Metabolic remodeling of white adipose tissue in obesity

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Although lack of exercise is an undeniable risk factor for weight gain (21, 27, 50), excessive caloric intake appears to be one of the key factors fueling the obesity epidemic. In the past three decades, the average consumption of calories in the US has increased by ≥200 kcal/day per person, which could be partly attributable to an increase in the intake of energy-dense foods (7, 29, 40, 77). Such poor dietary habits negatively affect metabolic homeostasis, which could not only promote obesity but hasten the development of obesity-related comorbidities as well. Despite the simplicity of the apparent remedy (i.e., decreasing caloric intake), treatment of obesity remains a challenging crisis facing the health care system. The efficacy of losing weight via caloric restriction is limited by multiple challenges. These include an evolutionarily engendered guard against starvation and low fat mass (70, 78) and a propensity to increase caloric efficiency during dieting (34, 47). Although drugs with anorectic and antiobesogenic properties are in clinical use, many of these show marginal long-term efficacy or have unacceptable or overtly dangerous side effects. Thus, recent strategies to modulate obesity have begun to target tissues that naturally regulate energy metabolism (70).

Increasing energy expenditure by modulating adipose tissue activity could be an attractive target for therapy. Because adult humans maintain small depots of brown fat that are capable of burning significant amounts of caloric energy (16, 53, 72, 75), multiple studies have focused on the physiological and molecular mechanisms regulating the thermogenic capacity of adipose tissue. These studies have shown that adaptive thermogenesis in brown fat can be a powerful regulator of systemic energy metabolism. However, the relatively small amount of brown adipose (<0.4% of body weight) compared with white adipose tissue (WAT; which can comprise ≥40% of the body weight of an obese human) suggests that WAT may be a more tangible target. Interestingly, white adipose depots, which typically function to esterify free fatty acids and store excess lipids, have the capacity to develop into brown adipose-like tissue that is capable of modulating systemic metabolism and preventing obesity and insulin resistance (65).

Although the phenomenon of adipose tissue “browning” is an active area of research, there is also considerable interest in understanding the metabolic changes that occur in WAT with obesity. It is becoming increasingly clear that conditions of nutrient excess promote “whitening” of adipose tissue characterized by a decrease in mitochondrial abundance (6, 14, 68). Hence, although promoting browning is one way to positively modulate metabolism, decreasing adipose tissue whitening could be another strategy to prevent dysregulation of systemic metabolism during obesity. Indeed, the beneficial metabolic changes induced by drugs such as rosiglitazone and pioglitata-
zone have been suggested to be due in part to their ability to prevent loss of mitochondria or increase mitochondrial function in WAT (6, 79). Nevertheless, mitochondrial changes in WAT during the development of obesity have not been thoroughly examined, and it remains unclear how changes in adipocyte metabolism affect adipocyte hypertrophy and metabolic homeostasis.

In this study, we examined WAT-specific changes in metabolism in a mouse model of diet-induced obesity. Our data indicate extensive metabolic remodeling of WAT that precedes both the infiltration of inflammatory cells and overt decreases in mitochondrial abundance. This renovation of adipocyte metabolism involved changes in glucose and lipid pathways that appear to favor fat storage and prevent excessive lipid oxidation. The metabolite profile also yields evidence of osmotic stress and adipocyte inflammation. Rerstructuring of intermediary metabolism was accompanied by biochemical and ultrastructural evidence of autophagy, which may promote degradation of mitochondria. These findings have important implications for understanding the metabolic effects of obesity on adipose tissue metabolism and suggest that targeting metabolic pathways that contribute to adipose tissue whitening or hypertrophy could form the basis for novel therapies to combat metabolic disease.

METHODS

Animal studies. All procedures were approved by the University of Louisville Institutional Animal Care and Use Committee. C57BL/6J [wild-type (WT)] mice were purchased from The Jackson Laboratory (Bar Harbor, ME). At 8 wk of age, male mice were placed on either a 10% low-fat diet (LFD) (no. D12450B; Research Diets) or a 60% high-fat diet (HFD) (no. D12492; Research Diets) for 6 or 12 wk. Water and diet were provided ad libitum. Body weights were recorded weekly.

Metabolic phenotyping. Body composition was measured by dual-energy X-ray absorptiometry using a mouse densitometer (PXiImus2; Lunar, Madison, WI), and whole body energy expenditure, respiratory exchange ratio, food consumption, and locomotion, ambulatory, and rest energy were measured using a physiological/metabolic cage system (TSE PhenoMaster System, Bad Homberg, Germany) (54). Glucose and insulin tolerance tests and plasma levels of insulin were performed. Most analyses were performed using Metaboanalyst 2.0 software (http://www.metaboanalyst.ca/) (82); z-score plots were constructed in GraphPad 5.0 software.

Adipose tissue bioenergetic measurements. The oxygen consumption rate (OCR) of intact WAT explants was measured using a Seahorse XF24 analyzer (Seahorse Bioscience, Billerica, MA), as described previously (54). At least two replicates from each animal were used for the assay. After baseline measurements, the maximal OCR was measured by exposing the explants to FCCP (10 μM). The nonmitochondrial OCR was measured following injection of antimycin A (25 μM) and rotenone (5 μM).

Citrate synthase activity assay. Citrate synthase assay was performed in 100 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 1 mM 5′,5′-dithiobis-2-nitrobenzoic acid, and 10 mM acetyl-CoA. The reaction was initiated by the addition of 10 mM oxaloacetate. Cuvettes were warmed to 37°C, and upon addition of 10 μg of protein from WAT lysates, absorbance at 420 nm was measured for 10 min. Activity is expressed as nanomoles per minute per microgram of protein.

