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Heterogeneity among white adipose tissue depots in male C57BL/6J mice

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Abstract

The widespread prevalence of obesity has lead to extensive research on white adipose tissue (WAT), which frequently uses the C57BL/6J mouse strain as a model. In many studies, results obtained in one WAT depot are often extrapolated to all WAT. However, functional differences among WAT depots are now becoming apparent. Thus, to identify the molecular mechanisms responsible for WAT depot-specific differences under “normal” conditions, four C57BL/6J mouse WAT depots (inguinal, mesenteric, epididymal and retroperitoneal) were analyzed. Depot proteomic profiles, along with weights, protein contents, adipocyte sizes and oxidative stress were determined. Mesenteric WAT had almost twice the protein content of the other depots analyzed. Mean adipocyte size was highest in epididymal and lowest in mesenteric and inguinal depots. The proteome of inguinal WAT displayed low levels of enzymes involved in ATP generation, glucose and lipid metabolism, and antioxidant proteins. Higher levels of these proteins were observed in mesenteric and epididymal WAT, with variable levels in the retroperitoneal depot. Some of these proteins showed depot-specific correlations with plasma levels of insulin, leptin and adiponectin. In agreement with the proteomic data, levels of the antioxidant protein HSP β 1 also were lower in inguinal WAT when analyzed by western blotting and immunohistochemistry. Also, lipid peroxidation products showed similar trends. Our results are consistent with lower triglyceride turnover and lower oxidative stress in inguinal than mesenteric and epididymal WAT. The observed WAT depot-specific differences provide clues as to the mechanisms leading to these depots’ respective diverse functions.

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Disclosure

The authors have no conflicts of interest to declare.

Supplementary Material

Supplementary Material is available at www.nature.com/obesity.

Summary of Supplementary Material

Supplementary Methods and Procedures.doc – Detailed procedures carried out for mass spectrometry.

Figure S1.doc – Plots of intensity for all spots that displayed significant differences among WAT depots.

Table S1.pdf - MS and MS/MS data for all spots showing significant intensity differences among WAT depots.

Introduction

White adipose tissue (WAT) fulfills a wide range of functions (including metabolic regulation, glucocorticoid and steroid hormone synthesis, blood pressure regulation, and others (1)). While WAT was historically considered a homogeneous organ, intraabdominal (IA) WAT is now recognized to have a more negative impact on health than subcutaneous (SC) WAT (2). In fact, fundamental differences among WAT depots have been reported, including their influence on metabolism (3), endocrine function (2), preadipocyte characteristics (4), response to high fat feeding (5), expression of developmental genes (6), etc. Still, data from an individual depot is often inappropriately assumed to reflect the status of all WAT (7). We and others (reviewed in 8) have reported striking differences in WAT depot enlargement in *GHR*^{-/-} mice (growth hormone receptor gene-disrupted mice), which present exaggerated fat deposition predominantly in the SC, but also in the retroperitoneal depot. Despite their elevated fat mass, these mice remain insulin sensitive and live longer than their wild-type littermates. A thorough characterization of the molecular mechanisms dictating WAT depot differences in wild-type mice is essential to be able to evaluate the corresponding differences in *GHR*^{-/-} mice (or any other mouse line, for that matter). The present study was performed to address that need.

The levels of several proteins have been measured and compared among WAT depots in humans and rodents, with many showing WAT depot-specific expression (9–13). In most studies of WAT, isolated adipocytes are separated from stromovascular cells (preadipocytes, macrophages, endothelial cells, etc) in a process that disrupts the complex WAT microenvironment. In this regard, a recent report showed that proteomes of visceral and SC WAT in humans display more differences in stromovascular cells than in isolated adipocytes (13), suggesting that the cellular heterogeneity in WAT might dictate physiological differences among depots. Therefore, for a more comprehensive analysis of the characteristics of WAT depots in vivo, whole tissue samples should be analyzed, preserving the mixture of cell populations and the tissue architecture.

Most obesity studies in mice involve mouse models of the C57BL/6J strain (*ob/ob*, *db/db*, diet-induced obesity, etc.) (14). Thus, it is of particular importance to understand the physiological differences among WAT depots (and the molecular mechanisms behind them), particularly in the C57BL/6J strain. In the current study, we used a proteomic approach to obtain a broad view of the protein profiles of various WAT depots. Previous proteomic studies comparing SC and IA WAT have focused on obese humans (12) or, as stated above, have used collagenase treated WAT samples to analyze isolated adipocytes separately from stromovascular cells (13). However, a proteomic characterization of intact tissue evaluating depot differences in protein profiles under “normal” physiological conditions has not been performed. Furthermore, analyses of WAT rarely include the mesenteric depot, even though many consider it a true visceral pad as, unlike epididymal or retroperitoneal, the mesenteric depot drains into portal circulation. Given that expansion of the visceral WAT is closely associated to human diseases such as type 2 diabetes and cardiovascular disease, in many respects, the mesenteric depot is directly relevant to human pathophysiology. In this study, we used two-dimensional gel electrophoresis (2DE) followed by mass spectrometry (MS) to study one SC (inguinal) and three IA (visceral: mesenteric; non-visceral: epididymal and retroperitoneal) WAT depots in wild-type male C57BL/6J mice. Specific proteins were also assayed by western blotting and immunohistochemistry (IHC). In addition, products of lipid peroxidation, correlations to plasma levels of insulin, leptin and adiponectin, and several phenotypic characteristics among WAT depots were evaluated. Our data reveal differences in metabolism and oxidative stress that likely reflect molecular mechanisms leading to unique physiological states in individual WAT depots. This information adds to our

