). While the levels of full-length OxPAPC species (HETE/HPETE and isoprostanate groups) and the γ-keto/hydroxyl-PCs increased with the duration of HFD feeding, interestingly the level of carboxyl species significantly dropped (Fig. 4C and D and SI Appendix, Fig. S3C). Overall, the oxidized fraction of PAPC (i.e., the sum of all OxPAPC species including LysoPC in relation to native PAPC) increased from 14% in lean to 26% and 35% after 9 and 17 wk of HFD, respectively (Fig. 4E). The relative level of full-length OxPAPC species increased at the expense of the truncated species (Fig. 4F), resulting in a strong positive correlation of the HETE/HPETE-PCs with body weight, while the carbonyl-PCs showed a negative correlation (Fig. 4G).

HFD Feeding Leads to a Disproportional Increase of Full-Length Compared with Truncated OxPAPC Species in Adipose SVF. Increased oxidative stress is a hallmark of metabolically active tissue, resulting in the formation of lipid oxidation products (10). Furthermore, HFD-feeding is known to cause oxidative stress and increased lipid oxidation (53). However, it is not known if OxPLs are present in adipose tissue and whether HFD-feeding affects the absolute or relative abundance of individual OxPL species. Immunostaining of adipose tissue from obese mice using the E06 antibody that recognizes oxidized phosphatidylcholines (54, 55) demonstrates that oxidation-specific epitopes derived from phospholipid oxidation are abundant in macrophage-containing crown-like structures (Fig. 5A). Next we analyzed the composition of native, nonoxidized, and oxidized PC species in the stromal compartment of adipose tissue, after isolating the SVF from the epididymal fat pads of lean or obese mice fed a HFD for 9 or 17 wk. HFD-fed mice had increased fat pad weights and numbers of stromal cells (SI Appendix, Fig. S4 B and C), and as expected, as the adipose tissue expands in response to HFD, the total PC content in the SVF also increased (SI Appendix, Fig. S4D). Analysis of PAPC and its oxidation products revealed that the total amount of PAPC increased during HFD, as did the PAPC-derived oxidation products (SI Appendix, Fig. S4E). Even in lean mice, 7.7% of the total PAPC in the SVF was oxidized, which doubled to 14.2% at 9 wk and dropped to 8.3%, after 17 wk of HFD (Fig. 5B). Interestingly, the degree of PAPC oxidation in SVF did not correlate with body weight ($R^2 = 0.0019$; slope was effectively zero, $P = 0.8798$), but significantly correlated with fat pad weight ($R^2 = 0.3145$; slope was significantly nonzero, $P = 0.0369$) (Fig. 5C).

The changes in abundance of individual OxPAPC species in adipose SVF during HFD were different for the four classes (Fig. 5D and SI Appendix, Fig. S3E). At 9 wk of HFD, members of all four groups were significantly increased, while after 17 wk of HFD we found, in addition to LysoPC, a shift toward full-length species (HETE/HPETE-PCs and isoprostanate-PCs). Both truncated and full-length PAPC-derived species are present in SVF of lean mice; however, the relative amounts of full-length species increased at a greater rate than truncated species as the adipose tissue expanded (Fig. 5E). These results show that phospholipid oxidation is a feature of lean adipose tissue, and HFD feeding induces significant changes in the phospholipidome in the adipose SVF, characterized by a shift in relative abundance from truncated OxPL species to full-length species.

Truncated OxPAPC Species Inhibit Macrophage Mitochondrial Function and Promote Antioxidant Gene Expression, While Full-Length Species Promote Inflammatory Gene Expression. We have recently demonstrated that OxPAPC depresses OCR in macrophages (3). To identify the OxPL species within OxPAPC that are responsible for...
inhibiting OCR in macrophages, we used solid-phase extraction (56) to separate OxPAPC into fractions highly enriched for either full-length (Fraction FL) or truncated (Fraction T) OxPAPC species (Fig. 6A). Quantification of each fraction using LC-MS/MS showed that we successfully enriched for both full-length (Fig. 6B) and truncated species (Fig. 6C) in their respective fractions. Treatment of bone marrow-derived macrophages (BMDMs) with each fraction demonstrated that the truncated, but not the full-length, OxPAPC species inhibited macrophage respiratory capacity, as measured by maximal OCR (Fig. 6D). This finding is corroborated by data we have previously published demonstrating that individually synthesized truncated species present in OxPAPC, including PGPC, POVPC, and LysoPC, significantly inhibited respiratory capacity (3). In contrast, full-length, but not truncated OxPAPC species, induced the glycolytic capacity of BMDMs, as measured by stressed ECAR (Fig. 6D).