Expression analyses. For quantitative RT-PCR, RNA was extracted from tissues using the RNeasy lipid tissue kit (Qiagen), followed by cDNA synthesis. Real-time PCR amplification was performed with SYBR Green quantitative PCR (qPCR) Master Mix (SA Biosciences) using a 7900HT Fast Real-Time PCR System (Applied Biosystems) and primers for Il1, Tnfα, Il6, Arg1, Il10, Ym1, Hif1α, Emr1, Pgc1a, Cytb, Sirt1, Sirt3, Pdk4, Cpt1a, Cpt1b, Cox7a1, Hprt, and Idh3α (IDT Bioscience). Relative expression was determined by the 2−ΔΔCT method. Mitochondrial cytochrome c oxidase (CM COX1) M1 macrophages in WAT were measured by flow cytometry, as described previously (22).

For measuring protein abundance, WAT homogenates were prepared as described in Horrillo et al. (25). Equal amounts of protein were separated by SDS-PAGE, electroblotted to PVDF membranes, and probed using primary antibodies according to the respective manufacturers’ protocols. The following antibodies were used: aldehyde dehydrogenase 2 (ALDH2; Abcam), sirtuin 3 (Sirt3; Cell Signaling Technology), MitoProfile Total OXPHOS Rodent WB Antibody Cocktail (Mitosciences), COX4I1 (Cell Signaling Technology), GAPDH (Cell Signaling Technology), Parkin (Abcam), Pink1 (Cell Signaling Technology), p62 (Cell Signaling Technology), LC3 (Cell Signaling Technology), MitoProfile Total OXPHOS Rodent WB Antibody Cocktail (Mitosciences), CYC (Bioscience). Relative expression was determined by the 2−ΔΔCT method.

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Relative mitochondrial DNA measurements. Mitochondrial abundance in adipose tissue was estimated by measuring mitochondrial DNA (mtDNA) abundance relative to nuclear DNA (nDNA) (73). Total DNA was isolated from WAT using a QIAamp DNA Mini Kit (Qiagen). A 25-ng aliquot of the tissue was homogenized, followed by overnight digestion in proteinase K at 55°C. Following isolation, relative amounts of mtDNA and nDNA were compared using quantitative real-time PCR, using 2 ng of the isolated DNA. Primers for cytochrome b (mtDNA) and β-actin (nDNA) were used; the sequences are cytochrome b, 5′-TCGGTTTGTTGTATCGTTCG-3′ and 5′-CTTGCTTTTCCATCTCATCTACC-3′ and β-actin, 5′-CAGGATGCTCTTCTTGCCTCT-3′ and 5′-CGTCTCCTCCTTTC-3′.

Electron microscopy. Adipose tissues were fixed with 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 4 h at room temperature (25°C). The tissues were then postfixed with 1% osmium tetroxide for 1 h, dehydrated, and embedded in Embed-812 plastic (Electron Microscopy Sciences). Ultrathin sections were stained with uranyl acetate and Reynolds lead citrate, and electron micrographs were taken using a Philips CM10 transmission electron microscope operating at 80 kV.

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Statistical analyses. Data are means ± SE. Unpaired Student’s t-test was used for direct comparisons. Statistical analyses for metabolomic data sets were performed using Metaboanalyst 2.0 software. A P value of <0.05 was considered significant.

RESULTS

HFD increases adiposity and alters systemic metabolism. WT C57BL/6J mice were placed on a LFD or HFD for 6 wk. Significant weight gain occurred as early as 1 wk on HFD, and the change in total body mass was nearly 10 g by 6 wk on the diet (Fig. 1A). Food and water intake were not significantly different between groups (Fig. 1, B and C). Dual-energy X-ray absorptiometry scan analysis showed a twofold increase in percent fat mass and a concomitant decrease in percent lean mass in HFD-fed mice (Fig. 1, D and E). These results are typical of this commonly utilized model of diet-induced obesity (30, 54).

To determine how diet affects systemic metabolism, mice fed either LFD or HFD for 6 wk were placed in metabolic chambers, and their oxygen consumption (VO2), carbon dioxide production (VCO2), and physical activity were measured. As shown in Fig. 1, F and G, average VO2 and VCO2 values decreased in HFD-fed mice compared with mice fed a LFD. The respiratory exchange ratio (RER) was also decreased in HFD mice vs. mice fed LFD (Fig. 1H). Physical activity, measured by total beam breaks (Fig. 1I), ambulatory counts (Fig. 1J), and fine movements (Fig. 1K), was not significantly different between groups, although the group fed a HFD...
appeared to show a trend toward decreased activity. Consistent with our previous results (54), mice fed a HFD also demonstrated worsened glucose and insulin tolerance (Fig. 2) as well as a significant increase in plasma insulin levels (WT LFD 151 ± 71 pg/ml vs. WT HFD 2,690 ± 593 pg/ml; n = 3/group, P < 0.05).

