Factors regulating subcutaneous adipose tissue storage, fibrosis, and inflammation may underlie low fatty acid mobilization in insulin sensitive obese adults.

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ABSTRACT

Although the rate of fatty acid release from adipose tissue into the systemic circulation is very high in most obese adults, some obese adults maintain relatively low rates of fatty acid release, which helps protect them against the development of systemic insulin resistance. The primary aim of this study was to identify factors in adipose tissue that may underlie low vs. high rates of fatty acid mobilization in a relatively homogeneous cohort of obese adults. We measured systemic fatty acid rate of appearance (FA Ra) via $^{13}$C-palmitate isotope dilution, and we obtained subcutaneous abdominal adipose tissue samples from 30 obese adults (BMI: 38±1 kg/m$^2$, age: 30±2 yr) after an overnight fast. We then measured insulin sensitivity using a hyperinsulinemic-euglycemic clamp. Confirming our previous work, insulin sensitivity was inversely proportional to FA Ra ($R^2=0.50; p<0.001$). Immunoblot analysis of subcutaneous adipose tissue samples revealed that, compared with obese adults with high FA Ra, those with low FA Ra had lower markers of lipase activation and higher abundance of glycerol-3-phosphate acyltransferase (GPAT), which is a primary enzyme for fatty acid esterification. Microarray and pathway analysis provided evidence of lower fibrosis and lower SAPK/JNK pathway activation in obese adults with low FA Ra compared to those with high FA Ra. Our findings suggest that alterations in factors regulating triglyceride storage in adipose tissue, along with lower fibrosis and inflammatory pathway activation, may underlie maintenance of a relatively low FA Ra in obesity, which may help protect against the development of insulin resistance.
Obesity is associated with insulin resistance, which is central to the development of many cardio-metabolic diseases. However, up to one-third of obese adults remain metabolically healthy (i.e., not insulin resistant) (52); these “insulin sensitive” obese adults have fewer metabolic health complications, and their mortality rates are similar to lean, healthy individuals (6, 19). While it remains unclear why some obese adults remain insulin sensitive, mounting evidence suggests adipose tissue plays an important role.

Much of the insulin resistance observed in obesity is a consequence of excessive release of fatty acids into systemic circulation (fatty acid mobilization [FA Ra]) and the resultant ectopic lipid deposition that disrupts insulin signaling in peripheral tissues. In fact, several studies report profound insulin resistance in healthy, lean subjects after short-term lipid and heparin infusion to mimic the high FA Ra found in obesity (24, 25). Conversely, drugs that lower systemic fatty acid availability in obese adults can reverse insulin resistance (3, 41). Our lab and others have demonstrated that the degree of FA Ra dictates the degree of insulin resistance in obesity and that obese adults who maintain a relatively low basal FA Ra remain insulin sensitive (28, 50). However, while it is clear that the rate of FA Ra from adipose tissue can greatly impact insulin resistance, little is known about factors that may help sequester excess fatty acids in adipose tissue, and thereby limit ectopic lipid deposition.

The accumulation of visceral adipose tissue is often linked with the severity of cardio-metabolic disease risk (40); however, the excess accumulation of visceral fat most likely results from the subcutaneous adipose tissue’s inability to effectively store excess nutrients. Nielsen et al. (33) demonstrated that approximately 90% of circulating fatty acids are derived from subcutaneous adipose tissue, with nearly 70% of these fatty acids coming from abdominal
subcutaneous adipose tissue. Moreover, despite the anatomical proximity of visceral adipose tissue to the liver, the vast majority of fatty acids in the hepatic circulation are also derived from abdominal subcutaneous adipose tissue (33). Human studies using deuterated water to track long-term turnover and storage of triacylglycerol (TG) found that TG synthesis and storage are impaired in the subcutaneous adipose tissue of insulin-resistant obese individuals compared with obese adults who maintain normal insulin sensitivity (1). These data demonstrate the importance of subcutaneous adipose tissue in the control of FA Ra and subsequently, the control of ectopic lipid deposition and insulin sensitivity.

The factors responsible for differences in FA Ra in obesity are not clear, and although the enzymes regulating TG hydrolysis and esterification are well described, the regulation of fatty acid release from adipose tissue is far more complex. Various adipose tissue factors have been postulated to be important mediators of adipose tissue function, including cell size (30), lipid storage capacity (49), adipogenesis (39), angiogenesis (8), extracellular matrix (ECM) dynamics (10), and inflammation (29). Advancing our understanding about mechanisms that allow some obese adults to maintain low FA Ra could lead to targeted interventions which could markedly improve metabolic health. The primary purpose of this study was to determine factors that protect some obese adults from developing high FA Ra from adipose tissue.