OxPLs induce different cellular programs in macrophages, including induction of proinflammatory as well as antioxidant gene expression (30, 57). However, the structural requirements of OxPLs for induction of these diverse cellular programs in...
macrophages are not known. Following treatment of BMDMs with either Fraction FL or Fraction T, we found that full-length, but not truncated, OxPL species induced proinflammatory gene expression in macrophages, demonstrated by increased expression of Il1β, Il6, and Cxcl1 (Fig. 6E). In contrast, truncated, but not full-length OxPL species induced antioxidant gene expression, as evidenced by increased expression of Hm1, Tnxrd1, and Gclm (Fig. 6F). Furthermore, we observed that the fraction containing truncated OxPL species significantly induced Glut1 gene expression, suggesting that the truncated OxPLs contribute to the metabolic reprogramming of macrophages to support antioxidant production. Interestingly, mRNA expression of cyclooxygenase 2 (Cox2) was induced by both the full-length and truncated OxPL fractions.

Together, these data show that the truncated species of OxPL induce reprogramming of macrophage metabolism to support antioxidant production, while the full-length OxPL species induce proinflammatory gene expression. These findings suggest that the relative abundance of truncated and full-length OxPL species in oxidatively damaged tissue determines the metabolic and inflammatory polarization of macrophages.

Discussion

We have previously shown that antioxidant macrophages (Mox) require suppression of regular energy metabolism to produce the antioxidant glutathione (3). The switch to aerobic glycolysis (referred to as the Warburg effect) in response to classic proinflammatory stimuli has been shown to be necessary for cytokine production by M1 macrophages (34). In adipose tissue, M1 macrophages are thought to be essential for the pathogenesis of obesity. However, the phenotypic and bioenergetic changes of macrophages in lean and obese adipose tissue are poorly understood.

We show here that specific OxPL differentially reprogram macrophage metabolism. We found that truncated OxPL inhibit macrophage respiratory capacity, an indicator of mitochondrial function and oxidative phosphorylation. Additionally, truncated OxPL induce expression of genes involved in redox homeostasis. In contrast, the full-length OxPL induce proinflammatory gene expression and do not inhibit respiration, instead promoting aerobic glycolysis. Together, these experiments clearly show the varying effects that different OxPL classes have on macrophage phenotype and bioenergetic polarization.

Numerous studies have confirmed that adipose tissue, being a highly metabolic tissue, experiences oxidative stress. The majority of these studies focus on oxidative stress that develops during obesity (11, 58); however, some studies suggest that there is already a notable basal level of oxidative stress in lean adipose tissue of healthy mice (11). As a product of oxidative stress, OxPL have been shown to be present on oxidized LDL particles (55), on the surface of apoptotic cells (59, 60), in multiple sclerosis (61), in atherosclerotic lesions (62), and in the plasma of patients with cardiovascular disease (63–66), demonstrated by using the E06 antibody, a mouse IgM antibody that recognizes oxidized phosphatidylcholines (54, 55, 67, 68). To identify individual OxPL species and to measure their abundance during disease progression, we developed an LC-MS/MS method based on previously published works (18, 44, 45, 69, 70). We grouped individual OxPL species based on the functional oxidized groups and biological activity (30, 48, 57). Similar to the findings of Podrez et al. (70), we found micromolar levels of OxPL species in murine whole blood, and a significant increase in response to...
high-fat feeding. As has been previously reported, HFD feeding induces PLA2 activity in blood (71) and it has been found that PLA2 preferentially cleaves the sn-2 moiety from truncated OxPL (72), offering an explanation for the increased level of LysoPC at the cost of carbonyl-PC species. Additionally, it was previously demonstrated that during obesity adipocytes significantly up-regulate two isoforms of secretory PLA2, specifically PLA2G5 and PLA2G2E (73). On the other hand, LysoPC is also an end product of phospholipid oxidation (52). Alternatively, it is possible that the truncated OxPL are covalently adducting to proteins, as was shown before in the case of POVPC binding to lysine residues on proteins (74).