Robust macrophage infiltration does not occur with 6 wk of HFD. To examine the effects of HFD on macrophage infiltration, we placed mice on LFD or HFD for 6 or 12 wk and measured adipocyte size, crown-like structures that are indicative of macrophage infiltration, and the expression of inflammatory genes. As shown in Fig. 3, A–C, mice fed a HFD for 6 and 12 wk showed a three- to fourfold increase in adipocyte size when compared with LFD controls. Whereas sections of WAT derived from mice fed a HFD for 12 wk showed obvious increases in crown-like structures, WAT from mice fed HFD for 6 wk showed minimal increases in such structures. These observations suggest that with 6 wk of HFD there is minimal macrophage accumulation. Indeed, the expression of Emr1, a marker of macrophages, as well as that of other inflammatory genes was not changed with 6 wk of HFD (Fig. 3D). Moreover, the abundance of M1 macrophages in adipose tissue stromal vascular fractions was not different between mice fed different diets (F4/80+/CD11c−/CD301− cells as %F4/80− cells: LFD 37.4 ± 3.5, HFD 45.9 ± 1.8; n = 9–10/group, P > 0.05). Most likely, the modest, insignificant increase in Tnfa is due to adipocytes, which have been shown to be capable of producing TNFα (26, 60). In addition, no increase in plasma levels of inflammatory mediators such as IL-6 were identified at 6 wk of HFD [IL-6 (pg/ml): LFD, 23.6 ± 7.5; HFD, 18.8 ± 4.2].

Collectively, these data show that adipocyte size was increased after 6 wk of HFD, without significant changes in infiltrating inflammatory cells.

Obesity alters the metabolite profile of adipose tissue. To examine the effect of obesity on adipose tissue metabolism, epididymal WAT from mice fed a LFD or HFD for 6 wk was subjected to unbiased metabolomic analysis. The relative concentration of adipose metabolites was measured by mass spectrometry and queried against the Metabolon reference library. It is important to note that the composition of adipose tissue derived from mice fed a LFD differed from mice fed a HFD. Compared with the LFD group, the protein yield per milligram wet weight was 43% lower in the adipose tissue of the HFD group (µg protein/mg wet wt: 6 wk LFD, 10.86 ± 1.70; 6 wk HFD, 6.21 ± 1.28; n = 10–12/group). Hence, for metabolomic analyses, we corrected for this difference in protein content. Partial least squares-discriminant analysis (PLS-DA) with the corrected data showed that the LFD samples separate clearly from HFD samples (Fig. 4A), and cluster analysis showed that the abundance of several metabolites was associated with diet (Fig. 4B). Of the 191 metabolites measured, 49 were found to be significantly different (P < 0.05) in the WAT of mice fed a HFD compared with the adipose tissue from mice fed a LFD. Volcano plot analysis, using data corrected for protein content, showed significantly higher levels of 34 metabolites and significantly lower levels of 15 metabolites in the adipose tissue of HFD-fed mice (Fig. 4C and Table 1). Volcano plot analysis based on uncorrected raw data showed a large skew toward decreased abundance of most metabolites (Fig. 4C, inset). To visualize the data in the biological context of metabolic pathways, metabolites that were statistically different in each group were analyzed using the MetPA tool of Metaboanalyst 2.0 software. The pathways were calculated as the sum of the importance measures of the matched metabolites normalized by the sum of the importance measures of all metabolites in each pathway (55, 81). As shown in Fig. 4D, the highest pathway impact values were related to linoleic acid metabolism and Phe, Tyr, and Trp metabolism. Branched-chain amino acids (BCAA), i.e., Val, Leu, and Ile, and pathways related to taurine metabolism, glycerolipid metabolism, Ala, Asp, and Glu metabolism, carbohydrate metabolism, and pyrimidine metabolism also showed relatively high pathway impact and significant values.

To delineate changes in metabolites further, metabolites were categorized based on superfamilies, and z-score plots were constructed. As shown in Fig. 5, the abundance of multiple members of the lipid superfamilies, including those involved in glycerolipid metabolism and linoleic acid metabolism, was higher. Several carnitine derivatives, with the sole exception of 3-dehydrocarnitine, were higher in abundance as well. In the amino acid superfamilies, the levels of nonessential amino acids Glu, Gln, and Asp were elevated, and those of essential amino acids Phe, Leu, and Val were lower. Notably, the organic osmolytes taurine, hypotaurine, and creatine were more abundant. In the HFD group, levels of the Krebs cycle intermediate succinate were higher; levels of malate were higher as well, although pantothenate, a precursor to CoA, was less abundant. Although both glucose and maltose levels were higher, phosphorylated sugars were diminished. Plasma 1,5-anhydroglucitol (1,5-AG), which is present in most organs and tissues and has been shown to decrease with loss of glycemic...
control (67, 83), was diminished in abundance, which is in agreement with insulin resistance in skeletal muscle and liver occurring at 6 wk of HFD (30). Interestingly, the pyrimidine monophosphates cytidine-5'-monophosphate (CMP) and uridine monophosphate (UMP), as well as the purine inosine-5'-monophosphate (IMP), were elevated in WAT from HFD-fed mice. A graphic illustration of these changes, placed into context of known pathways of intermediary metabolism, is shown in Fig. 6.

**Effects of obesity on oxygen consumption and mitochondrial remodeling in WAT.** The changes in energy metabolism found in our metabolomic analyses suggested that HFD might have altered adipose tissue bioenergetics. Importantly, these changes occurred in the absence of inflammatory cell infiltration (see Fig. 3), which could otherwise confound adipocyte-specific changes in metabolism. To determine how obesity affects mitochondrial function, WAT explants from mice fed a LFD or HFD were subjected to extracellular flux analysis. As shown in Fig. 7, A and B, the apparent basal mitochondrial OCR of adipose tissue derived from mice fed a LFD was approximately twofold higher when compared with adipose explants derived from HFD-fed mice ($P < 0.05$); however, after correction for protein content, there was no difference in mitochondrial oxygen consumption. Nevertheless, explants derived from mice fed a HFD responded more strongly to FCCP (Fig. 7C). Although citrate synthase activity was lower by $>50\%$ in WAT derived from these mice (Fig. 7D), relative abundance of mtDNA, as assessed by qPCR of mtDNA normalized to nDNA, was not affected with 6 wk of HFD (Fig. 7E).