MATERIALS AND METHODS

Subjects

Thirty sedentary, premenopausal women (n=25) and obese men (n=5) (BMI: 30-45 kg/m²) ages 18-40 years were recruited for this study. Participants with coronary heart disease, type 2 diabetes, hypertension, or clinically significant hypertriacylglycerolemia (plasma TG
participants were not taking regular medications known to effect metabolic processes, and some women were taking contraceptive medication. All subjects were non-smokers, weight stable (±2 kg) for 6 months, and had not participated in regular exercise for at least 6 months. Body composition was assessed using dual energy X-ray absorptiometry (Lunar DPX DEXA Scanner). Written, informed consent was obtained from all subjects before initiating participation and all study procedures were approved by the University of Michigan Institutional Review Board.

**Experimental Procedures**

Subjects were admitted to the Michigan Clinical Research Unit at 0700h after an overnight fast. We obtained a baseline blood sample and an ~100-200 mg subcutaneous adipose tissue sample from the abdominal region approximately 6 cm lateral to the umbilicus. The sample was cleaned with saline, blotted dry, and quickly frozen in liquid nitrogen. At ~0900h, we began a constant-rate infusion of [1-13C]-palmitate (0.04 µmol/kg/min); after 45 min of infusion, three arterialized blood samples were obtained from a heated hand vein in 5-min intervals for determination of FA Ra. At ~1000h, we began a hyperinsulinemic-euglycemic clamp to assess peripheral insulin sensitivity using a primed 2-h insulin infusion at a rate of 100 mU/m²/min (9). This insulin infusion rate was selected to inhibit hepatic glucose production, even in insulin resistant subjects (4), allowing us to assess insulin sensitivity largely independent of the liver. Plasma glucose concentration was monitored every 5 minutes during the clamp study with a glucose autoanalyzer (Yellow Springs Instruments, Yellow Springs, OH), and glucose was infused at a variable rate to maintain plasma glucose concentration at each participant’s baseline fasting glucose concentration. Stability of glucose concentration was
achieved during the last 20 minutes of insulin infusion. On average, plasma glucose concentration during the final 20 minutes of the clamp were 98±5% of baseline fasting glucose concentration values, across all 30 subjects (mean±SD). Insulin sensitivity was defined as the glucose infusion rate (GIR: mg/min) during the last 20 minutes of the hyperinsulinemic-euglycemic clamp (steady-state) divided by fat-free mass (FFM, kg).

Analytical Procedures

Plasma fatty acid kinetics

The tracer-to-tracer ratio for plasma palmitate was determined by gas chromatography–mass spectrometry (MSD 5973, Agilent Technologies, Wilmington, DE) as previously described (32). Palmitate rate of appearance (Ra) into plasma was calculated using the Steele equation for steady-state conditions (47). FA Ra was calculated by dividing palmitate Ra by the ratio of plasma palmitate to total plasma fatty acid concentration.

Subject stratification

Subjects were divided into tertiles based on the magnitude of their FA Ra. For our main comparisons, we examined differences between subjects that maintained a relatively low FA Ra despite being obese (LOW-FA; n=10) to those with a high FA Ra (HIGH-FA; n=10).

Plasma measurements

Plasma glucose (Thermo Fisher Scientific, Waltham, MA), fatty acids (Wako Chemicals, Richmond, VA), TG (Sigma Aldrich, St. Louis, MO), and total cholesterol (Wako Chemicals, Richmond, VA) concentrations were measured using commercially available colorimetric assay
kits. Plasma insulin concentration was measured by radioimmunoassay (EMD Millipore, St.
Charles, MO).

mRNA expression
RNA was isolated from subcutaneous adipose tissue (~50mg) using a commercially
available kit (Aurum total RNA fatty and fibrous tissue kit, Bio-Rad, Hercules, CA), quantified
spectrophotometrically, and reverse transcribed (High-Capacity cDNA Reverse Transcription
Kit, Thermo Fisher Scientific). Predesigned PrimeTime qPCR Assays (Integrated DNA
Technologies, San Diego, CA) were used for mRNA analyses. Real-time quantitative PCR data
was normalized to peptidylprolyl isomerase A (PPIA) and beta-2-microglobulin (B2M)
expression using the -ΔCt method (44).

Microarray Analysis
Microarray analysis of adipose tissue gene expression was performed by the University
of Michigan DNA Sequencing Core following manufacturer recommendations. RNA from the 5
subjects with highest and lowest FA RA was hybridized to Human Gene ST 2.1 strips
(Affymetrix, Santa Clara, CA). The fold difference in gene expression between LOW-FA and
HIGH-FA was determined with ArrayStar version 12.1 (DNASTAR, Rockville, MD). The
Upstream Regulator Module of Ingenuity Pathway Analysis (IPA) software (Qiagen, Redwood
City, CA) was used to identify upstream transcriptional regulators potentially explaining
differences in microarray gene expression. We performed gene set enrichment analysis with
Pathway Analysis using Logistic Regression (LRPath: http://lrpath.ncibi.org/)(42) to test for
predefined biologically relevant gene sets containing more significant genes than expected by
chance between LOW-FA and HIGH-FA. The data discussed in this publication have been
deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession
number GSE95777.

