Diet-induced obesity regulates adipose-resident stromal cell quantity and extracellular matrix gene expression

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ABSTRACT

Adipose tissue expansion during periods of excess nutrient intake requires significant turnover of the extracellular matrix (ECM) to allow for maximal lipid filling. Recent data suggest that stromal cells may be a primary contributor to ECM modifications in visceral adipose. The purpose of this study was to investigate the capacity for high fat diet (HFD)-induced obesity to alter adipose-derived stromal cell (ADSC) relative quantity and ECM gene expression, and determine the extent to which exercise training can mitigate such changes. Male C57BL/6J mice were placed on control or HFD for 8 weeks prior to and following initiation of a 16 week treadmill exercise program. ADSCs (Sca-1+CD45−CD146−CD31+CD34+) were isolated from epididymal adipose tissue and mRNA was evaluated using high throughput qPCR. Stromal cells were also obtained from skeletal muscle (MDSC). HFD decreased the quantity of ADSCs and markedly altered gene expression related to ECM remodeling (Col1α1, MMP2, MMP9, Timp1). Exercise did not reverse these changes. MDSCs were minimally altered by HFD or exercise. Overall, the data from this study suggest that ADSCs decrease in quantity and contribute to ECM remodeling in response to obesity, and exercise training does not significantly impact these outcomes.

1. Introduction

Excess nutrient availability results in deposition of lipid into preexisting adipocytes (adipose hypertrophy) or newly formed adipocytes (adipose hyperplasia). Adipocyte filling or preadipocyte expansion, however, cannot occur without supportive changes to the microenvironment, including extracellular matrix (ECM) and vascular remodeling (Cawthorn et al., 2012; Cinti et al., 2005; Strissel et al., 2007; Sun et al., 2011). The stromal vascular fraction (SVF) is a heterogeneous mixture of non-adipocyte cells in the adipose tissue that allow for tissue plasticity. The SVF includes immune cells, fibroblasts, preadipocytes, and important vascular-associated stem cells, including endothelial progenitor cells (EPCs) (CD45−CD31+CD34+), adipose-derived stromal cells (ADSCs) (predominantly mesenchymal stem cells (MSCs)) (CD45−CD31−CD146−NG2−), and pericytes (CD45−CD31−CD34−CD146+NG2+) (Qin et al., 2014; Sun et al., 2011). The complex cell-cell interactions that occur within the SVF to allow for appropriate tissue remodeling and expansion during conditions of healthy weight gain or obesity are not fully understood.

Stem cell antigen-1 (Sca-1) is a glycosyl phosphatidylinositol-anchored cell surface protein that was originally used as a marker to identify stem cells from bone marrow in the mouse, and is subsequently expressed by a variety of murine stem and progenitor cells in multiple tissues, including MSCs (Sharon & Lehto, 2002; van de Rijn et al., 1989). Recent studies suggest that ADSCs and adipocyte progenitors express Sca-1 (Berry et al., 2014; Ong et al., 2014; Schulz et al., 2011) and that the lineage negative (Lin−) fraction (CD45−CD31−Ter119−) in white adipose tissue specifically represents MSCs. Sca-1high stromal cells extracted from subcutaneous and visceral fat express a gene signature that reflects significant contribution to the synthesis and degradation of ECM molecules, including matrix metalloproteinases (MMPs) (Tokunaga et al., 2014), and widespread downregulation of MSC gene expression is observed in Zucker diabetic fatty rats (Ferrero-Lorente et al., 2014). Interestingly, Sca-1+/− mice display extensive skeletal muscle fibrosis following injury as a result of a deficiency in MMP activity (Kafadar et al., 2009) and develop insulin resistance and elevated blood glucose in response to a HFD (Staszekiewicz et al., 2012). Thus, these studies suggest that ADSCs provide an essential role in adipose tissue remodeling, and engagement of ADSCs in this event may be...
impaired with obesity as a result of long-term high fat feeding. However, to our knowledge, the impact of long-term HFD-induced obesity on ADSC quantity and gene expression has not been evaluated.

Endurance exercise training results in adipose lipolysis and attenuation of fibrosis (Kawanshiti, et al., 2013) and inflammation (Linden, et al., 2014; Vieira, et al., 2009) in adipose and skeletal muscle, independent of weight loss. Thus, participation in an endurance training program is a well-established recommendation for individuals diagnosed with Type 2 diabetes. Currently, the impact of an endurance training on stromal cell quantity and function in both adipose and skeletal muscle has not been investigated, particularly in the context of obesity. We have previously established that muscle-derived MSC (mMSC) relative quantity is increased in skeletal muscle in response to an acute bout of eccentric exercise, and that mMSC transplantation can facilitate improvements in myofiber growth and strength in response to training (Valero, n.d.). Thus, we speculate that resident stromal cells may provide the basis for positive changes in the structure and function of a variety of tissues, including both skeletal muscle and adipose, observed as a result of exercise.