understanding of WAT function and should be considered when interpreting results of WAT studies using individual depots.

Methods and Procedures

Mice

Male 12-month-old wild-type C57BL/6J mice were kept on a 14/10-hour light/dark cycle at 22 °C with food and water provided ad libitum. Because these are usual housing conditions, we considered these mice as “normal”. Procedures were approved by the Ohio University Animal Care and Utilization Committee.

WAT depot samples

Mice were sacrificed by cervical dislocation and inguinal, retroperitoneal, mesenteric and epididymal WAT (including corresponding lymph nodes and blood vessels) were collected and weighed. Regarding the retroperitoneal depot, we dissected WAT located behind the kidney, along the back of the abdomen (we consider WAT around the kidney to be ‘perirenal’, which in our experience, is negligible in lean mice). WAT samples for proteomics (n=6 mice) were snap-frozen in liquid nitrogen and stored at –80°C until processing; samples for histology (n=6 mice) were fixed in 10% phosphate buffered formalin.

Tissue homogenization

Complete epididymal, retroperitoneal and inguinal WAT depots from the right flank of each mouse and half of the mesenteric depot were separately thawed and diluted in buffer containing 7 M urea, 2 M thiourea, 1% w/v sulfobetaine-10, 3% w/v CHAPS, 0.25% v/v Bio-Lyte 3/10 ampholytes (Bio-Rad Laboratories, Inc., Hercules, CA), and 1.5% v/v protease inhibitor cocktail (Sigma, St. Louis, MO). Protein solubilization was achieved through mechanical homogenization followed by brief sonication, incubation at 35 °C for 15 min, and centrifugation at 16,000 g for 45 min at room temperature. Floating lipid layers were removed and protein solutions were transferred to clean tubes. Protein concentrations were measured using Bio-Rad Protein Assay. Based on the amount of protein obtained and the initial weight of each WAT sample, protein content per gram of tissue was estimated for each depot.

Proteomics

These procedures, described below, were based on those described previously (15–17).

a) 2DE—Aliquots containing 150 µg of protein were further diluted to 350 µl in sample buffer; disulfide bonds were reduced with tributylphosphine and sulfhydryls were alkylated with iodoacetamide. For the first dimension (isoelectric focusing, IEF), 350 µl of each reduced and alkylated protein solution was loaded onto an immobilized pH gradient (IPG) strip (17 cm, pH 3–10 linear, Bio-Rad) and passively rehydrated for two hours at room temperature. Then, IPG strips were placed into a PROTEAN IEF cell (Bio-Rad) for IEF consisting of 12 hours of active rehydration at 50 V and separation at 10,000 V with slow voltage increase for 3 h followed by rapid voltage increase up to 60,000 Vh. Next, strips were equilibrated for 45 min in buffer containing 0.375 M Tris-HCl pH 8.8, 6 M urea, 2% w/v SDS, 20% v/v glycerol and bromophenol blue. After cutting 4.5 cm from both sides of each strip, the middle 8-cm segment (pH 5 to 8) was loaded on a 15% polyacrylamide gel with 4% stacking. SDS-PAGE was performed in a Mini-PROTEAN 3 cell (Bio-Rad) at 25 mA/gel for 270 Vh. After fixing and washing, gels were stained using SYPRO Orange

(Sigma) and scanned in a PharosFX Plus Molecular Imager (Bio-Rad). Excitation was performed at 488nm, and emission was detected at 605 nm.

b) Image analysis—Spot matching was performed using the image analysis software PDQuest Advanced (version 8.0, Bio-Rad), and all matches were examined manually. Spot intensities were normalized to total image density in each gel. Protein spots displaying significant intensity changes among depots were manually excised from the gels and sent to Protea Biosciences Inc., Morgantown, WV for analysis by MS and tandem MS (MS/MS).

Mass spectrometry (Performed by Protea Biosciences Inc.)

MS and MS/MS was performed on selected protein spots using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and MALDI-TOF-TOF, respectively. These procedures are described in Supplementary Methods and Procedures online.