**Western blotting**

Adipose tissue was homogenized in tissue lysis buffer (Cellytic MT cell lysis reagent,
Sigma-Aldrich) with commercially available proteinase and phosphatase inhibitors (P8340,
P5726, and P0044, Sigma-Aldrich). Protein concentration was determined using the
bicinchoninic acid method (Thermo Fisher Scientific). Fifteen to twenty micrograms of protein
were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were
Ponceau stained to confirm equal loading. Membranes were blocked, incubated in primary
antibodies overnight at 4°C, washed, and incubated with appropriate secondary antibodies for 1
h. Blots were developed using enhanced chemiluminescence (Clarity western ECL substrate,
Bio-Rad), imaged, and quantified via densitometry (Image Lab software, Bio-Rad). The primary
antibodies used were phosphorylated extracellular signal-regulated kinase (p-ERK1/2Thr202/Tyr204)
(Cell Signaling #9101), extracellular signal-regulated kinase (p-ERK1/2Thr202/Tyr204) (Cell
Signaling #9102), phosphorylated hormone sensitive lipase (p-HSLser660) (Cell Signaling #4126),
hormone sensitive lipase (HSL) (Cell Signaling #4107), p-HSLser563 (Cell Signaling #4139), p-
HSLser565 (Cell Signaling #4137), adipose triglyceride lipase (ATGL) (Cell Signaling #2138),
glycerol-3-phosphate acyltransferase 1 (GPAT1) (Sigma-Aldrich, PRS4613), diacylglycerol
acyltransferase 1 (DGAT1) (Novus Biologicals, NB110-41487), phospho-signal transducer and
activator of transcription 3 (p-Stat3Tyr705) (Cell Signaling, #9131), signal transducer and activator
of transcription 3 (Stat3) (Cell Signaling, #4904), phospho-p38 MAPKThr180/Tyr182 (Cell Signaling
#9211, p38 MAPK (Cell Signaling #9212), monocyte chemotactant protein 1 (MCP1) (Cell Signaling #2720), Galectin-3 (MAC2) (Abcam Inc., ab2785), and fatty acid translocase (FAT/CD36) (Abcam Inc., EPR6573).

**Statistical Analysis**

Simple linear regression was used to examine the relationship between FA Ra and insulin sensitivity. Unpaired student’s t-tests were used to test for between group differences (LOW-FA vs. HIGH-FA) in all measured outcome variables. The linear regression and student’s t-tests were processed using Sigmaplot 13.0. Statistical significance was defined as p<0.05. Gene expression differences in the microarray data were determined in ArrayStar version 12.1 using a moderated t-test (45). The fold difference values and p-values generated from ArrayStar were then used for directional tests in LRpath gene ontology analysis. For all immunoblot and gene expression analyses, we used Grubb’s outlier test (18) to detect outliers in the data sets using GraphPad statistical software (https://graphpad.com/quickcalcs/). Using this test, we found one outlier value in each of the immunoblot analyses of pHSL/HSL, HSL, and CD36 and removed these data points. We also found and removed one outlier from each of the mRNA expression analyses for SORL1 and SPP1.

**RESULTS**

**FA Ra and cohort stratification**

As expected, FA Ra varied widely among our subjects (Figure 1A). Subjects were stratified into FA Ra tertiles (Figure 1A), allowing for direct comparisons between subjects with
the highest FARa values (“HIGH-FA”; ≥12.8 μmol/min/kg FM; n=10) vs. subjects with the
lowest FARa values (“LOW-FA”; ≤8.7 μmol/min/kg FM; n=10). Subjects with
intermediate/moderate FARa values (“MOD-FA”; 8.8-12.7 μmol/min/kg FM; n=10) were
excluded from our primary analyses (i.e., HIGH-FA vs. LOW-FA; Figure 1B), but were included
in our correlational analyses across the entire cohort. Expressing FARa relative to fat mass
(FM) allowed us to evaluate differences in FARa independently of adipose tissue mass.
Importantly, however, FARa remained significantly different (p<0.05) between HIGH-FA and
LOW-FA even when not normalized (Figure 1C) or when expressed relative to FFM (Figure
1D).

