Dynamic differences in oxidative stress and the regulation of metabolism with age in visceral versus subcutaneous adipose

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Once thought only as storage for excess nutrients, adipose tissue has been shown to be a dynamic organ implicated in the regulation of many physiological processes. There is emerging evidence supporting differential roles for visceral and subcutaneous white adipose tissue in maintaining health, although how these roles are modulated by the aging process is not clear. However, the proposed beneficial effects of subcutaneous fat suggest that targeting maintenance of this tissue could lead to healthier aging. In this study, we tested whether alterations in adipose function with age might be associated with changes in oxidative stress. Using visceral and subcutaneous adipose from C57BL/6 mice, we discovered effects of both age and depot location on markers of lipolysis and adipogenesis. Conversely, accumulation of oxidative damage and changes in enzymatic antioxidant expression with age were largely similar between these two depots. The activation of each of the stress signaling pathways JNK and MAPK/ERK was significantly more resistant than visceral-derived cells to cell death caused by oxidative stress. Cellular respiration in visceral-derived cells was dramatically higher than in cells derived from subcutaneous adipose despite little evidence for differences in mitochondrial density. Together, our data identify molecular mechanisms by which visceral and subcutaneous adipose differ with age and suggest potential targetable means to preserve healthy adipose aging.

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1. Introduction

Adipose tissue shows dramatic alterations in growth and metabolism that are modulated by development, diet, environment and age. While adipose tissue has historically been thought to exist largely as a benign storage vesicle, there is now clear evidence that adipose has varied physiological roles that include regulation of metabolism, immunity and endocrine function among others. In addition, it is increasingly clear that cellular lineage and/or anatomical location has dramatic effect on the basic biology of adipose with a classic example being the differences between brown adipose tissue (BAT) and white adipose tissue (WAT). However, even amongst adipose tissue broadly characterized as WAT, adipose depots that develop in different locations of the body may dramatically differ in physiological function. For example, excess accumulation of visceral adipose in the abdominal cavity is associated with increased risk of metabolic dysfunction, diabetes, cardiovascular disease and mortality [1, 2]. On the other hand, subcutaneous adipose, primarily located in depots under the skin, appears to be relatively benign and may in some cases actually be beneficial for overall health [3, 4]. Moreover, transplantation of subcutaneous adipose tissue to visceral locations can improve glucose metabolism, including increasing insulin sensitivity in rodent models [5]. Therefore, understanding how to promote maintenance of “healthy” subcutaneous adipose over visceral may have significant effects in improving health.

With age, WAT undergoes significant changes in growth regulation, metabolism, and inflammation that may drive the pathophysiology of some age-related diseases. Human fat mass is thought to typically increase throughout a large percentage of life in humans, though there is significant redistribution of these stores preferentially towards visceral adipose [6–8]. Many of the normal functions of WAT, including those that modulate glucose metabolism, also become dysregulated with age in part due to the accumulation of senescent adipocytes [9, 10]. The accumulation of...
these senescent adipose cells may also contribute to the increased pro-inflammatory state associated with mammalian aging. Adipose tissue may play a significant role in the regulation of the aging process itself as the beneficial effects of caloric or dietary restriction have been thought to be promoted in part by a reduction in adipose tissue throughout life [11]. More directly, surgical removal of visceral adipose tissue can extend lifespan in rodents as well as improve insulin sensitivity [5,12]. Interestingly, ablation of the insulin receptor in adipose tissue in mice (FIRKO) causes local insulin resistance in adipose tissue but tends to improve whole animal insulin sensitivity and extends median and maximum lifespan [13].

The metabolic consequences of changes in adipose mass are generally clear, though the mechanisms by which these are driven remain elusive. Over the last decade, there has been growing evidence that oxidative stress caused by the accumulation of adipose tissue may have a role in the etiology of metabolic dysfunctions commonly associated with obesity [14,15]. While accumulation of visceral adipose is clearly associated with increased oxidative stress, this relationship in other adipose depots is less clearly defined [16]. Some have suggested that fat accumulation may accelerate mammalian aging, at least at the tissue/organ levels, through oxidative stress-mediated processes [17,18]. How these processes are altered by the normal dynamics of aging in different adipose depots has yet to be determined. In this study, we first addressed how visceral and adipose tissue differed in their changes in oxidative stress, antioxidant function and oxidant response in young, aged, and old C57BL/6 mice. We then addressed the potential that differential metabolic and mitochondrial demands may play in this process using primary cell culture models. Together, our data identify substantial differences between visceral and subcutaneous adipose depots that suggests these tissues have differential means to deal with the metabolic and oxidative demands of aging.