Protein identification (performed at Ohio University)

Protein identities obtained by Protea Biosciences were verified or revised using the online software Mascot (www.matrixscience.com). Search parameters included: MS: database: NCBIInr; taxonomy: *Mus musculus*; enzyme: trypsin; missed cleavages allowed: 1; fixed modifications: none or carbamidomethyl (C); protein mass: none; peptide tolerance: $\pm 0.1 - 1.2$ Da; mass values: MH⁺; monoisotopic/average: monoisotopic. MS/MS: database: NCBIInr; taxonomy: *Mus musculus*; enzyme: trypsin; missed cleavages allowed: 1; fixed modifications: none or carbamidomethyl (C); quantitation: none; peptide tolerance: $\pm 0.1 - 1.2$ Da; MS/MS tolerance: $\pm 0.1 - 0.6$ Da; peptide charge: 1⁺; monoisotopic/average: monoisotopic; precursor m/z: none; instrument: MALDI-TOF-TOF. Variable modifications that were included in separate and combined submissions for both MS and MS/MS were Acetyl (K), Acetyl (N-term), Carbamidomethyl (C), Oxidation (M), Deamidated (NQ), Phospho (ST), and Phospho (Y).

Western blot

Ten micrograms of each homogenized WAT sample was diluted in buffer containing 0.25 M Tris, 1% SDS, 10% glycerol, 1.5% v/v tributylphosphine and 1.5% v/v protease inhibitor cocktail. SDS-PAGE was performed in the same way as for the proteomic analysis. After transfer to a PVDF membrane, equal loading of samples was confirmed by Ponceau S staining. Blocking was performed with 5% bovine serum albumin. Primary antibodies [rabbit polyclonal anti-enolase (ENO) (sc-15343), rabbit polyclonal anti-triosephosphate isomerase (TIM) (sc-30145), goat polyclonal anti-HSP27 (heat shock protein 27, also called HSP β 1) (sc-1048), and rabbit polyclonal anti-phosphoenolpyruvate carboxykinase (PEPCK) (sc-32879)] and secondary antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Protein bands were observed by addition of Pierce ECL western blotting substrate (Thermo Scientific, Waltham, MA) and exposure to HyBlot CL autoradiography film (Denville Scientific, Inc., Metuchen, NJ). For quantification of band intensities, Quantity One version 4.4.0 (BioRad) was used.

Plasma insulin, leptin and adiponectin

Blood from the tail vein was collected in heparinized tubes after mice (n=10) were fasted for twelve hours. Plasma was then separated by centrifugation at 7,000 g for 10 min and stored at -80°C until processing. Insulin levels were measured using an ELISA kit from ALPCO Diagnostics, Salem, NH (80-INSMSU-E01). Leptin levels were quantified using an ELISA kit from R&D Systems, Inc., Minneapolis, MN (MOB00). High molecular weight (HMW) and total adiponectin levels were measured using an ELISA kit from ALPCO Diagnostics (47-ADPMS-E01).

Histology and adipocyte sizing

Formalin-fixed samples were embedded in paraffin, sliced into 5- μ m sections, mounted, and stained with hematoxylin and eosin. Stained slides were examined under a 20X objective with a Nikon Eclipse E600 microscope equipped with a SPOT RT digital camera. For each mouse WAT depot, cell size was determined as described by Chen and Farese (18) based on the cross-sectional area of all the adipocytes present in three non-overlapping microscope fields. Adipocyte number in each depot was estimated by converting cross-sectional areas into cell volumes [$4/3 \times \pi \times (\text{area}/\pi)^{3/2}$], calculating adipocyte masses by multiplying cell volumes by the density of triolein (0.915 g/ml), and dividing each depot's weight by mean adipocyte mass in that location (19).

Immunohistochemistry (Performed by Cutting Image Histology, Ann Arbor, MI)

Five-micrometer sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked. After heat-induced antigen retrieval with Antigen Decloaker (Biocare Medical, LLC, Concord, CA), HSP β 1 was detected with goat polyclonal anti-HSP27 (sc-1048). For PEPCK, Diva Retrieval (Biocare) was used for heat-induced epitope recovery, and rabbit polyclonal anti-PEPCK (sc-32879) was applied. Biotinylated secondary antibodies (Vector Laboratories, Inc., Burlingame, CA), avidin-biotin-horseradish peroxidase complex and diaminobenzidine were added for detection. Slides were counterstained with hematoxylin. WAT samples of four depots from five mice were stained in all cases, including negative controls using normal goat (sc-2028) and rabbit (sc-2027) IgG as primary antibodies.

Products of lipid peroxidation

Levels of 4-hydroxynonenal (HNE)-His protein adducts were measured in homogenized WAT samples. HNE-protein adducts are considered to be more cytotoxic and a more specific indicator of lipid peroxidation than malondialdehyde (20). An OxiSelect HNE-His Adduct ELISA kit (Cell Biolabs) was used according to the manufacturer's instructions.