The observed changes in metabolite profile, increased mitochondrial responsiveness to FCCP, and diminished citrate synthase activity suggested that HFD might affect the expression of genes associated with metabolic activity. Indeed, the expression of Cox7a1, a subunit of cytochrome oxidase, was more than twofold higher in adipose tissue from mice fed a HFD, whereas the expression of Pgc1a, Sirt3, and Pdk4 was diminished (Fig. 8A), indicating mitochondrial remodeling despite preserved basal mitochondrial activity.

Fig. 3. Effect of HFD on adipose tissue expansion and inflammation. Morphological and molecular changes in adipose tissues. A: representative hematoxylin and eosin stains of epididymal adipose tissue from mice fed a LFD or HFD for 6 or 12 wk; inset shows a crown-like structure (CLS) in the 12-wk HFD group. B: average area of adipocytes. C: adipocyte size distribution. D: quantitative RT-PCR analyses of markers of inflammation in adipose tissues from mice fed a LFD or HFD for 6 wk; $n = 4–5$/group. *$P < 0.05$ vs. indicated groups.
To further understand how WAT mitochondria change with obesity, we assessed the relative abundance of several mitochondrial complex proteins as well as mitochondrial matrix proteins. Although no changes in mitochondrial protein abundance were observed at 6 wk of HFD, the protein levels of NDUFB8, SDHB, and COX4I1 subunits of complexes I, II, and IV, respectively, were diminished significantly by 12 wk of HFD (Fig. 8, B–E). The levels of the matrix proteins ALDH2 and Sirt3 showed similar trends, with ALDH2 being significantly lower after 12 wk of HFD.

Assessment of adipose tissue ultrastructure. To determine subcellular changes that occur in adipose tissue of nutrient-stressed mice, we examined adipocyte ultrastructure using electron microscopy. As shown in Fig. 9A, adipose tissue from mice fed a LFD showed mitochondria with three distinct morphologies: a round morphology of small size that was located near the nucleus (Fig. 9A, images i and ii), a typical elongated shape up to ~0.7 μm in length located in small protrusions along the adipocyte cell membrane (Fig. 9A, image iii), and long mitochondria (3 μm and above), which were located in juxtaposition to the fat locule (Fig. 9A, image iv). In adipocytes derived from HFD-fed mice, autophagosomes, defined by a double-membrane and comprising cytoplasmic constituents, were found next to mitochondria (Fig. 9B, image i), and large vacuoles of electron-dense material were found adjacent to autophagosomes (Fig. 9B, images ii and iii). In addition, many mitochondria in adipose tissue from HFD-fed mice appeared to be undergoing fission (Fig. 9B, images iv and v).

Fig. 4. Metabolomic analyses of adipose tissue. Analyses of epididymal adipose tissue metabolites from WT mice fed a LFD or HFD for 6 wk. A: multivariate analysis; partial least squares-discriminant analysis (PLS-DA). Inset shows PLS-DA analysis from the uncorrected data set. B: hierarchial clustering; heat map and dendogram. C: univariate analysis; volcano plot of metabolites. Those metabolites that were significantly higher in abundance are in the quadrant shaded red, and those that were significantly lower are shaded green (P < 0.05, t-test). The identity of these metabolites is found in Table 1. The inset shows volcano plot analysis using data that were not corrected for protein content. D: metabolites found to be significantly different were subjected to pathway impact analysis using Metaboanalyst MetPA and the Mus musculus pathway library. Fisher’s exact test was used for overrepresentation analysis, and relative betweenness centrality was used for pathway topology analysis; n = 14 animals (7 WT LFD and 7 WT HFD).
Effects of HFD on autophagy. Changes in mitochondrial proteins, along with the ultrastructural alterations found to occur in the adipose tissue, suggest that HFD may promote mitochondrial remodeling and activate mitophagy in WAT. To examine this possibility, we measured markers of mitophagy and autophagy in adipose tissues from mice fed a LFD or HFD for 6 wk. Levels of the E3 ubiquitin ligase Parkin, which has been shown to accumulate in mitochondria destined for degradation (24), were 2.3-fold higher with 6 wk of HFD and nearly twofold higher after 12 wk of HFD (Fig. 10, A and B). Furthermore, the kinase Pink1, critical for identifying mitochondria destined for autophagy (24), was also nearly 40% higher in the HFD groups. In combination with the presence of autophagosomes and mitochondrial alterations observed by EM (Fig. 9), this observation suggests that metabolic remodeling of adipocytes in the expanding adipose organ may be related to autophagy. To address this possibility, we measured changes in protein indicators of autophagy. As shown in Fig. 10, C–H, levels of p62 and LC3-I were diminished significantly, and the LC3-II/LC3-I ratio was more than twofold higher in mice fed a HFD for 6 wk compared with those placed on LFD. However, there was no significant difference in total protein abundance of protein-ubiquitin and LC3-II.