2. Methods

2.1. Animals

All mice used in this study were male C57BL/6J either born and reared in house or obtained from the aged rodent colony maintained by the National Institute on Aging. For body and tissue weights, only mice from in-house colonies were used. Molecular assays were performed on tissues from both groups of mice. All mice were sacrificed and tissues were collected in the morning (09:00–12:00) after receiving food and water ad libitum through the night and were euthanized by CO2 asphyxiation followed by cervical dislocation. At sacrifice, tissues were weighed and frozen immediately in liquid N2 then stored at −80 °C until use. For the purposes of this study, mice classified as Young were between ~6–9 months of age, those classified as Aged were ~15–19 months of age, and those classified as Old were ~24–30 months of age.

2.2. Assessment of protein content and activity

Frozen samples from visceral (perirenal depots) and subcutaneous (both inguinal and subscapular depots) adipose depots were homogenized in RIPA buffer with added protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL, USA) then centrifuged at 13,000g at 4 °C for 15 min. The total protein content of supernatants from this procedure was measured by Pierce BCA (Thermo Scientific) and equal amounts of protein were prepared in a loading buffer containing 2-mercaptoethanol, heated, then separated electrophoretically by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Primary antibodies to individual proteins used in this study were: phospho-HSL, total HSL, PPARγ, phospho-JNK, total JNK/SAPK, cytochrome c (Cell Signaling, Beverly MA), LPL (Santa Cruz Biotechnologies, Santa Cruz CA), GAPDH, Sod1, Sod2, Gpx1 (Abcam, Cambridge MA). We also used the Mitosciences OxPhos cocktail (Abcam) to determine relative levels of mitochondrial proteins NDUF88, CII-30, CII-core2, and CV-I simultaneously. All alkaline phosphatase-conjugated secondary antibodies (anti-rabbit and anti-mouse) were purchased through Santa Cruz Biotechnologies. Individual protein bands on immunoblots were detected using ECL reagent (Thermo Scientific) and analyzed using ImageJ. To measure levels of protein-bound 4-HNE, we separated total cellular protein homogenates by SDS-PAGE with subsequent Western blot for 4-HNE (Abcam). The total signal of all visible bands within a lane was determined using ImageJ. Kinetic enzyme assays for superoxide dismutase and glutathione peroxidase activity were performed using kits for each as per manufacturer’s instructions (Cayman Chemicals, Ann Arbor MI). Kinetic enzyme activity assays were performed using adipose lysates prepared in RIPA buffer and triplicate samples for each were measured in sample sizes of 50 μg/protein per assay. For phospho ERK and total ERK measurements, protein homogenates in RIPA buffer measured by ELISA assays performed as per manufacturer’s instructions (Abcam).

2.3. Pre-adipocyte (PA) isolation and growth

Visceral (epididymal) and subcutaneous (both inguinal and subscapular) adipose depots were collected from young (3–6 month) C57BL/6 mice. Pre-adipocytes were isolated and cultured using methods previously described [19,20]. In brief, fresh adipose depots were minced and digested in media containing collagenase Type II for 30 min, then filtered through sterile mesh. After centrifugation, the resultant cell pellet was washed with 1X DPBS and suspended in DMEM + 10% fetal calf serum + antibiotics (Life Technologies, Carlsbad CA). The entire pellet was plated in 60 mm tissue culture dishes and placed in water-jacketed, humidified incubator maintained at 37 °C with 5% CO2 in air, and ambient (21%) O2 in air. Cells were monitored for confluence, then trypsinized, counted and re-seeded into T25 or T75 flasks at a cell density of 10,000 cells/cm² flask area. All cells were tested after the second or third passage using this protocol. To assay cell stress resistance, we utilized a modified version of a protocol previously described for fibroblast cultures [21,22]. In brief, 25,000 cells/well were seeded into 96 well plates in 100 μL DMEM + 10% fetal calf serum + antibiotics and allowed to adhere for 48 h. Wells were then washed and fresh DMEM (+ FCS and antibiotics) containing either paraquat or tert-butyl hydroperoxide was added for a period of 6 h after which cells were washed and fresh DMEM (+ FCS and antibiotics) was replaced. After 18 h, cell viability was measured using WST-1 as per manufacturer’s instruction (Roche Life Science, Indianapolis IN).