Analyzing the macrophage-containing fraction of adipose tissue (the SVF) from mice fed chow or HFD, we found that the full-length OxPL species increase disproportionately compared with the truncated OxPL after HFD, providing an endogenously formed class of compounds that promote inflammation. Although we find full-length species to constitute a majority of the OxPL content in lean adipose SVF, it is possible that the truncated OxPL after HFD, providing an endogenously formed class of compounds that promote inflammation. Although we find full-length species to constitute a majority of the OxPL content in lean adipose SVF, it is possible that the truncated OxPL are covalently adducting to proteins, as was shown before in the case of POVPC binding to lysine residues on proteins (74).

Fig. 6. Truncated OxPAPC species promote a quiescent metabolism and antioxidant gene expression while full-length stimulate metabolism and proinflammatory gene expression. (A) Schematic representing the generation of OxPAPC from native PAPC followed by solid-phase extraction-based separation of full-length (FL) and truncated (T) OxPAPC species. (B) Fraction FL, is shown using a pie chart for relative species abundance (Left) and a precursor ion scan for 184 (Right). (C) Fraction T, is shown using a pie chart for relative species abundance (Left) and a precursor ion scan for 184 (Right). (D) MST of BMDMs treated with vehicle (RPMI media), 10 μg/mL OxPAPC, Fraction FL, or Fraction T for 4 h. Maximal OCR (respiratory capacity) and stressed ECAR (glycolytic capacity) also presented, with the results summarized in a bioenergetics chart (Right). (E) mRNA expression of proinflammatory genes Iliβ, Il6, Cxcl1, and Cox2 measured by qPCR in BMDMs treated with vehicle (RPMI media), 10 μg/mL OxPAPC, vehicle FL/T (combined eluents from column loaded with only chloroform, dried down and resuspended with RPMI media), 10 μg/mL Fraction FL, or Fraction T for 4 h. **P < 0.01; *P < 0.05; ns, nonsignificant. (F) mRNA expression of redox homeostasis genes Ho1, Txnrd1, and Gclm, and glucose transporter Glut1 measured by qPCR in BMDMs treated with vehicle (RPMI media), 10 μg/mL OxPAPC, vehicle FL/T (combined eluents from column loaded with only chloroform, dried down and resuspended with RPMI media), 10 μg/mL Fraction FL, or Fraction T for 4 h. "P < 0.05; ***P < 0.001.
macrophages (43, 77). Furthermore Kratz et al. (13) recently labeled ATMs found in obesity as “metabolically activated,” described as macrophages that result from the metabolic environment of obesity, lending them to have both pro- and antiinflammatory characteristics. Here, we identify two distinct populations of ATMs (CD45 + F4/80 + CD11b +) previously observed and coined F4/80lo (CX3CR1lo) and F4/80hi (CX3CR1hi), based on differential F4/80 and CX3CR1 expression (40, 78). CX3CR1 is not thought to play a role in diet-induced obesity, but it is considered a marker of monocyte-derived macrophages. CX3CR1 does not seem to be expressed on the F4/80hi population, indicating that these cells would not be subject to generic modification by CX3CR1-driven Cre recombination.

We find that the F4/80lo population describes the resident ATMs, constituting the majority of macrophages in lean mice, and does not change in abundance after high-fat feeding. The F4/80hi ATMs significantly increase in number after high-fat feeding, identifying them as the infiltrating population. Unexpectedly, we find that the predominant ATM phenotype from lean mice appears to be Max (F4/80hi HO-1 “Tmx1Δ1”), with a fraction being of the M2 phenotype (F4/80lo CD206+). Over the course of HFD feeding and development of obesity, we find that the Max macrophages become outnumbered by macrophages that are M2 and M1/M2 hybrids (F4/80+CD11c+CD206+). This was accompanied by a relative loss of truncated OxPL species, we discovered to promote redox homeostasis after HFD feeding, and a disproportional increase of full-length OxPL species. We demonstrate to promote an inflammatory macrophage profile.

Taking these data together, we show that ATM cellular metabolism changes dramatically during obesity. We show that this shift during obesity from a quiescent to an energetic metabolic profile correlates with two more changes: shift from (i) truncated to full-length OxPL species and (ii) antioxidant Mox to activated M1/M2 ATMs. Finally, we connect the OxPL microenvironment of SVF to the ATM polarization state by showing that: (i) truncated OxPL induce antioxidant gene expression and quiescent metabolism and (ii) full-length OxPL induce proinflammatory gene expression and an energetic metabolism. Further study into modulating the metabolism of macrophages in obesity is warranted, as it may offer an important tool for addressing whole-body metabolism-linked pathologies.