Table 1. List of adipose tissue metabolites that changed significantly in high-fat-fed mice

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Fold Change</th>
<th>P Value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stearoyl sphingomyelin*</td>
<td>2.8</td>
<td>0.000542</td>
<td>0.010414</td>
</tr>
<tr>
<td>Glycerophosphoethanolamine*</td>
<td>2.7</td>
<td>0.000000</td>
<td>0.000014</td>
</tr>
<tr>
<td>Succinate*</td>
<td>2.34</td>
<td>0.000139</td>
<td>0.006385</td>
</tr>
<tr>
<td>Inosine 5'-monophosphate</td>
<td>2.27</td>
<td>0.016131</td>
<td>0.088492</td>
</tr>
<tr>
<td>Uridine monophosphate (5' or 3')</td>
<td>2.26</td>
<td>0.009442</td>
<td>0.067442</td>
</tr>
<tr>
<td>Dihomo-linolenate (20:2n6)</td>
<td>2.03</td>
<td>0.011868</td>
<td>0.071209</td>
</tr>
<tr>
<td>Pelargone (9:0)</td>
<td>2.02</td>
<td>0.030984</td>
<td>0.14203</td>
</tr>
<tr>
<td>Cytidine 5'-monophosphate*</td>
<td>1.95</td>
<td>0.000214</td>
<td>0.006839</td>
</tr>
<tr>
<td>Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate, myo-inositol 1,4 or 1,3-diphosphate*</td>
<td>1.87</td>
<td>0.000166</td>
<td>0.006385</td>
</tr>
<tr>
<td>Maltose</td>
<td>1.86</td>
<td>0.03107</td>
<td>0.14203</td>
</tr>
<tr>
<td>Docosapentaenoate (22:5n3)</td>
<td>1.83</td>
<td>0.033432</td>
<td>0.14928</td>
</tr>
<tr>
<td>Glutamate*</td>
<td>1.81</td>
<td>0.000111</td>
<td>0.006385</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.8</td>
<td>0.005764</td>
<td>0.046111</td>
</tr>
<tr>
<td>Azelate (nonanediol)</td>
<td>1.75</td>
<td>0.001215</td>
<td>0.01795</td>
</tr>
<tr>
<td>Dihomo-linolenate (20:3n3 or n6)</td>
<td>1.75</td>
<td>0.006864</td>
<td>0.052713</td>
</tr>
<tr>
<td>Stearate (18:0)</td>
<td>1.74</td>
<td>0.003972</td>
<td>0.038131</td>
</tr>
<tr>
<td>Arachidonate (20:4n6)</td>
<td>1.68</td>
<td>0.001536</td>
<td>0.021059</td>
</tr>
<tr>
<td>Deoxycarnitine</td>
<td>1.64</td>
<td>0.000539</td>
<td>0.010414</td>
</tr>
<tr>
<td>Glycerophosphorylcholine</td>
<td>1.64</td>
<td>0.005243</td>
<td>0.045087</td>
</tr>
<tr>
<td>Hypotaurine</td>
<td>1.62</td>
<td>0.024921</td>
<td>0.12592</td>
</tr>
<tr>
<td>12-Hydroxyeicosatetraenoic acid</td>
<td>1.61</td>
<td>0.036945</td>
<td>0.16121</td>
</tr>
<tr>
<td>Acetylcarnitine</td>
<td>1.54</td>
<td>0.000362</td>
<td>0.00923</td>
</tr>
<tr>
<td>Margarate (17:0)</td>
<td>1.51</td>
<td>0.046138</td>
<td>0.18455</td>
</tr>
<tr>
<td>Aspartate</td>
<td>1.47</td>
<td>0.003862</td>
<td>0.038131</td>
</tr>
<tr>
<td>Creatine</td>
<td>1.46</td>
<td>0.000666</td>
<td>0.01162</td>
</tr>
<tr>
<td>Malate</td>
<td>1.45</td>
<td>0.008981</td>
<td>0.063867</td>
</tr>
<tr>
<td>Linoleate (18:2n6)</td>
<td>1.42</td>
<td>0.026561</td>
<td>0.13076</td>
</tr>
<tr>
<td>Palmitoyl ethanolamide</td>
<td>1.38</td>
<td>0.024441</td>
<td>0.12592</td>
</tr>
<tr>
<td>Propionylcarnitine</td>
<td>1.37</td>
<td>0.048868</td>
<td>0.18902</td>
</tr>
<tr>
<td>Taurine</td>
<td>1.33</td>
<td>0.001968</td>
<td>0.024159</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1.32</td>
<td>0.000372</td>
<td>0.008923</td>
</tr>
<tr>
<td>Choline phosphate</td>
<td>1.32</td>
<td>0.011199</td>
<td>0.06936</td>
</tr>
<tr>
<td>Citrulline</td>
<td>1.29</td>
<td>0.002367</td>
<td>0.025244</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.28</td>
<td>0.002251</td>
<td>0.025244</td>
</tr>
<tr>
<td>Leucine*</td>
<td>0.79</td>
<td>0.005401</td>
<td>0.045087</td>
</tr>
<tr>
<td>Tyrosine*</td>
<td>0.78</td>
<td>0.042828</td>
<td>0.18273</td>
</tr>
<tr>
<td>Pantothenate</td>
<td>0.73</td>
<td>0.049223</td>
<td>0.18902</td>
</tr>
<tr>
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<td>0.000986</td>
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FDR, false discovery rate. Wild-type mice were fed a low-fat (LFD) or high-fat diet (HFD) for 6 wk. Epididymal adipose tissue was then subjected to liquid chromatography or gas chromatography mass spectrometric analysis. Raw spectral values from white adipose tissue (WAT) of HFD-fed mice were corrected on the basis of mean difference in protein content (per mg wet wt) compared with WAT from LFD-fed mice. Adipose tissue metabolites in HFD-fed mice that were ≥25% changed in abundance and significantly different (P < 0.05) compared with LFD-fed mice are shown; n = 7 mice/group. *Metabolites that were significant even when ±2 SE was applied in protein correction.
Fig. 5. Z-score plot analysis of metabolite changes in adipose tissue from HFD-fed mice. Data are shown as standard deviations from the mean of LFD. Z-scores of some metabolites were not plotted due to lack of detectable signal in either the LFD or HFD group, e.g., glyceylleucine, glycerate. Each point represents 1 metabolite in 1 sample; n = 7/group.