Subject characteristics

Age, body mass, waist circumference, hip circumference, and BMI were not different
between groups (Table 1). However, LOW-FA had a significantly higher percentage of body fat
(p<0.01) and lower FFM (p<0.01) compared with HIGH-FA (Table 1). Although the HIGH-FA
group contained 3 males and 7 females, while the LOW-FA contained only females (n=10), the
between-group differences in body composition were still evident even when comparing only the
female subjects in HIGH-FA vs LOW-FA (Body fat (%): 47±6 vs 53±3; FFM (kg): 54±9 vs
46±4, respectively; both p<0.05) while all other all other physical characteristics remained
similar (data not shown). There were no significant differences in fasting plasma concentrations
of insulin, glucose, fatty acids, TG, or total cholesterol (Table 1).

FARa and insulin sensitivity
Subjects exhibited a wide range of insulin-mediated glucose uptake (i.e., insulin sensitivity) during the hyperinsulinemic-euglycemic clamp. In agreement with our previous findings (50), we found a highly significant negative correlation between insulin sensitivity and FA Ra across the entire cohort (p<0.001; Figure 2A), and 50% of the variability in insulin sensitivity among subjects was explained by the magnitude of FA Ra ($R^2=0.50$). In addition, mean insulin sensitivity was nearly 85% greater in LOW-FA vs. HIGH-FA (p=0.003; Figure 2B). While the insulin sensitivity data presented in Figure 2 was normalized per FFM, it is important to note that insulin sensitivity was also significantly higher in LOW-FA compared with HIGH-FA when not normalized to FFM (GIR (mg/min): 610±128 vs. 446±182; p<0.05).

Furthermore, because the steady-state insulin concentration in plasma (SSI) during the clamp was not different between LOW-FA and HIGH-FA (SSI (µU/ml): 287±64 vs. 324±78; p=0.28)), insulin sensitivity also remained significantly different between the groups when normalized to SSI (GIR/SSI; 2.2±0.5 vs. 1.4±0.5; p=0.005). To assess whether differences in the sex distribution in our cohorts (HIGH-FA: 7 women and 3 men; LOW-FA: 10 women and 0 men) influenced our findings, we also compared insulin sensitivity between only the female subjects in our groups, and found the differences in insulin sensitivity remained significant when the 3 men were removed from the HIGH-FA group (GIR (mg/min/kg FFM): 13.3±3.0 vs. 7.8±2.2, for LOW-FA and HIGH-FA, respectively; p=0.002); Therefore, it appears that the inclusion of men in the HIGH-FA group was not responsible for the observed differences in insulin sensitivity between the HIGH-FA and LOW-FA groups.

Adipose tissue

Lipolysis and esterification markers
We found a lower abundance (p=0.02) of phosphorylated hormone sensitive lipase (HSL) at serine 660 (HSL_{ser660}), a marker of increased HSL activity, in LOW-FA compared with HIGH-FA (Figure 3A and 3C). There were no differences in phosphorylated HSL at serine 563 (p-HSL_{ser563}) (p=0.27), another marker of increased HSL activity, or phosphorylation of serine 565 (p-HSL_{ser565}) (p=0.47), a marker of inhibition of HSL activity (data not shown). Interestingly, abundance of p-HSL_{ser660}/HSL was significantly correlated to FA Ra across the entire cohort (R^2=0.31, p=0.003). There was no difference in HSL mRNA expression between groups (Figure 3D). In contrast to HSL, neither protein abundance nor mRNA expression of adipose triglyceride lipase (ATGL) were different between groups (Figures 3A, 3C, and 3D). Extracellular signal-regulated kinase (ERK) pathway activation, which increases lipolysis, was also significantly lower in LOW-FA vs. HIGH-FA (p-ERK^{Thr202/Tyr204}/ERK; p=0.05; Figures 3A and 3C). We also found 3-fold greater protein abundance of glycerol-3-phosphate acyltransferase 1 (GPAT1) in LOW-FA compared with HIGH-FA (p=0.02; Figures 3B and 3C), despite no difference in GPAT1 mRNA expression (Figure 3D). There were no differences in protein abundance or mRNA expression of diacylglycerol acyltransferase 1 (DGAT1) (Figures 3A-D).

Markers of lipogenesis, adipogenesis, lipid storage, and transport

mRNA expression of factors involved in lipid droplet storage (PLIN1 and CIDEA) was not different between LOW-FA and HIGH-FA (Figure 3D). mRNA expression of factors involved in lipogenic and adipogenic processes (i.e., PPARG, CEBPA, and SREBP1C) was also similar between LOW-FA and HIGH-FA (Figure 3D). Lastly, we found no differences between
LOW-FA and HIGH-FA in protein abundance of CD36, a primary fatty acid transporter (Figure 3B and 3C).