Materials and Methods

**Mice.** C57BL/6 mice were obtained from Jackson Laboratories and housed in the Pinn vivarium, at the University of Virginia, according to standard animal care guidelines established by the University of Virginia’s Institutional Animal Care and Use Committee. Diet-induced obesity mice and controls were purchased from Jackson Laboratories after 8–17 wk of HFD feeding, depending upon the experiment.

**Air Oxidation of Phospholipids.**

**Chemical reagents.** PAPC and PLPC were obtained from Avanti Polar Lipids. As previously described, PAPC (1 mg) was dried down in a glass tube, covered loosely with aluminum foil, and allowed to oxidize by air for 5 to 12 d (3). PLPC (1 mg) was dried down in a glass tube, covered loosely with aluminum foil, and allowed to oxidize by air for 3 to 4 wk. The oxidation status was monitored by qualitative direct inject-MS analysis and quantitative LC-MS quantification. The final mixture of phospholipids is referred to as OxPAPC or OxPLPC.

**Classification.** We broadly defined species as either “full-length” or “truncated” based on the extent of arachidonic acid oxidation in the sn-2 position and previously defined conventions (48, 57). Species that we classified in the full-length group include: HETE-PC, HETPE-PC, PEPC, PEIPC, H2-IP-PC, and F2-IP-PC. Species we classified in the truncated group include: POCP, PGCP, PONPC, PAzPC, HOA-PC, KOA-PC, HODia-PC, KODia-PC, and LysoPC. Of the 13 species of OxPAPC and 2 species of OxPLPC we detected, all have been previously identified, allowing us to assign them to one of four classes based on the structure of the oxidized moiety and previously characterized biological activity (30, 48, 49). Of the full-length species, we defined the class of “HETE/HETPE” to encompass species of arachidonic acid containing either a hydroxide or hydroperoxide functional group, such as the species HETE-PC and HPETE-PC. These species have been shown to be produced through action of lipoxygenases (for HPETEs) and subsequent action of glutathione transferases (for HETEs), but are also thought to be products of simple air oxidation (40). These species have a variety of biological activities, including but not limited to regulating macrophage response to apoptotic cells (79) and inducing platelet aggregation (80). Next, of the full-length species measured, we defined a class based on the oxidized moiety’s structural similarity to prostaglandins, which we refer to as “isoprostane.” Members of this group include F2-IP-PC, H2-IP-PC, PEIPC, and PEPC. Watson et al. (81) first described PEPC, shown to be a proinflammatory mediator (23) thought to activate the NLRP3 inflammasome in a caspase-11–dependent manner (16). PEPC, containing a cyclopentenone group and bearing a strong structural resemblance to Prostaglandin J2, has been reported to be an activator of Nrf2 (76, 82, 83).

Of the truncated species, the “carbonyl” class denotes the presence of an aldehyde or carboxylic acid on the oxidized moiety, and contains POVPC, PGPC, PONPC, and PAzPC. The species in the carbonyl group have previously been described to have mild proinflammatory activity (84, 85), LPS-inhibiting activity (86, 87), and Nrf2-activating ability. Another class of truncated OxPLs we define as “γ-keto/hydroxyl” are αβ unsaturated γ-keto- or γ-hydroxy containing species, including HOA-PC, HOdIA-PC, KOA-PC, and KODia-PC. The γ-keto/hydroxyl compounds were discovered by Podrez and et al. (25, 50), who coined them as OxPC_{α,β} for their ability to bind the CD36 scavenger receptor on macrophages to promote foam cell formation and atherogenesis.

**Separation of OxPL Classes.** Using a solid-phase extraction procedure of separating lipid classes (56), we separated full-length and truncated OxPAPC species. We loaded 1 mg of OxPAPC in chloroform on Hypersep amino-propyl cartridges. First, the column was washed with 4 mL of hexanes. Next, 2 mL of 5 mg/mL chloroform was added to the column. The column was then eluted with 4 mL methanol to recover Fraction FL (containing predominantly full-length species), followed by 4 mL of 420:350:100:50:0.5 hexanes: isopropanol:ethanol:water:formic acid with 3.75% phosphoric acid to recover Fraction T (containing predominantly truncated species). The fractions were then dried down under argon and suspended in solvent A (69% methanol, 31% water, 10 mM ammonium acetate) for LC-MS analysis or in cell culture media (RPMI with 10% FBS, 2% Hepes, and 1% anti-anti for cell culture.