DISCUSSION

The results of this study demonstrate coordinated restructuring of adipose tissue metabolism during adipocyte hypertrophy. Using metabolomics analysis, we identified several HFD-induced changes in key metabolites involved in energy, carbohydrate, nucleotide, lipid, and amino acid metabolism. Importantly, these changes were relatively independent of inflammatory cell infiltration, and the data were corrected for adipocyte content, both of which help eliminate confounding features related to tissue and adipocyte composition. Our analysis showed an early decrease in citrate synthase activity that coincided with reduced expression of Pgc1a, although mitochondrial abundance was not affected. Ultrastructural and immunological evidence suggested that whitening of the adipose tissue, evident after 12 wk of HFD, may also be related to autophagy, which appears to occur early after adipocytes attain near-maximum size. Taken together, these results reveal that conditions of nutrient excess promote progressive metabolic remodeling, decreased capacity for mitochondrial biogenesis, and potential activation of the autophagic program in WAT.

The major goal of this study was to understand how HFD affects adipose tissue biology and metabolism. This is important because key metabolic changes that occur during adipocyte hypertrophy could be targets for antiobesity or insulin-sensitizing therapies. We found that 6 wk of HFD feeding led to a profound increase in fat mass, which was accompanied by a decrease in systemic VO₂, VCO₂, and RER and insulin resistance. Although adipose tissue inflammation was evident by the 10th to 12th wk of HFD (41, 66), we found no significant increase in inflammatory cell infiltration with 6 wk of HFD (e.g., see Fig. 3 and Refs. 30 and 41). This lack of extensive tissue infiltration by energetic inflammatory cells allowed us to examine HFD-induced changes specific to the adipose tissue.

Moreover, in our metabolic measurements, we accounted for changes in the composition of the adipocytes themselves. Because white adipocytes expand by storing triglycerides in the central lipid droplet, an equivalent tissue mass of WAT from a HFD-fed mouse comprises fewer cells (and more triglyceride) than adipose tissue from a LFD-fed mouse. Thus, normalization of metabolite levels by wet weight alone could lead to erroneous conclusions. An example of this is shown in Figs. 4 and 6, where uncorrected values indicate that most metabolites are decreased in WAT from HFD-fed mice (Fig. 4C, inset) and that mitochondrial OCR is diminished in adipocytes from the obese mice (Fig. 7, A and B). Correcting for protein content in samples from HFD-fed mice normalized the distribution of metabolic changes and rectified the leftward skew caused by decreased adipocyte content. Similarly, when values were normalized for protein content (rather than mg wet wt), there was no difference in tissue oxygen consumption between explants from LFD and HFD groups. This indicates that with 6 wk of HFD there was no change in basal mitochondrial oxygen consumption per adipocyte.

Our metabolic profiling indicates that HFD affects several metabolic pathways. In mice placed on HFD for 6 wk, we found significant changes in lipid, amino acid, energy, nucleotide, and carbohydrate metabolism. Higher levels of several fatty acids and lipid metabolites belonging to the linoleic acid metabolism subfamily were observed in WAT from HFD-fed mice. In this tissue there was also a higher abundance of arachidonic acid as well as metabolites involved in arachidonic acid synthesis, including linolein acid and dihomo-linolenic acid, which indicates a coordinated upregulation of this pathway. WAT from HFD-fed mice also contained higher levels of the eicosanoid 12-hydroxyeicosatetraenoic acid (12-HETE), which is formed from arachidonate by 12/15-lipoxygenase (12/15-LO). Interestingly, 12/15-LO is upregulated in white adipocytes of both HFD-fed C57BL/6J mice (10) and Zucker obese rats (11). Thus, upregulation of 12/15-LO and increased...
production of its product (12-HETE) are consistent features of hypertropic WAT. This may be particularly significant in mediating adipose tissue dysfunction because two separate studies have shown that genetic deletion of 12/15-LO prevents HFD-induced adipose tissue inflammation and insulin resistance (42, 59).

The elevated levels of glycerophosphoethanolamine in WAT from obese mice could be due to an increase in the breakdown of phosphatidylethanolamine or a decrease in its rate of hydrolysis. Previous studies have shown that glycerophosphoethanolamine is hydrolyzed by enzymes such as glycerophosphodiester phosphodiesterase to form glycerol 3-phosphate (G3P) (84), which is required for triglyceride synthesis and thus would likely be in high demand in expanding adipocytes (46). Glycerophosphorylcholine, which was elevated in WAT from HFD-fed mice, could also be utilized to form G3P. However, a major function of glycerophosphorylcholine in the cell is to act as an organic osmolyte (19), suggesting that there might be an increase in osmotic stress in hypertrophic adipocytes. This view is supported by our observation that the levels of several major osmolytes such as taurine, hypotaurine, and creatine were higher in WAT from obese mice. Changes in osmolality could trigger a form of cell death called pyroptosis (4), which has been linked with inflammasome activity (57). Interestingly, the NLRP3 inflammasome is activated by cholesterol crystals, and, consistent with previous observations in hypertrophied adipocytes (20, 85), our analyses also showed an elevation of cholesterol in adipose tissue of obese mice. Collectively, these observations suggest an early increase in key metabolic instigators that could contribute to osmotic stress and inflammation in adipose tissue.