LOW-FA vs. HIGH-FA gene expression profile

We performed gene ontology (GO) analyses on the microarray data from LOW-FA and HIGH-FA to identify novel alterations in biological pathways that may underlie the observed differences in FA Ra between LOW-FA and HIGH-FA. Table 2 summarizes the three most highly upregulated and downregulated GO terms in LOW-FA compared to HIGH-FA that were statistically significant and biologically relevant. LOW-FA had lower enrichment of pathways related to ECM structure, organization, and disassembly. Surprisingly, the most highly upregulated GO processes in LOW-FA were antigen receptor-mediated signaling, defense response, and lymphocyte differentiation, suggesting greater immune activity in LOW-FA adipose tissue. Of the 413 statistically significant differentially (>2-fold) expressed genes in LOW-FA compared with HIGH-FA (Figure 4A), 210 genes were upregulated and 203 were downregulated in LOW-FA vs. HIGH-FA. We examined the most biologically relevant genes to identify those with novel involvement in adipose tissue metabolism. Out of the 47 most relevant genes (Table 3), 40 were related to the adipose tissue immune response, while other candidate genes were related to fatty acid metabolism, ECM, and mitochondria (Supplementary Material).

Follow-up qPCR validation analyses performed for Sortilin Related Receptor 1 (SORL1), Secreted Phosphoprotein 1 (SPP1), Pyrin Innate Immunity Regulator (MEFV), B And T Lymphocyte Associated (BTLA), Killer Cell Lectin Like Receptor K1 (KLRK1), and C-X-C Motif Chemokine Receptor 1 (CXCR1) confirmed significant differences in mRNA expression between HIGH-FA and LOW-FA for these 6 genes (p<0.05; Figure 4B). The observed
differences in SORL1 between groups was of high interest to us considering its role in enhancing cellular insulin signaling (43), which in turn could impact lipolytic rate.

Inflammation and fibrosis

IPA analysis predicted significant (p=0.04) downregulation of the stress/inflammatory SAPK/JNK pathway in LOW-FA vs. HIGH-FA (Figure 5A). Follow-up western blot analysis confirmed significantly lower JNK pathway activation (p-JNK\textsuperscript{Thr183/Tyr185}/JNK) in LOW-FA compared with HIGH-FA (p=0.04; Figures 5B and 5C). We found no differences between groups for other stress-related signaling pathways (i.e., p38 MAPK or STAT3; Figures 5B and 5C). There were also no differences in protein expression of MCP-1 or MAC2, a crude marker of macrophage abundance (Figures 5B and 5C). qPCR analysis revealed no differences in mRNA expression of some canonical markers of inflammation (TNFA and MCP1) or COL1A1 between LOW-FA and HIGH-FA (Figure 6A). However, in agreement with the GO analysis, COL6A1 mRNA expression was lower in LOW-FA vs. HIGH-FA (p=0.009; Figure 6A), and COL6A1 expression was positively correlated with FA Ra (R\textsuperscript{2}=0.29; p=0.003; Figure 6B).

DISCUSSION

Our observation that FA Ra from subcutaneous adipose tissue is an important mediator of insulin resistance in obesity is consistent with previous work from our lab (50) and others (28). Our findings suggest lower lipase activation, greater fatty acid esterification capacity, and alterations in ECM organization and fibrosis in subcutaneous adipose tissue may all contribute to the attenuated fatty acid release in our LOW-FA cohort, perhaps through an enhanced ability to
sequester and store fatty acids as TG within adipocytes. In contrast to our hypothesis, our microarray data revealed upregulated markers of immune activity in subcutaneous adipose tissue from LOW-FA vs. HIGH-FA. Because insulin sensitivity was relatively high in our LOW-FA subjects, these findings from our microarray conflict with the overly simplistic notion that upregulated adipose tissue immune activity directly underlies the development of insulin resistance.

The inability of subcutaneous adipose tissue to adequately sequester excess fatty acids, which may lead to excessive FA Ra, is causally linked with a host of cardio-metabolic complications, including insulin resistance (2). The importance of storing fatty acids in adipose tissue and preventing excessive exposure of fatty acids to peripheral tissues is demonstrated clinically in patients with lipodystrophy, a condition characterized by an extraordinarily low capacity to store fatty acids in subcutaneous adipose tissue, leading to extreme insulin resistance (14). Lipodystrophic mice develop similar metabolic complications; however, these complications (e.g., insulin resistance, ectopic lipid deposition, etc.) can be reversed with transplantation of well-functioning subcutaneous adipose tissue (15). Although it may seem counter-intuitive, the ability to expand subcutaneous adipose tissue to accommodate nutrient oversupply may actually help prevent insulin resistance by enhancing the capacity to sequester fatty acids – thereby preventing excess fatty acid release and ectopic lipid deposition in insulin sensitive tissues (17). Along these lines, this is one of the main mechanisms of action for thiazolidinedione (TZD) treatment for type 2 diabetes (5). Our data suggest that LOW-FA subjects may have a naturally enhanced ability to expand and store excess nutrients as TG in subcutaneous adipose tissue. While our data captures only a snapshot of the fat storage capacity in our subjects, other human studies that tracked long-term TG turnover and storage using
Deuterated water reported impaired TG synthesis and storage in subcutaneous adipose tissue of insulin-resistant obese adults (1). Our data further support the importance of subcutaneous adipose tissue in the control of FA Ra and, subsequently, peripheral tissue insulin sensitivity.