Statistical analysis

Proteomic spot intensity data were log-transformed, and all data were checked for normality using Shapiro-Wilk's test ($P < 0.05$). Normal distributions were analyzed by within-subjects one-way ANOVA, using Greenhouse-Geisser's correction if data were not spherical (Mauchly's test $P < 0.05$); Tukey's HSD post-hoc tests; and Pearson correlations. For non-normal distributions, Friedman's tests, Bonferroni-Dunn post-hoc tests and Spearman's correlations were applied. Significance cutoffs were $P < 0.01$ for spot intensities (given the intrinsic variability of the 2DE technique) and $P < 0.05$ for all other data. The software used was SPSS v14.0, and post-hoc tests were performed manually in Excel spreadsheets.

Results

Depot weights and protein contents

The epididymal depot ($1,683 \pm 106$ g, mean $\pm$ SE) was significantly larger and the retroperitoneal depot (439 ± 19 g) significantly smaller than the others (inguinal = 881 ± 106 g; mesenteric = 804 ± 58 g; $n=12$; $P < 0.001$). Also, the mesenteric depot (9.5 ± 0.5 mg/g tissue) had significantly higher protein content than the other fat pads (inguinal = 4.9 ± 0.6 mg/g; retroperitoneal = 4.8 ± 0.2 mg/g; epididymal = 4.9 ± 0.3 mg/g; $n=6$; $P=0.002$).

Depot-specific proteomes and protein identification by mass spectrometry

A total of 166 spots were resolved by 2DE, and 38 showed significant differences among WAT depots ($P < 0.01$, Figure 1 and Supplementary Figure S1 online). Protein identification

of these 38 spots was performed by MS and MS/MS (Supplementary Table S1 online) and included 15 proteins, with many spots sharing the same protein identity. Varying post-translational modifications might explain why one protein can show subtle changes in its isoelectric point (pI) and/or molecular weight (MW), leading to its appearance in different spots on a 2D-gel. Overall, spots containing the same protein tended to display similar trends in the four WAT depots (Supplementary Figure S1 online). Table 1 summarizes the proteins identified, their intensities in the WAT depots, and their functions.

Overall, proteins related to ATP generation, such as creatine kinase type B (CKB) and ATP synthase subunit d, were increased in mesenteric WAT, and enzymes involved in glucose metabolism (ENO and TIM) were higher in the epididymal depot. Carbonic anhydrase 3 (CA-III) showed higher levels in IA depots than in inguinal WAT, and antioxidant proteins (HSP β 1 and peroxiredoxin 2) also were lower in the inguinal depot. Other proteins identified are involved in cytoskeleton structure, lipid transport in plasma, and other functions (Table 1).

Correlations between spot intensity and circulating hormone levels

Fasting plasma levels of insulin (3.16 ± 0.58 ng/ml), leptin (31.4 ± 5.1 ng/ml), total adiponectin (29.1 ± 2.0 μ g/ml) and HMW adiponectin (10.6 ± 1.4 μ g/ml) were measured and correlations between these levels and spot intensities in WAT depots were evaluated. The HMW form of adiponectin recently has gained attention in terms of its ability to act as an insulin sensitizer, as it has been suggested to represent the active form of adiponectin (21). In this study, levels of HMW adiponectin correlated closely to total adiponectin levels ($r^2=0.89$, $n=10$, $P<0.001$).

When evaluating possible correlations between the intensities of the 38 proteins that changed significantly among WAT depots and the levels of the four hormones measured, isoform- and depot-specific correlations were found for a subset of the proteins (Table 2). There were evident overlaps in the proteins that correlated to insulin and those that showed significant correlations to leptin, HMW and total adiponectin. However, some of these overlaps involved different WAT depots or different isoforms of the same protein. For example, insulin levels correlated negatively with TIM levels in the retroperitoneal depot, whereas leptin and total adiponectin correlated negatively with TIM levels in the epididymal depot. Also interesting was the fact that some actin isoforms correlated positively with insulin and total adiponectin, while other actin isoforms correlated negatively with these hormones.

Western blotting and IHC

Differential expression of proteins involved in specific metabolic pathways and stress-resistance emerged from the proteomic data. Thus, we selected proteins involved in these processes to further characterize their expression in the WAT depots by western blotting and IHC staining. Total levels of ENO showed no differences among depots when measured in western blots (Figure 2A). However, detection by western blotting normally does not differentiate among forms of a target protein that in 2D-gels might appear as separate spots; and in fact, in our 2D-gels there were two additional spots containing ENO (MW ~50, pI ~6.9 and 6.7, respectively) that were not significantly different among depots (data not shown). Therefore, the combined intensities of the three ENO spots were compared among WAT depots. Consistent with the western blotting results, no differences among WAT depots were found when the intensities of the three ENO spots were combined [spot 6 (MW ~50, pI ~7.3) plus the two additional ENO spots] (Figure 2B). Western blots of TIM and PEPCK also showed no differences among WAT depots (Figure 2C–D). It should be noted that there was visible variability in band intensity among different mice, particularly for

TIM (Figure 2C); however, the relative intensity from one depot to another showed similar values in all mice. Finally, IHC staining of PEPCK showed marked variability in WAT depots, with no clear differences (data not shown).