**OxPL Measurement by LC-MS/MS.** Lipids from samples were extracted by a modified Bligh-Dyer extraction. The tissue was manually homogenized and then mixed in a glass tube with 750 μL HPLC-grade chloroform and 250 μL HPLC-grade methanol supplemented with at least 0.01% butylated hydroxytoluene (BHT; Sigma) and 189 nM of the internal standard for phosphatidylcholines (PC), 1-nanomol-phosphatidylcholine (DNPC, Avanti Polar Lipids). One milliliter of HPLC-grade water was added and the mixture was vigorously vortexed for 60 s. Next, the mixture was centrifuged (200 g x 10 min) to separate the fractions and the organic layer (bottom) was removed and placed into a fresh glass tube. One milliliter of chloroform was added to the aqueous fraction and the extraction was performed once more. The organic layer of the second extraction was combined with the first extraction, and the mixture was dried down under nitrogen. Upon complete evaporation of the organic solvent, the lipids were suspended in 300 μL of Solvent A (69% water, 31% methanol, 10 mM ammonium acetate), vortexed vigorously, and stored at −80 °C. The determination and quantification of both nonoxidized and oxidized PC species was performed by LC-linked electrospray ionization (ESI) mass spectrometry using a Sciex 4000 QTrap (see SI Appendix, Table S1 for species specific parameters). Of the oxidized species listed, the isoprostane-PC species H2-IP-PC and F2-IP-PC have isobaric species, consisting of multiply hydroxylated and peroxylated PAPC, which are currently unable to be resolved by fragmentation or LC. Separation of the phospholipids was achieved by loading samples onto an EVO C18 column (Kinetex 5 μm, 100 × 4.6 mm; Phenomenex). Elution of the phospholipids was achieved using a binary gradient with Solvent A (69% water, 31% methanol; 10 mM ammonium acetate) and Solvent B (50% methanol, 50% isopropanol; 10 mM ammonium acetate) as the mobile phases, with the oxidized species eluting earlier than the nonoxidized species. Detection for PC species was conducted using multiple reaction monitoring in positive mode by identification of two transition state ions for each analyte. Quantification of each PC analyte was performed based on the peak area of the positively charged 184 m/z fragment ion.

**Bone Marrow Isolation and Culture.** BMDDMs were cultured as previously described (3). Briefly, the bone marrow from the hind legs of mice was extracted and incubated for 5 min with 0.83% ammonium chloride, to clear erythrocyte progenitors. The bone marrow was cultured with RPMI media
supplemented with 10% FBS (Atlanta Biologicals), 5% Hepes (Gibco), 1% antibiotic-antimotic (Gibco), and 10% L929-conditioned media (L929 cells purchased from ATCC). The culture continued for 7 d, with media changes every 3–4 d, after which the media was exchanged for one lacking L929-conditioned media. On day 7, the BMDMs were gently separated from Petri dishes using 0.25% trypsin (Gibco), followed by centrifugation and media replacement. Finally, BMDMs were plated on nontissue culture treated Petri dishes for various treatments and analyses.

Isolation of SVF from Adipose Tissue. The SVF was collected as previously described (88). The adipose tissue was excised from the mouse and minced with scissors. The tissue was then digested for 30 min in a solution containing 2 mg/ml collagenase type II solution at 37 °C. After digestion, the tissue was filtered through a nylon mesh (100 μm) and washed with buffer containing high salt concentration and 1% BSA. The filtered cells were allowed to separate, with the adipocytes floating on top of the buffer. The infranatant was collected, centrifuged (450 × g for 5 min) and incubated with 0.83% NH₄Cl for 5 min to remove red blood cells. Finally the cells were washed, centrifuged, and resuspended in various buffers or media, depending on the subsequent analysis.