2.4. Mitochondrial bioenergetics

Mitochondrial respiration was measured in intact pre-adipocyte cell lines using a Seahorse Bioscience XF24 Extracellular Flux Analyzer (North Billerica, MA, USA). For each experiment, cells were seeded at a density of 40,000 cells per well. We used a standard running medium of unbuffered DMEM medium + 4.5 g/L glucose and l-glutamine, with 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA). The BOFA mitochondrial function assay was used as described by Seahorse Biosciences and utilized sequential injections of Oligomycin, FCCP and Antimycin A. All cell culture experiments were performed at ambient (21%) O2 and immediately following each assay, cells were lysed to quantify total protein...
using Pierce BCA. We found only minor variations in protein content and no significant difference in protein between visceral and subcutaneous derived lines. Oxygen consumption measurements are expressed as pmole/min and normalized to protein content. Integrated area under the curves were determined using the included Seahorse analysis software.

2.5. Statistical analyses

For aging studies, data were analyzed by two factor ANOVA testing the effect of age (group) and depot. When applicable, post-hoc analyses (Holm-Sidak) were used to assess multiple comparisons for significance. Multiple linear regressions were used to assess correlations between tissue weights and age of mice at sacrifice. For pre-adipocyte studies, data were analyzed by Student’s t-test comparing cells derived from either visceral or subcutaneous adipose depots. For all, significance was assigned for analyses that reached \( p < 0.05 \).

3. Results and discussion

Using data from Jackson Labs regarding the growth rate of C57BL/6 mice [23], we determined that male mice of this strain reach adulthood at \( \sim6–9 \) months based on their general plateauing of body weight and size. Among mice that reached at least 6 months of age, we show here that further advancing age is correlated with a significant decline in body weight (Fig. 1). The mean weight (and SEM) of young males in our study was 31.0 ± 2.7 g and that of old males was 28.5 ± 1.9 g. To eliminate potential cofounds, all animals with obvious neoplasia were censored from this study. Upon sacrifice, the wet weight of all tissues was collected and nearly all visceral and subcutaneous adipose depots showed a significant decline in weight with age suggesting that all were lost with age to relatively similar degrees (Fig. 1). Brown fat weight did not significantly correlate with age, though this may not be surprising as its content and lineage may be more similar to tissues such as muscle rather than WAT. For further biochemical and cellular experiments, we chose to focus on two groups of adipose depots: visceral (including epidydimal or perirenal depots) and subcutaneous (including both inguinal and subscapular depots).

Maintenance of adipose tissue is a dynamic process as lipids are mobilized for energy utilization through regulation of adipose breakdown or lipolysis and also stored for future use through the regulation of adipogenesis. Even in young animals, we found evidence that the regulation of these processes differs greatly between subcutaneous and visceral adipose depots. Hormone sensitive lipase (HSL) and lipoprotein lipase (LPL) hydrolyze triglycerides stored in adipose tissue to their free fatty acid forms and are rate-limiting steps in lipolysis. We found that the phosphorylated (active) form of HSL was relatively high in visceral adipose as compared to subcutaneous adipose, though there was no specific effect of age (Fig. 2). Similarly, LPL was high in visceral adipose, though levels of this enzyme declined with age in both depots. However, levels of the transcription factor PPAR\( \gamma \), which regulates