Regarding antioxidant proteins, and consistent with the proteomic results, HSP β 1 levels were lower in inguinal WAT than in the other depots in both western blots and IHC slides (Figure 2E–F). Interestingly, IHC staining seemed more intense in large adipocytes compared to smaller ones. As a note, HSP β 1 was not only present in adipocytes but was also present in relatively high amounts within blood vessel walls of all depots.

Lipid peroxidation

To establish whether the differences in antioxidant protein levels represented variations in stress levels among WAT depots, we measured products of lipid peroxidation (HNE-His protein adducts). Interestingly, levels of HNE-His protein adducts in the epididymal depot correlated significantly with plasma insulin levels ($r^2=0.96$; $n=5$; $P=0.004$). No other significant correlations were found between HNE-His protein adducts in WAT depots and insulin or other hormone levels. No significant differences were found in HNE-His protein adduct levels among WAT depots, but there was a trend suggesting oxidative damage was lower in the inguinal than the epididymal WAT depot (Figure 3).

Adipocyte size and number

Adipocyte cross-sectional areas measured in all depots are shown in Figure 4. Mean adipocyte sizes of the inguinal and mesenteric depots were significantly smaller than that of the epididymal depot ($P=0.003$, Figure 4C). The mean adipocyte size in retroperitoneal fat showed a trend towards increase, but was not significantly higher than that found in inguinal and mesenteric WAT depots (Figure 4C). Additionally, no significant correlations were found between the intensity of each spot and mean adipocyte size in each depot. Regarding adipocyte number estimation, the retroperitoneal depot displayed the lowest value ($\sim 2.2 \times 10^6$), due to its small mass and large adipocytes. Also, the inguinal depot, that displayed small adipocytes and intermediate weight, had the largest number of adipocytes ($\sim 1.3 \times 10^7$) among all depots. The remaining depots had intermediate adipocyte numbers (mesenteric: $\sim 8.2 \times 10^6$; epididymal: $\sim 6.8 \times 10^6$).

Discussion

This study analyzed the proteomic and several phenotypic differences among four WAT depots of healthy, non-obese wild-type male C57BL/6J mice, including the corresponding mixture of cell populations, the tissue architecture and the microenvironment. The WAT depots analyzed included one SC (inguinal) and three IA (retroperitoneal, mesenteric and epididymal) WAT depots, among which the mesenteric depot is the only “true visceral” pad, given its portal drainage. Because visceral fat is closely associated to human morbidities, the inclusion of this WAT depot provides greater relevance to our study. As expected, several differences among the four WAT depots were found, including depot weight, total protein content, mean adipocyte size, and expression profiles of numerous proteins. The WAT depot weights were consistent with those reported recently by Palmer et al. (22). Notably, the mesenteric WAT depot showed twice the protein content per gram of tissue when compared to the other three depots. This is probably related to the higher amount of blood vessels and lymph nodes observed by histology in mesenteric WAT (data not shown). In fact, higher blood flow in mesenteric compared to retroperitoneal, inguinal and epididymal WAT depots has been described for rats (23).

In the proteomic analysis, we found 38 protein spots that varied among WAT depots. Two proteins related to ATP generation (CK and ATP synthase subunit d), were increased in mesenteric WAT. In humans, visceral WAT displays higher rates of triglyceride turnover (via lipogenesis and lipolysis) than SC fat (24), which may account for a higher energetic need in mesenteric WAT. However, immunoreactive CKB was recently found only in nerves and blood vessels of adipose tissue and not in adipocytes (25). Therefore, elevated CKB levels in mesenteric WAT could reflect a higher density of blood vessels and nerves in this depot.

Enzymes involved in glycolysis and glyceroneogenesis (TIM and ENO) showed higher levels in epididymal WAT. However, western blots of these two enzymes showed no difference in total levels among WAT depots. This situation is not uncommon when measuring individual protein spots in 2D-gels (representing a particular form of the protein with a specific MW and pI) versus total levels by classic immunoassays and highlights the resolving power of the 2DE technique. Normally, antibodies do not distinguish between the slightly modified forms of a protein that in 2DE appear as separate spots, and therefore immunoassays can only detect total levels of the target protein (which often include different spots). In our 2D-gels there were three spots of ENO, and only one of them (spot 6) showed significant differences among WAT depots. Therefore, it was not surprising to find no differences in the total levels of this enzyme both by western blotting and when comparing the intensities of the three spots combined. Similarly, several TIM spots have been identified in 2D-gels of various tissues (26), which if also true for WAT, might explain why total levels of this enzyme showed no significant differences among depots in western blots. On the other hand, it was interesting to observe high variability among mice, particularly in TIM western blots. Although the trends across depots within each mouse were similar from mouse to mouse, some mice had relatively low TIM levels in all depots, while others showed higher levels in all depots.