Differences in the expression, abundance, and activation of enzymes controlling lipolysis and esterification among our subjects may contribute to the variability in their FA Ra and may impact whole-body insulin resistance. Partial inhibition of lipolysis in obese mice through pharmacological or genetic manipulation of HSL resulted in lower fatty acid availability and improved insulin sensitivity without affecting fat mass (16). Lower markers of lipolysis are also correlated with reduced indices of insulin resistance in obese humans, independently of fat mass (16). In agreement, our LOW-FA group had lower markers of HSL activation (p-HSL<sup>ser660</sup>/HSL) and ERK (p-ERK<sup>Thr202/Tyr204</sup>/ERK) signaling, which are indicative of lower lipolytic activation (7, 27). Systemic fatty acid release from subcutaneous adipose tissue can also be attenuated by upregulated esterification, which can synthesize TGs from locally released fatty acids before they enter the circulation. Our finding that GPAT protein abundance (i.e., the enzyme catalyzing the first committed step of the esterification pathway (23)) was 3-fold greater in LOW-FA than HIGH-FA suggests greater esterification capacity may have also contributed to a lower systemic FA release. Together, our findings suggest that obese adults who maintain relatively low FA Ra (LOW-FA) may have reduced activation of basal lipolysis and an increased capacity for fatty acid esterification compared with obese adults with high FA Ra.

Our finding that markers of ECM organization and assembly were lower in LOW-FA vs. HIGH-FA is very intriguing and among the most novel findings of our study. Furthermore, to our knowledge, this is the first study demonstrating a strong direct correlation between FA Ra and collagen VI expression in human adipose tissue. Therefore, ECM-mediated alterations in
adipose tissue fatty acid metabolism may be an important contributor to the well-described relationship between excess accumulation of certain adipose tissue ECM components (including collagen VI) and insulin resistance in obesity (11). Subcutaneous adipose tissue collagen VI expression has been found to be positively correlated with BMI, fat mass, and insulin resistance, and is upregulated during short-term overfeeding (37). In agreement, mice lacking collagen VI were protected from high-fat diet induced insulin resistance (26). The maintenance of insulin sensitivity in these mice was accompanied by larger, more hypertrophied adipocytes despite similar fat mass, indicating the lack of collagen VI may have allowed the adipocytes to expand with less constraint, providing improved capacity for fatty acid storage. Having less subcutaneous adipose tissue fibrosis in our human subjects may also have allowed for an increased adipocyte expansion, with potential for enhanced adipocyte lipid storage capacity, lower mobilization of fatty acids, and a resultant lower ectopic lipid deposition. This would likely be advantageous for maintaining insulin sensitivity in obese humans as well.

Fibrotic adipose tissue is also often accompanied by an elevated inflammatory profile (29, 46), which is a key contributor to metabolic dysfunction in obesity (48). However, despite evidence for higher adipose tissue fibrosis and lower insulin sensitivity in HIGH-FA vs. LOW-FA, our finding that adipose tissue immune activity was lower in HIGH-FA compared with LOW-FA conflicts with this notion. The adipose tissue immune response is very complex, and inflammatory status clearly cannot be classified simply as “pro-inflammatory” and “anti-inflammatory”. The regulation of macrophages, neutrophils, lymphocytes, and T cell activity has many redundant, overlapping mechanisms controlling whether these cells confer pathogenic or protective functions. While macrophages are commonly linked to the development and maintenance of fibrosis, they are also involved in the inhibition and reversal of fibrosis (12, 31),
which corroborates our data. Although overall immune activity may be elevated, there could be a compensatory rise in mechanisms that mitigate the detrimental effects of increased immune activity on adipose tissue function, such as Pyrin Innate Immunity Regulator (MEFV) and B And T Lymphocyte Associated (BTLA). MEFV plays a role in the degradation of several inflammasome components (36), while BTLA is involved in inhibiting the Th1 “pro-inflammatory” T cell response (51). Furthermore, our LOW-FA group had higher mRNA expression of C-X-C Motif Chemokine Receptor 1 (CXCR1), a powerful chemoattractant factor that activates neutrophils (22), and lower expression of Secreted Phosphoprotein 1 (SPP1), which is related to induction of the inflammatory response in obesity (34). Unfortunately, without specific sorting and analysis of the adipose tissue immune cells, we can only speculate on the “protective” vs. “pathogenic” immune cell abundance and activity in the LOW-FA group. Nevertheless, these data suggest an uncoupling of the immune inflammatory response and the development of insulin resistance in obese, yet otherwise healthy, individuals.