The HFD-induced increase in stearoyl sphingomyelin could further prime adipocytes for inflammation and metabolic dysfunction. Sphingomyelin (d18:1/18:0), which in humans is the only membrane phospholipid not derived from glycerol, is a type of sphingolipid found in cell membranes that consists of oleic acid attached to the C1 position and stearic acid attached to the C2 position. Genetic deletion of enzymes involved in sphingomyelin synthesis has been shown to protect against diet-induced obesity and insulin resistance (35, 38), and the
breakdown of sphingomyelin could yield significant amounts of ceramide, which inhibits insulin signaling (12). Hence, the elevated levels of sphingomyelin observed in our study could prime adipocytes to release significant amounts of ceramide, which has been shown to be increased by 300% in both the plasma and the adipose tissue by HFD (61). Although ceramide levels were not measured in our analyses, we did observe a decrease in 

Pgc1a

expression and a decrease in citrate synthase activity, both of which can be regulated by ceramide (56, 71). However, further studies are required to determine whether changes in mitochondrial biogenetic capacity and citrate synthase activity are related to high levels of stearoyl sphingomyelin.

Our metabolomic data suggest significant changes in intermediary metabolism related to energy expenditure and fat storage. We found that many carnitine derivatives were increased in WAT from HFD-fed mice. These include acetyl carnitine, propionyl carnitine, and deoxycarnitine, although 3-dehydrocarnitine, an intermediate in carnitine degradation, was diminished. These changes suggest the presence of a “flooded” pool of adipocyte metabolites, which could be used either for energy provision or, more likely, as a temporary repository for substrates for fatty acid synthesis. In other tissues such as the heart, acetyl carnitine functions as a buffer of the acetyl-CoA pool, although it could be used as an energetic substrate as well (58). Similarly, the propionyl carnitine could buffer the propionyl CoA pool (63). Thus, it appears that, in expanding adipocytes, short-chain acylcarnitine pools could help buffer excess carbon until it is assimilated into fatty acids for triglyceride storage.

The observation that tissue levels of BCAAs such as leucine and valine were decreased, whereas propionylcarnitine and succinate levels were increased with HFD, suggests an increase in BCAA catabolism. Catabolism of BCAAs results in the formation of both acetyl-CoA and succinyl-CoA, and the latter can be converted to succinate, which in our analysis was elevated in WAT from HFD-fed mice. Thus, it is likely that in hypertrophic adipocytes there is increased utilization of BCAAs. In contrast to the decrease in BCAAs, we found that levels of Gln and Glu were increased with HFD. This increase may be particularly important given the multifaceted uses of glutamine, e.g., in anaplerotic metabolism, as a nitrogen donor.
in anabolic processes as well as a precursor for glutathione synthesis (69, 80). In addition, Gln and Asp are required for synthesis of several nucleotides, such as IMP, UMP, and CMP, all of which were increased in these studies. Interestingly, adipose tissue is a major site of glutamine synthesis, and its uptake can be regulated by changes in osmolality as well as by insulin (49).

Although there is a clear relationship between amino acids, insulin resistance, and obesity in animal models and humans (1, 13, 39), the mechanistic basis of this link is less clear. BCAA metabolism in adipose tissue modulates plasma BCAA levels (23), and interestingly, increasing circulating BCAAs in mice by preventing BCAA catabolism prevents diet-induced obesity and insulin resistance in mice (62), whereas feeding BCAAs to HFD-fed rats increases insulin resistance (39).

Although further studies are required to test the full significance of such metabolic changes in BCAAs and other amino acid pathways, it is possible that changes in amino acid catabolism and downstream metabolites have novel roles in adipose tissue biology such as the regulating of lipid storage and release. This possibility is supported by a recent report showing that succinate, which can be derived from BCAA breakdown, inhibits lipolysis by activating Gpr91 (a succinate receptor) (48).

Metabolites related to glucose metabolism were also markedly affected by HFD. We found a nearly 50% decrease in the abundance of 1,5-AG in WAT samples from HFD-fed mice. Plasma 1,5-AG is a validated marker of short-term glycemic control and has been suggested to be in equilibrium in tissue and plasma pools (67, 83). Hence, although 6 wk of HFD does not appear to significantly affect fasting blood glucose levels (54), the decreased abundance of 1,5-AG is consistent with systemic insulin resistance and poor glycemic control (30). With respect to glycolysis, glucose 6-phosphate was decreased, and adipose tissue glucose was increased, suggesting that hexokinase might be a major rate-limiting step in the provision of glucose carbons required for triglyceride synthesis. Further evidence of changes in glucose metabolism is shown by diminished pdk4 expression, which regulates pyruvate dehydrogenase activity. This decrease in pdk4 could potentially promote glyceroconversion of G3P through cataplerosis (45).