The exact function of the specific forms of ENO and TIM identified (as opposed to the forms of these proteins present in other spots) is not known. Others have previously found glyceroneogenesis and glucose oxidation rates in rats to be higher within IA WAT depots compared to inguinal fat (27, 28). In addition, TIM was increased in omental vs. abdominal SC WAT in a recent proteomic study of obese humans (12). To evaluate if the identified ENO and TIM forms participated mainly in glyceroneogenesis, we tested the levels of PEPCK, the rate limiting enzyme in the pathway. However, assuming that PEPCK levels correlate with the enzyme's activity, our data indicate that glyceroneogenesis is not activated in any one depot more than in the others. Further research might indicate if the differences observed are related to the rate of glycolysis in each WAT depot and might shed light on the observed negative relationships between plasma hormones and TIM levels in the retroperitoneal and epididymal depots.

The intensities of protein spots containing CA-III were low in SC (inguinal) and higher in IA depots. Although its function in WAT is not well understood, CA-III has been proposed to regulate intracellular pH upon lipolysis and donate bicarbonate ions for glyceroneogenesis and fatty acid synthesis (29). Therefore, lower levels of CA-III in inguinal fat are consistent with lower triglyceride turnover in this depot vs. IA pads. In addition, positive correlations observed between CA-III and the four plasma hormones tested could suggest a role of this enzyme in the regulation of lipid turnover in WAT.

Depot differences detected in apolipoprotein A-I spots might indicate that the rate of cholesterol transport from WAT to the liver is higher in retroperitoneal and mesenteric fat. Another protein that transports lipids in the blood, α -fetoprotein, was increased in the mesenteric depot. Nevertheless, high levels of plasma proteins in mesenteric WAT might

reflect high blood flow in this depot. Several actin spots were elevated in mesenteric WAT. Unfortunately, it was not possible to discriminate between α -, β - or γ -forms of actin with the MS data obtained (Supplementary Table S1 online). We suspect at least a few of these to contain α - and γ -actin forms from smooth muscle in blood vessel walls. Also, mesenteric WAT was the only depot where transgelin and myosin regulatory light polypeptide 9 (MYL9) spots were detected. Because MYL9 and transgelin are known constituents of smooth muscle (30, 31), the presence of these proteins in mesenteric WAT emphasizes the increased density of blood vessels in this fat depot compared to the others.

One of the most interesting findings in our study was the difference among WAT depots in proteins related to cells stress. Of these proteins, we focused on HSP β 1, which protects the cell against heat shock, oxidative stress and chemotherapy drugs (32) and has been shown to mediate pro-inflammatory cell signaling in response to interleukin-1 and tumor necrosis factor in HeLa cells and fibroblasts (33). Our data showed HSP β 1 was low in inguinal and higher in the remaining WAT depots when measured in 2D-gels (spots 9a and 9b), western blots and IHC stained slides. We also observed negative correlations between plasma insulin and the levels of this protein in certain WAT depots. Additional data related to this protein and adipose tissue are very limited although several other proteomic studies have shown differences in HSP β 1. For example, HSP β 1 was higher in SC WAT of obese subjects when compared to lean individuals (34), and within obese subjects, higher HSP β 1 levels were observed in IA compared to SC WAT (12). These data are consistent with IA depots displaying enhanced inflammatory capacity than SC WAT depots (35) and might be a consequence of increased metabolic rate in IA WAT, generating more reactive oxygen species (ROS). This is in agreement with a previous study in lean Zucker rats, showing that epididymal WAT might be subject to higher oxidative stress than inguinal WAT (36). In fact, when HNE-His adducts were measured as an indicator of lipid peroxidation, we found a trend for lower levels in inguinal WAT and higher levels in IA fat, especially the epididymal depot. Reciprocal to the negative relationship between insulin levels and HSP β 1, a positive correlation was found between plasma insulin and lipid peroxidation products in the epididymal depot. Seemingly, insulin action might relate to higher ROS production and lower antioxidant capacity in epididymal WAT.