Quantitative Real-Time PCR. Quantitative real-time PCR was performed as previously described (3). Briefly, an RNaseasy Mini Kit (Qiagen) was used to isolate RNA from 300 to 500 thousand cells. The iScript cDNA Synthesis Kit (Biorad) was used to produce cDNA libraries. SensiMix SYBR & Fluorescein Kit (Bioline) was used in conjunction with 250 ng of cDNA for quantitative real-time PCR. Primer pairs used were described previously or designed using Primer Blast and validated using melt curve analysis. Sequences for each primer pair can be found in SI Appendix, Table S2.

Flow Cytometry Analysis of SVF. Cell preparation for flow cytometry was performed as previously described (88). Cells were suspended in FACS buffer (0.5% BSA, 2 mM EDTA, and 0.18% NaN₃ in PBS). Cells were first stained with Live/Dead Yellow dye, then fixed using 4% paraformaldehyde. Cell surface Fc receptors were blocked with CD16/32 for 20 min in FACS buffer to prevent nonspecific binding of antibodies to surface receptor. Cells were washed in FACS buffer and stained for surface markers (CD45, F4/80, CD11b, CD11c, and CX3CR1) for 30 min. Cells were washed and permeabilized with 0.1% saponin. Cells were then stained for intracellular markers (HO1, TXNRD1, and CD206) in FACS buffer containing 0.1% saponin for 30 min. Cells were washed three to four times with FACS buffer containing 0.1% saponin to dilute and remove nonbound antibodies. Finally, cells were washed and suspended in FACS buffer for final analysis. The flow-cytometer used was the BD LSRFortessa, and data were analyzed using FlowJo v10 (Flowjo). Compensation was performed in FlowJo using single stain and fluorescence-minus-one controls, as well as compensation beads.

Mitochondrial and Glycolytic Stress Tests. Extracellular flux analysis was performed as previously described (3). Briefly, cells were seeded into a Seahorse 24-well plate (Agilent Technologies). The cultures adhered for a minimum of 1 h before treatment. For assessing respiratory capacity, cells were subjected to a mitochondrial stress test (MST) as established previously (89), briefly described here. At the beginning of the assay, the media was changed to DMEM with pyruvate (Thermo-Fisher Cat#:12800017; pH = 7.35 ± 3 °C) and cells equilibrated for 30 min. OCR from the cell media was measured using 4-min measurement periods, represented in units of pmol O₂/min, using a Seahorse XF24 Flux Analyzer (Agilent Technologies). After three basal OCR measurements, compounds to modulate cellular respiratory function [1 μM Oligomycin (Sigma-Aldrich); 2 μM BAK15 (Cayman Chemical Company); 1 μM Antimycin A and 100 nM Rotenone (Sigma-Aldrich)] were injected into the plate, after every three measurements, in order. Basal respiration was calculated by subtracting the average of the post-Antimycin A and Rotenone measurements from the average of the first three measurements. Maximum respiratory capacity was calculated by subtracting the average of the post-Antimycin A and Rotenone measurements from the average of the post-BAM15 measurements. The reserve capacity was calculated by subtracting the average of the basal measurements from the average of the post-BAM15 measurements. For assessing the glycolytic capacity of the cells, a glycolytic stress test (GST) was performed to measure ECAR, representing the secretion of lactate into the extracellular media. The GST was used in lieu of measuring ECAR concurrently during an MST. The media used for the GST (unbuffered, glucose-free DMEM; Sigma-Aldrich Cat#: D5030; pH = 7.35 ± 3 °C; supplemented with 143 mM NaCl and 2 mM glutamine) has fewer buffering agents than the media used for the MST, allowing for greater sensitivity in measuring ECAR. After three basal ECAR measurements, compounds to modulate glycolysis [1 μM Oligomycin (Sigma-Aldrich); 20 mM GM6001 (Calbiochem); 20 mM D-Deoxyglucose (Sigma-Aldrich)] were injected after every four measurements and ECAR was measured using 3-min measurement periods. Basal glycolysis was calculated by subtracting the average of the post–2-Deoxyglucose measurements from the average of the post-Glucose measurements. Glycolytic capacity was calculated by subtracting the average of the post–2-Deoxyglucose measurements from the average of the post-Oligomycin measurements. Finally, XF Phenotrons (Agilent Technologies), or bioenergetic fingerprints were created using the maximum respiratory capacity from MST (y-axis) and the glycolytic capacity from GST (x-axis) from parallel experiments in which the cells received identical treatments.

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