Interestingly, although we observed no change in basal mitochondrial OCR when corrected for protein content, WAT

Fig. 8. Obesity-related changes in mitochondrial protein abundance in white adipose tissue. Analysis of gene expression and protein abundance in adipose tissue from mice fed a LFD or HFD for 6 or 12 wk. A: expression of metabolic genes after 6 wk of diet. B: representative Western blots of mitochondrial matrix proteins and respiratory chain subunits. C: quantification of aldehyde dehydrogenase (ALDH2). D: quantification of sirtuin 3 (Sirt3). E: quantification of respiratory subunit abundance. All blots were normalized to ATP5A, which showed no change in abundance in any group; n = 4/group. *P < 0.05 vs. 6-wk LFD; #P < 0.05 vs. 12 wk LFD. NDUFB8, NADH dehydrogenase-Iβ subcomplex subunit 8; SDHB, succinate dehydrogenase complex subunit B; UQRC2, ubiquinol-cytochrome c reductase core protein 2; MTCO1, cytochrome c oxidase subunit 1; COX4I1, cytochrome c oxidase subunit 4 isoform 1; ATP5A, ATP synthase H+ transporting mitochondrial F1 complex α-subunit.
from HFD-fed mice showed an increased response to FCCP. We speculate that once metabolic restraint due to Δρ is lifted (by addition of uncoupler), the increased substrate available for oxidative phosphorylation, e.g., succinate, malate, glutamate, or short-chain acylcarnitines, results in increased rates of oxygen consumption. Also, in data sets corrected for protein content, there was a trend toward an increased rate of nonmitochondrial oxygen consumption, which might suggest an increase in adipocyte superoxide production due to cytosolic oxidase activity (9, 24). This possibility is consistent with previous studies showing an increase in oxidative stress in adipose tissue of obese mice, which is caused predominantly by an increase in NADPH oxidase and downregulation of antioxidant enzymes (15, 18, 36, 43).

Further support for coordinated remodeling of mitochondrial and cytosolic metabolism is provided by the observation that expression of Cox7a1 was increased in the adipose tissue of obese mice. A similar increase in Cox7a1 gene has been shown in the WAT of fattening cattle (3), although the implications of this increase are currently unclear. Cox7a1 is considered to be a heart- and muscle-specific subunit of cytochrome oxidase, but it is also present in brown adipose tissue; these tissues are highly metabolic organs capable of utilizing large amounts of substrate. Thus, it is possible that the increase in Cox7a1 may be an adaptive response to dissipate excessive energy in the adipocyte. The decrease in citrate synthase activity, which provides additional evidence for remodeling of mitochondrial metabolism during adipocyte hypertrophy, is similar to that observed in db/db mice, where citrate synthase activity is diminished remarkably with no change in mtDNA/nDNA (52). Why citrate synthase decreases in expanding adipocytes is currently unclear; however, it could be an adaptation to prevent excessive influx of carbon into the Krebs cycle, which would be consistent with the view that during obesity there is an overall shift in metabolism from energy provision to lipid storage.

The notion that HFD remolds mitochondrial metabolism in adipocytes is further supported by diminished mitochondrial biogenetic capacity and later decreases in electron transport chain protein abundance. With 6 wk of HFD, mitochondrial
abundance per se was not affected; however, we did find a significant decrease in Pgc1a expression, which was followed by loss of mitochondrial protein abundance after 12 wk of HFD. This relatively late decrease in mitochondrial protein abundance may also be due to early changes in mitochondrial metabolism that promote removal of mitochondria by autophagy. Autophagy has been shown previously to be increased in adipocytes from obese humans and mice (28, 33, 44). Consistent with those studies, our biochemical results show an increase in the LC3-II/LC3-I ratio and a decrease in p62, the combination of which would appear to be consistent with an increase in autophagic flux (5, 31). Ultrastructural data showing autophagosomes and vacuolated structures containing electron-dense material further suggest changes in autophagy consistent with its activation. However, a limitation of this study is that autophagic flux was not measured directly, making it difficult to ascribe with certainty directional changes to the autophagy pathway. Nevertheless, an activation of autophagy in adipose tissue would be expected to decrease the abundance of both LC3-I and p62 (32, 64, 76, 86), which is consistent with our results. Hence, our cumulative data are supported by evidence in the literature and suggest that autophagy is activated by HFD. Although we cannot clearly distinguish whether mitochondria are removed by autophagy, the increase in key proteins involved in mitophagy, Parkin and Pink1 (24, 74), would be consistent with a significant role of mitophagy in mediating mitochondrial remodeling and turnover in the WAT of obese mice.

In summary, in this study we identified key metabolic changes that occur during WAT expansion. These coordinated changes occur before the infiltration of inflammatory cells and include loss of mitochondrial biogenetic capacity, dysregulation of lipid, amino acid, glucose, nucleotide, and amino acid metabolism, changes in mitochondrial gene expression and protein abundance, and evidence of alterations in autophagy and possibly mitophagy. Our results are consistent with the view that constant nutrient excess shifts adipocyte metabolic activity from energy provision to lipid storage. We speculate that the decrease in mitochondrial biogenetic capacity and later removal of mitochondria may also be a response to limit lipid storage.

Fig. 10. Evidence for activation of mitophagy in white adipose tissue of obese mice. Immunoblot analysis of markers of mitophagy and autophagy. A: Western blots of Parkin and Pink 1 in adipose tissues from mice fed a LFD or HFD for 6 (left) or 12 wk (right). B: quantification of Parkin and Pink1 abundance from E; n = 4/group. C: representative Western blots of ubiquitinated proteins, p62, and LC3 in mice fed a LFD or HFD for 6 wk. D–H: quantification of protein-ubiquitin abundance, p62, LC3-I, LC3-II abundance, and LC3-II/LC3-I ratio; n = 10/group. *P < 0.05 vs. LFD.
oxidation and oxidative stress and to promote fat storage. Further studies are required to assess the significance of each of the metabolic alterations and to test whether therapeutically targeting these pathways is a gainful strategy for preventing obesity and its unfortunate metabolic consequences.

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DISCLOSURES
The authors declare no competing or relevant financial interests.

AUTHOR CONTRIBUTIONS

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