Despite evidence of higher overall adipose tissue immune activity, SAP/JNK pathway activation was lower in adipose tissue from LOW-FA compared with HIGH-FA. The SAPK/JNK pathway is activated in multiple tissues in obesity, including adipose tissue, and is implicated in orchestrating the relationship between inflammation and poor metabolic outcomes (35). While the SAPK/JNK pathway can interfere with insulin signaling and promote insulin resistance (21), SAPK/JNK pathway activation in macrophages may also be important for controlling basal fatty acid release from adipose tissue. For example, reducing JNK pathway activity in macrophages alters macrophage polarization and lowers the expression and release of pro-inflammatory cytokines that may cause excess FA Ra (20, 38). Therefore, the lower SAPK/JNK pathway activation in LOW-FA may explain why this group can maintain a lower
FA Ra despite elevated overall immune activity. Our findings demonstrate that preserving low JNK pathway activation in adipose tissue in obesity may be important for maintaining low FA Ra and preserving whole body insulin sensitivity in humans.

Another novel finding was the greater mRNA expression of sortilin-related receptor (SORL1) in adipose tissue from LOW-FA vs. HIGH-FA. SORL1 is best known for its role in the neurodegenerative processes involved in Alzheimer’s disease (13). However, Schmidt et al. demonstrated an important role for SORL1 in modulating adipose tissue metabolic processes (43). For example, SORL1 overexpression in mice reduced TG hydrolysis, while inactivation of SORL1 increased TG breakdown and fatty acid release from adipose tissue (43). The proposed mechanism for SORL1 action on adipose tissue lipolysis is to enhance cellular insulin signaling (43), and thereby increase the antilipolytic response to insulin. This enhanced effect on insulin signaling may be particularly relevant at basal (i.e., relatively low) insulin concentrations, which is when FA Ra was measured in our study. Perhaps the elevated SORL1 expression we found in subcutaneous adipose tissue from our LOW-FA subjects may be beneficial for sequestering fatty acids, reducing their release into the systemic circulation.

While our data provide important insight into possible mechanisms protecting some obese adults from developing insulin resistance, there are some limitations to this study. First, although our findings identify several factors that may underlie differences in FA Ra among our cohort of obese subjects, our study does not directly address causation, which is always very challenging in human studies. Second, because our measurements of FA Ra, gene expression, and protein abundance were performed on samples collected during the post-absorptive state, our conclusions do not address alterations in adipose tissue in the post-prandial state (i.e., insulin-stimulated). Relevant to this, we acknowledge that varying degrees of adipose tissue resistance...
to the anti-lipolytic effects of insulin could also contribute to differences in FA Ra among our subjects. However, because our measurements were made in the post-absorptive state (i.e., when plasma insulin concentration was at its lowest point), the contribution of differences in adipose tissue insulin resistance on FA Ra was likely rather small in our study. More importantly, we contend that ectopic lipid deposition (and its negative impact on whole-body insulin resistance) is primarily affected by the much higher FA Ra that occurs in the post-absorptive state, which is why we focused on assessing differences in FA Ra among our subjects after an overnight fast. Unfortunately, because we could not measure plasma catecholamines due to inadequate sample availability, we do not know if differences in circulating catecholamines may have also contributed to the differences in FA Ra between our groups. Finally, we did not have adequate adipose tissue samples to perform histological measurements, which could provide more information about adipose tissue fibrosis and inflammation. These measures will be incorporated in follow-up studies.

In conclusion, our findings support the importance of FA Ra from adipose tissue in modulating the degree of insulin resistance in obese adults. Obese adults who can maintain low FA Ra may do so via reduced activation of lipolytic pathways (ERK and HSL) and enhanced fatty acid esterification via increased protein content of GPAT. The reduced activation of lipolytic pathways may also be mediated, in part, by increased SORL1 expression and/or reduced SAPK/JNK pathway activation. Furthermore, obese adults who maintain low FA Ra have lower markers of fibrosis and ECM deposition, potentially enhancing their ability to store fatty acids as TG within adipocytes and protecting them from ectopic lipid deposition. Expanding our understanding about what dictates systemic fatty acid release from adipose tissue may lead to the
development of treatments or therapeutic interventions that can reduce and/or prevent insulin resistance in obesity.

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AUTHOR CONTRIBUTIONS

DVP and JFH designed the study. DVP, LMG, AYW, and JFH collected samples, performed experiments, and analyzed data. DVP, LMG, and JFH wrote the manuscript. All authors approved the final manuscript.
DISCLOSURES

The authors declare no conflict of interest.

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adipose tissue: composition, distribution, and link with lipid metabolism and fat mass loss.