It is worth noting that our proteomic results may be influenced by the presence of lymph nodes (particularly mesenteric, but also inguinal WAT), high density of blood vessels (particularly mesenteric WAT) and unique proportions of specific cell populations (immune cells, preadipocytes, etc.). This possibility has been discussed for some of the proteins detected (influence of blood vessel density on the levels of actin, CKB, etc.). However, the specific cell type(s) within WAT that produce most of the proteins found remain(s) to be established. Further research will help characterize the expression of these proteins in specific cell populations.

Finally, adipocyte size can influence protein expression in WAT, as has been reported for lipoprotein lipase, hormone-sensitive lipase, leptin, and many pro- and anti-inflammatory factors (37, 38). We found mean adipocyte size to be largest in epididymal WAT and smallest in the inguinal and mesenteric depots. These results are consistent with previous reports in mice and rats (39, 40). However, when correlations between mean adipocyte sizes and spot intensities or HNE-His adducts were tested in each depot, no significant relations were found. Given that the 2DE and HNE analyses were performed on separate groups of mice than the adipocyte sizing, these correlations could only be tested using group mean values. This may explain why none of the spot intensities correlated to mean adipocyte sizes in the depots. Thus, the apparently increased levels of HSP β 1 observed in larger adipocytes by IHC deserve further investigation.

In summary, marked differences among WAT depots of male C57BL/6J mice were found, including depot mass, total protein content, mean adipocyte size, and the expression levels of numerous proteins (Figure 5). A few of the variations were consistent with larger blood supply and higher blood vessel density in the mesenteric depot. Overall, the proteome of the inguinal depot showed major differences from those of mesenteric and epididymal WAT, suggesting differences at the molecular level, such as lower generation of ATP, glucose and lipid metabolism, and lower antioxidant protein levels in SC (inguinal) WAT. These results are consistent with slower triglyceride turnover and consequently lower ROS production in inguinal as compared to mesenteric and epididymal WAT, with intermediate levels in the retroperitoneal depot. This information enhances the understanding of the molecular mechanisms behind WAT physiology and the role of different WAT depots in metabolism and homeostasis and should be taken into account when studying individual WAT depots as representative of all WAT. Particularly, given the differences found in HSP β 1 levels among WAT depots, the role of this protein in stress resistance/oxidative damage in WAT should be determined. This study serves as a start point for future studies of SC and IA (including visceral) WAT depots in a variety of conditions. Aging, dieting, and WAT-related diseases (such as obesity and its comorbidities) are just some of them.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Protein spots showing significant intensity differences among WAT depots. A. Representative 2D-gel of WAT showing 38 spots (x) that displayed significant intensity differences among depots ($P < 0.01$). The dotted square delineates the gel area shown in B. Numbered labels correspond to protein identities shown in Table 1. B. Topographical representation of the intensity of an individual spot (9a) in different WAT depots of the same mouse. Images were obtained using the 3D-viewer tool of PDQuest Advanced v8.0 (Bio-Rad). The gel area shown corresponds to the dotted square delineated in A. Spot 9a was selected as a representative spot that displays intensity variations among WAT depots.

Figure 2.

Western blotting results for ENO (A), TIM (C), PEPCK (D) and HSPβ1 (E), sum of intensities of three ENO spots present in 2D-gels (B), and immunohistochemistry results for HSPβ1 (F). For western blotting results, each panel shows at the top a representative image of WAT samples from two mice and at the bottom the relative band intensities for all samples (n=6 mice). Numbering of samples on blot images is arbitrary and may not represent the same mice in all panels (i.e. mouse #1 in “A” is different from mouse #1 in “C”). Bars showing different letters are significantly different (P<0.05). I, ingual; R, retroperitoneal; M, mesenteric; E, epididymal. For immunohistochemistry images (F), low staining was detected for HSPβ1 in ingual WAT and higher staining in the intraabdominal depots. Scale bar = 50 μm.

Figure 3.
Concentration of HNE-His protein adducts in different WAT depots. Data shown are mean $\pm$ SE (n=5 mice; $P=0.564$ in Friedman's test).

Figure 4.

Adipocyte sizes in different WAT depots. A. Representative WAT images. Scale bar = 50 μm . B. Histograms of adipocyte sizes in the four WAT depots (areas measured in six different mice are grouped in each graph). C. Mean adipocyte size for each WAT depot ($n=6$). Different letters indicate significant differences in Friedman's test ($P=0.003$).

Figure 5.

Summary of the main characteristics of the WAT depots studied. Epididymal depots have been moved outside of the abdomen while conserving their attachment point. Inset image on the right shows the abdomen after dissection of the intestines, to view the kidney (K) and the retroperitoneal depot. Inset image on the left shows the inguinal depot before the abdominal cavity was open, to better show that this depot is subcutaneous. Among the intraabdominal depots, only mesenteric WAT drains into the portal vein (true “visceral” depot); the retroperitoneal depot is the only intraabdominal depot that lies outside the peritoneal cavity; the epididymal depot is the most studied WAT depot in rodents given its ease of dissection and considerable size.