![Fig. 1](image_url). Aging is associated with a loss of fat mass in C57BL/6 mice. Each plot shows the correlation between the mass of the indicated parameter (Y axis) and age of the animal at sacrifice (X axis). Y-axis is presented in grams and X-axis is in months of age. Each symbol is the value obtained from an individual mouse for the given parameter. Each plot also shows the least squares regression line and \( R^2 \) and \( p \) values for linear regression between the indicated parameters. Body—total body weight, Epigon—epigonadal visceral depot, Peri—perirenal visceral depot, Inguinal—inguinal subcutaneous depot, Subscap—subscapular subcutaneous depot, Brown—brown adipose depot.
fatty acid storage in adipocytes, were significantly higher in visceral adipose compared to subcutaneous in young and aged animals, but not old animals. Together, these data suggest relatively high levels of lipolysis and adipogenesis in visceral adipose that declines with age, in contrast to the relatively constant low levels in subcutaneous adipose.

As a potential mechanism behind this difference, we focused on oxidative stress and the accumulation of oxidative damage. Oxidative stress has been suggested to play an etiological role in the development of metabolic disorders due to actions within adipose tissue [14,24]. In addition, 4-hydroxynonenal (4-HNE), a product of lipid peroxidation in cells, has been associated with the lipolytic activation in adipocytes and impaired adipogenesis [24–26]. Thus, we predicted that the age- and depot-dependent differences in adipose dynamics described above could be driven by alterations in oxidative stress among these groups. In young mice, protein-bound 4-HNE adducts in subcutaneous adipose depots were significantly lower than in visceral adipose (Fig. 3). With age there was no effect on 4-HNE levels in visceral adipose overall, whereas there was a small, but significant, accumulation of 4-HNE adducts with age in visceral adipose. In old animals, there was no significant difference in 4-HNE between visceral and subcutaneous adipose tissues. One interpretation of these data may be that antioxidant defenses decline with age in subcutaneous adipose thereby allowing for increased oxidative damage. Cu/Zn superoxide dismutase (Sod 1) levels did decline with age, but there was no difference between depots (Fig. 3). On the other hand, levels of Mn superoxide dismutase (Sod 2) did not change with age, though subcutaneous fat had lower levels overall as measured by two-factor ANOVA. Similar to these expression results, the activity of superoxide dismutase (encompassing both Sod1 and Sod2) does not differ with age or depot. In contrast, we found both the activity of glutathione peroxidase 1 (Gpx1) to be significantly reduced in subcutaneous adipose, identifying a potential primary defect that could drive the relative accumulation of oxidative damage with age (Fig. 3).

However, these oxidative stress-related data only partially explain the age- and depot-specific differences in regulation of lipolytic and adipogenic modulators described in Fig. 2. The activation of stress-signaling pathways mediate much of the cellular response to oxidative stress and thus may better represent how the cell perceives these stresses rather than the accumulation of damage overall. Moreover, some stress signaling pathways, including jun N-terminal kinase (JNK) and mitogen-activated protein kinases (MAPK/ERK), have been associated with the regulation of PPARγ and HSL [27–30]. In young mice, we found that subcutaneous adipose had a significant reduction in phosphorylation of JNK, a marker of activation in this pathway, than did visceral adipose (Fig. 4). This difference persisted in aged animals, but in tissues from old animals there was no significant difference between depots. Similar to our results measuring 4-HNE levels, there was no significant effect of age in visceral adipose whereas there was a significant increase with age in subcutaneous adipose. Interestingly, phosphorylation of MAPK/ERK followed a different depot- and age-specific pattern (Fig. 4). Phosphorylation of this pathway increased with age; however, visceral and subcutaneous depots only differed in old animals with subcutaneous adipose showing a significant reduction in ERK activation. While these general findings agree with our findings regarding oxidative stress, they also cannot fully account for the extreme disparities in lipolytic and adipogenic markers we found that exist between adipose depots.

