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Research Paper

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

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ABSTRACT

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 relatively suppressed in subcutaneous adipose tissue suggesting reduced sensitivity to oxidative stress. Similarly, pre-adipocytes from subcutaneous adipose were 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.

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

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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 CO_2 asphyxiation followed by cervical dislocation. At sacrifice, tissues were weighed and frozen immediately in liquid N_2 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) were homogenized in RIPA buffer with added protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL, USA) then centrifuged at 14,000*g* 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 NDUFB8, CII-30, CIII-core2, and CIV-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 Image J. 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% CO_2 in air, and ambient (21%) O_2 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/cm^2 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%) O_2 and immediately following each assay, cells were lysed to quantify total protein