Table 1

Summary of protein identities^a, functions, and depot-specific relative intensities^b for identified spots^c

Spot	Protein identity	Relative intensity			Function
		Inguinal	Retroperitoneal	Mesenteric	Epididymal
ATP generation					
2a-c	Creatine kinase B-type	↓/↔	↓/↔	↑	↓/↔
13a-b	ATP synthase subunit d, mitochondrial ^d	↔	↔	↑	↓
Glucose/lipid metabolism					
8	Triosephosphate isomerase ^d	↓	↔	↔	↑
6	Enolase	↔	↓	↔	↑
7a-d	Carbonic anhydrase 3	↓/↔	↔/↑	↓/↔/↑	↔/↑
Lipid transport					
10a-c	Apolipoprotein A-I	↓/↔	↑	↔/↑	↓/↔
3	α-fetoprotein	↔	↓	↑	↔
Stress resistance					
1	Heat shock cognate 71 kDa protein	↑	↓	↓	↑
9a-b	HSPβ1	↓	↔/↑	↔/↑	↑
12a-b	Peroxioredoxin-2	↓	↔	↑	↔
Cytoskeleton					
5a-k	Actin (various forms)	↓/↔	↓/↔	↑	↓/↔
11	Myosin regulatory light polypeptide 9	-	-	↑	-
14	Transgelin	-	-	↑	-
Other					
15a-c	Hemoglobin subunit β-1	↓	↓/↔/↑	↑	↓/↔
4a-b	14-3-3 protein γ	↔	↔/↑	↓	↔/↑

^aProtein identities were obtained by MS and MS/MS (see Supplementary Table S1 online). Spot labels correspond to the gel locations shown in Figure 2A.

^bThe intensities of isoforms of a same protein were grouped into a single value using a qualitative scale. Intensity values marked “↓” (low) are significantly different from “↑” (high). Intensities marked “↔” are intermediate and not significantly different from “↓” or “↑”. Grouping of isoform intensities was possible as they displayed similar trends among WAT depots. Two exceptions were observed among isoforms of carbonic anhydrase 3 (7a-d) and of hemoglobin subunit β-1 (15a-c) where intensity values ranged from low (↓) to high (↑) in the mesenteric and retroperitoneal depots, respectively (see Supplementary Figure S1 online).

^cAll listed spots showed significant intensity differences among the studied WAT depots ($P < 0.01$).

Spot 13a also might contain a ferritin light chain; spot 8 also might contain carbonic anhydrase 3 (see Supplementary Figure S1 online).

Table 2

Significant correlations between circulating hormone levels and spot intensities in WAT depots

Protein	Positive correlation			Negative correlation		
	Spot	Depot	Correlation ^a	Protein	Spot	Depot
Insulin						
CKB	2b	Epi	$r^2=0.75$; $P=0.026$	TIM	8	Ret
CKB	2c	Epi	$r^2=0.81$; $P=0.015$	ApoA-I	10a	Ret
CA-III	7d	Ing	$r^2=0.78$; $P=0.020$	ApoA-I	10a	Epi
Actin	5h	Epi	$r^2=0.73$; $P=0.031$	ApoA-I	10b	Ing
				HSP β 1	9b	Ing
				HSP β 1	9b	Epi
				Actin	5b	Mes
				Actin	5c	Epi
				Actin	5k	Mes
				Transgelin	14	Mes
Leptin						
CA-III	7b	Ret	$r^2=0.89$; $P=0.005$	TIM	8	Epi
Actin	5b	Epi	$r^2=0.82$; $P=0.013$	ApoA-I	10a	Epi
Actin	5g	Ing	$r^2=0.71$; $P=0.034$	α -fetoprotein	3	Mes
Actin	5i	Ing	$r^2=0.75$; $P=0.026$			
Actin	5i	Ret	$r^2=0.70$; $P=0.038$			
Total adiponectin						
CA-III	7b	Ret	$r^2=0.88$; $P=0.006$	TIM	8	Epi
Actin	5i	Ing	$r^2=0.78$; $P=0.020$	α -fetoprotein	3	Mes
				Actin	5b	Mes
HMW adiponectin						
CKB	2c	Epi	$r^2=0.82$; $P=0.014$	ApoA-I	10a	Epi
CA-III	7b	Ret	$r^2=0.68$; $P=0.043$	Actin	5b	Mes
				Actin	5k	Mes
				Transgelin	14	Mes

^aResults correspond to ρ^2 and P values for Pearson correlation tests. Abbreviations: CKB, creatine kinase B; CA-III, carbonic anhydrase 3; TIM, triosephosphate isomerase; ApoA-I, apolipoprotein A-I; HSP β 1, heat shock protein β 1; Ing; inguinal; Ret, retroperitoneal; Mes, mesenteric; Epi, epididymal.