We next addressed the possibility that these depot-dependent effects could be driven by differences in metabolic demands that exist between these tissues. The effect of mitochondrial content and function in adipose tissue has been thought to play a
signifcant role in driving metabolic defects under conditions like obesity [31]. In humans, visceral adipose has been thought to have higher metabolic rate and greater mitochondrial density than subcutaneous fat [32]. To address the role of metabolism in the depot-specific differences we report, we utilized primary pre-adipocyte (PA) cultures derived from visceral or subcutaneous depots of young mice. We chose to use primary cultures of PA because: (1) PA contribute 15–50% of the total cellular content of adipose; (2) are significant contributors to chronic inflammation and oxidative stress in adipose tissue; (3) they retain in vivo characteristics of the depot from which they are derived; (4) their activity and function change with age and contribute largely to age-related functional changes of adipose tissue [10,20]. Moreover, changes in adipose mass occur in part through recruitment and differentiation of an existing pool of PA into mature adipocytes.

We found that visceral- and subcutaneous-derived PA differ dramatically in their mitochondrial bio-energetic function (Fig. 5). We found that basal respiration (oxygen consumption rate; OCR) of visceral depots was more than double that of pre-adipocytes isolated from subcutaneous depots (Fig. 5B). After treating with the mitochondrial uncoupler FCCP to assess maximal respiration rates, visceral-derived PA again exhibited a greater than two-fold higher rate of OCR as compared to subcutaneous-derived PA (Fig. 5C). Lastly, the oxidative phosphorylation-coupled OCR, that is the respiration tied directly to the production of ATP, was similarly higher in visceral PA compared to subcutaneous PA.
Despite this dramatic increase in cellular (i.e., mitochondrial) respiration in cells derived from visceral adipose depots, we found little evidence for differences in mitochondrial content as measured by the protein expression of cytochrome c or of different constituents of electron chain complexes I–IV in total protein homogenates from these PA lines (Fig. 6A). While not comprehensive studies of mitochondrial density, these data suggest fundamental differences in mitochondrial number is likely not the reason for the depot-specific differences in PA mitochondrial respiration. This raises interesting possibilities for the potential mechanisms including differences between depots in the formation of complexes, difference in pools of available energetic precursors, or possibly fundamental differences in the structure of the mitochondria themselves that limit effectiveness of the

Fig. 4. Activation of stress signaling pathways with age differs between adipose depots. A. Representative Western blot and quantified ratio of phosphorylated JNK to total JNK in indicated groups (effect of age $p = 0.71$, effect of depot $p < 0.001$). Y indicates samples from young mice, A indicates samples from aged mice and O indicates samples from old mice. B. Quantified ratio of phosphorylated ERK to total ERK as measured by ELISA in indicated groups (effect of age $p < 0.001$, effect of depot $p = 0.08$). Bars indicate average values ± SEM for ($n=4$–$5$) for each group. Asterisks represent significant difference between depots at indicated age group as measured by post-hoc analysis of ANOVA.

Fig. 5. Source of pre-adipocytes dramatically alters their mitochondrial respiration. A. Oxygen consumption rate (OCR) of pre-adipocytes derived from visceral (closed) and subcutaneous (open) depots as measured by BOFA assay using Seahorse bioanalyzer. Symbols represent average values ± SEM for ($n=8$) for each group at indicated time points. Letter A indicates addition of oligomycin, letter B indicates addition of FCCP and letter C indicates addition of antimycin A to the cellular chamber. B–D. Integrated area under the curve (AUC) for basal oxygen consumption (B.), maximum oxygen consumption (C.) and oxidative phosphorylation-coupled oxygen consumption (D.) measured during assay. Each circle represents AUC value calculated from an individual cell line; $p$ value is given for t-test comparing each group.

(Fig. 5D). Despite this dramatic increase in cellular (i.e., mitochondrial) respiration in cells derived from visceral adipose depots, we found little evidence for differences in mitochondrial content as measured by the protein expression of cytochrome c or of different constituents of electron chain complexes I–IV in total protein homogenates from these PA lines (Fig. 6A). While not comprehensive studies of mitochondrial density, these data
with both age and depot with evidence that subcutaneous adipose 
activation of the stress responsive signaling pathways differed 
previous study [33]. However, we discovered that the pattern of 
oxidant defense. Moreover, our data show that the effect of age on 
form of 4-HNE adducts) or in their changes in enzymatic anti-
pose. Again, the mechanisms responsible for this stress resistance 
are not yet clear, though reduced activation of stress signaling 
pathways in subcutaneous fat would be consistent with increased 
stress resistance of cells derived from this depot. This difference in 
stress sensitivity might also explain the relatively high rates of 
cellular senescence found in visceral adipose tissue both with 
aging and obesity [7,9,10].