17. Gray SL, and Vidal-Puig AJ. Adipose tissue expandability in the maintenance of metabolic homeostasis. *Nutrition reviews* 65: S7-12, 2007. doi:


FIGURE LEGENDS

Figure 1: FA Ra variability across all subjects. (A) FA Ra across the entire cohort (n=30) normalized to FM (B) in HIGH-FA (black circles) vs LOW-FA (white circles) normalized to FM (C) in HIGH-FA vs LOW-FA expressed as total FA Ra (D) in HIGH-FA vs LOW-FA normalized to FFM. Data expressed as MEAN ± SD. *P<0.05 vs HIGH-FA.

Figure 2: FA Ra and insulin sensitivity. (A) Correlation between insulin-mediated glucose uptake during the clamp (insulin sensitivity) and FA Ra (LOW-FA [n=10; white circles], MOD-FA [n=10; half black, half white circles], HIGH-FA [n=10; black circles]) (B) Insulin sensitivity in LOW-FA vs HIGH-FA. Data expressed as MEAN ± SD. *P<0.05 vs HIGH-FA.

Figure 3: Markers of lipolysis, esterification, and fatty acid uptake. (A) Relative protein abundance of proteins related to lipolysis normalized to HIGH-FA. (B) Relative protein abundance of proteins related to esterification and fatty acid uptake expressed relative to HIGH-FA. (C) Representative images for western blotting analysis of proteins related to lipolysis, esterification, and fatty acid uptake. (D) mRNA expression of factors related to lipolysis, esterification, lipogenesis, and fatty acid storage in LOW-FA (n=9; white circles) compared with HIGH-FA (n=9; black circles). Expression values were normalized to the mean of the housekeeping genes PPLA and B2M and then expressed relative to HIGH-FA. Data expressed as MEAN ± SD. *P<0.05 vs HIGH-FA.

Figure 4: Microarray and gene expression analysis of adipose tissue. (A) Heatmap representing genes 2-fold differentially expressed in LOW-FA compared with HIGH-FA. (B) qPCR validation of mRNA expression of 6 genes found to be greater than 2-fold different in LOW-FA (n=9, white circles) compared with HIGH-FA (n=9, black circles). Expression values were
normalized to the mean of the housekeeping genes *PPIA* and *B2M* and then expressed relative to HIGH-FA. Data expressed as MEAN ± SD. *P<0.05 vs HIGH-FA.

**Figure 5:** SAPK/JNK and stress/inflammatory pathway activation. (A) Ingenuity Pathway Analysis identified the SAPK/JNK pathway to be significantly downregulated in LOW-FA compared with HIGH-FA (p=0.04) (B) Relative protein abundance of proteins related to stress pathway activation and inflammation expressed relative to HIGH-FA (LOW-FA n=8, white circles; HIGH-FA n=9, black circles). Data expressed as MEAN ± SD. *P<0.05 vs HIGH-FA.

**Figure 6:** Markers of inflammation and fibrosis. (A) mRNA expression of factors related to inflammation and fibrosis in LOW-FA (n=7-9; white circles) compared with HIGH-FA (n=7-9; black circles). Expression values were normalized to the mean of the housekeeping genes *PPIA* and *B2M* and then expressed relative to HIGH-FA. Data expressed as MEAN ± SD. *P<0.05 vs HIGH-FA. (B) Correlation between *COL6A1* mRNA expression and FA Ra across 28 subjects.
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<tr>
<th>TABLE 1: Subject Characteristics</th>
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<td><strong>Age (y)</strong></td>
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<td><strong>Fat free mass (kg)</strong></td>
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<td><strong>Body Fat (%)</strong></td>
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<td><strong>Insulin (μU/mL)</strong></td>
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<td><strong>NEFA (μM)</strong></td>
<td>615±66</td>
<td>515±53</td>
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<tr>
<td><strong>Triglycerides (mg/dL)</strong></td>
<td>68±13</td>
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<td><strong>Total cholesterol (mg/dL)</strong></td>
<td>161±7</td>
<td>143±11</td>
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Values are Means ± SD. Plasma concentrations measured after an overnight fast. NEFA = “non-esterified fatty acids”. * significant difference compared to HIGH-FA, p<0.05
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<th>GO term</th>
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Table 3: Most biologically relevant genes over 2 fold different in LOW-FA compared with HIGH-FA

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<td>Mitochondria</td>
<td>YME1L1</td>
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</table>
A

mRNA expression ($2^{-\Delta Ct}$)
(relative to HIG-HF-FA)

TNFA  MCP1  COL1A1  COL6A1

B

Adipose tissue COL6A1 mRNA expression ($2^{-\Delta Ct}$)

Fatty acid mobilization
FA Ra ($\mu$mol/kg FM/min)

$R^2=0.29$
$P=0.003$