Contrary to our initial hypothesis, our data suggest no large 
difference between visceral and subcutaneous adipose tissue in 
their age-associated accumulation of oxidative damage (in the 
form of 4-HNE adducts) or in their changes in enzymatic anti-
oxidant defense. Moreover, our data show that the effect of age on 
both adipose depots is relatively minor, in line with at least one 
previous study [33]. However, we discovered that the pattern of 
activation of the stress responsive signaling pathways differed 
with both age and depot with evidence that subcutaneous adipose 
showed reductions in the activation of both JNK and MAPK/ERK 
signaling. One interpretation of these findings may be that sub-
cutaneous adipose requires a higher threshold of oxidative stress 
to initiate these stress responses. Our finding that pre-adipocytes 
from subcutaneous fat are more resistant to at least two forms of 
oxidative stress are supportive of this particular interpretation. How 
these stress responsive pathways directly respond to oxidative 
stress is still largely unknown, though there are clear roles for JNK 
and ERK in metabolic stress and in particular in response to mi-
chondrial stress [34–36]. A better understanding of how the cells 
respond to oxidative stress particularly in adipose tissue could 
certainly help delineate how the response to oxidative stress may 
mediate some of the other physiological distinctions between 
subcutaneous and visceral adipose tissue.

Mitochondrial function and oxidative phosphorylation are im-
portant to the homeostatic maintenance of WAT [37]. It was then 
quite surprising to us to find the dramatic differences in oxidative 
phosphorylation of pre-adipocytes derived from two different 
adipose depots. While using pre-adipocytes has some limitations 
on interpreting effects on the adipose tissue as a whole, many 
depot-specific characteristics are retained even after differentia-
tion to mature adipose cells [7,20]. Due to their requirement of 
ATP, the depot-specific alterations with age in lipolytic and adi-
pogenic markers may be driven in part by differences in mi-
chondrial oxidative phosphorylation between subcutaneous and 
visceral adipose tissue [38,39]. In comparison to other tissues, 
there is a relative dearth in information regarding changes in 
mitochondrial function with age in adipose tissue. Expression of 
mitochondrial proteins, including cytochrome C, Hsp60 and pyr-
uvate dehydrogenase have been reported to decline with age in 
mouse visceral fat [40]. However, a proteomic-based study has 
suggested that many mitochondrial/ATP-generating proteins (in-
cluding ATP synthase, pyruvate dehydrogenase and isocitrate de-
hydrogenase) are actually elevated in visceral adipose from 24 
month old C57BL/6 mice compared to 12 month old mice [33]. 
In line with the latter, oxygen consumption of mitochondria from 
visceral adipose of old mice has been reported to be elevated 
compared to that of young mice for glycolytic, though not fatty 
acid, substrates [41]. Further studies are needed to both clarify 
how aging affects adipose mitochondrial function and whether 
this is altered by the anatomical site of the adipose tested.

While not directly tested, our findings may also offer some 
insight into the purported metabolic benefits of subcutaneous 
adipose tissue versus its visceral counterpart. Our findings suggest 
less remodeling (i.e., breakdown and biogenesis) of subcutaneous 
adipose in mice at least until they become very old. Our data also 
suggest both relatively lower metabolic rates in cells derived from 
subcutaneous adipose and reduced activation of stress signaling in 
this adipose depot. An interpretation of these data together might 
be that subcutaneous adipose is far more stable than visceral 
adipose. This relative “stability” of subcutaneous adipose might 
then prevent lipid release, mitochondrial dysfunction, and cellular 
dysfunction thereby providing metabolic benefit in contrast to 
visceral adipose which has been identified as contributing to 
metabolic dysfunction. Thus, identifying ways to improve or en-
haul the benefits of maintaining, or promoting, subcutaneous 
adipose tissue with age fits in line with the goal of extending the 
period of healthy aging in humans.
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