In the present study, we hypothesized that ADSC quantity would be reduced (Ferrer-Lorente, et al., 2014), yet expression of genes related to ECM would be enhanced with HFD-induced obesity to sustain adipocyte growth. We also predicted that a 16 week endurance training program would prevent adipocyte growth and concomitant changes in ADSC quantity and gene expression. Using the same rationale, we hypothesized that muscle-derived stromal cell (MDSC) ECM gene expression would be elevated in skeletal muscle following endurance training to facilitate structural remodeling.

2. Materials and methods

2.1. Animals

Three month old male C57BL/6J mice (n = 20) were purchased from Jackson Laboratories (Bar Harbor, ME) and were group-housed (3–4 mice per cage). Mice were kept on a 12 h dark/light cycle (lights on 07:00 to 19:00 h) in a pathogen free, temperature-controlled facility and fed ad libitum. For the in vitro experiment, three male mice (mixed genetic background, SJL x C57BL/6) were used from our breeding colony. These mice were fed standard chow (Harlan-Teklad, 13% calories from fat). National Institutes of Health guidelines for the care and use of laboratory animals were strictly followed, and all experiments and procedures were performed by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

2.2. Design

Mice were randomly assigned to one of four groups: control diet-sedentary (no exercise) (Con-Sed, n = 5), control diet-exercise (Ex) (Con-Ex, n = 5), high fat diet-sedentary (HFD-Sed, n = 5) or HFD-exercise (HFD-Ex, n = 3). Five animals were originally assigned to HFD-Ex, but two died immediately before study completion and were omitted from the analysis. All diets were purchased from Research Diets Inc. (New Brunswick, NJ) and consisted of 10% or 60% of the calories from fat (OpenSource Diets D12450B or D12492, for Con or HFD respectively). Nutrient composition of these diets is matched and reported on the Research Diets website. Mice were fed specialized diets for 8 weeks, and then either remained sedentary or were subjected to a 16 week progressive aerobic exercise program.

2.3. Progressive aerobic exercise training

Exercise training was conducted on a motorized treadmill (Jog-a-Dog, Ottawa Lake, MI) for 60 min/day at 12–17 m/min, 5% grade, 5 days/week, for 16 weeks. Mice were introduced to treadmill exercise for a week, gradually running for 10–60 min at 10–12 m/min. Running speed was then increased every few weeks until the last two weeks of the study when running speed was 17 m/min. All animals complied with the exercise protocol. To control for stress associated with the training protocol, non-exercised control animals were exposed to similar noise and handling.

2.4. Hindlimb grip strength measurement

Grip strength was measured using a hindlimb pull bar on a 1027DM grip strength meter (Columbus instruments, Columbus, OH). Measurements were taken three weeks before the end of the study (week 21). All mice were habituated to the procedure for 3 consecutive days followed by a day of rest before being subjected to grip strength measurement (on the fifth day). To assess strength, mice were secured by their scruff and tail and allowed to grip the pull bar using their hindlimbs. The tester then gradually pulled the mouse back horizontally until the mouse grip was released. After 10 successful trials were performed only the 5 middle trials were used to record grip force in grams. The highest of these five values was used as peak force and the average of the five values was used as average force. Body weight was measured before each grip strength test and was used to express grip strength relative to weight (grip strength (g) divided by body weight (g)). All measurements were performed by the same evaluator.

2.5. Plasma analyses

Fasting plasma was collected in lithium-heparin coated conical tubes (Microvette CB300, Sarstedt, Nümbrecht, Germany) from the submandibular vein after 6 h fasting, 5 days before the end of the study. Samples were centrifuged for 15 min (2000g at 4 °C) and were stored in −80 °C until analysis. Plasma glucose was measured using a colorimetric assay (Cayman chemical, Ann Arbor, MI) and insulin was measured using an Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Downers Grove, IL), both according to the manufacturer instructions. The homeostasis model assessment method (HOMA–IR) was used to evaluate insulin resistance utilizing the following formula: Fasting glucose (mmol/l) × Fasting insulin (μU/ml) / 22.5 (Lee et al., 2008). Systemic inflammation was evaluated using a Mouse Serum Amyloid-A ELISA kit (Alpco diagnostics, Salem, NH). Colorimetric and ELISA assays were analyzed using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT).
2.6. Stromal cell isolation

Stromal cell isolation was performed under a laminar flow hood using sterile technique as previously reported (Zou et al., 2014). In brief, both epididymal fat pads and both gastrocnemius-soleus complexes were dissected from each individual mouse and extensively minced in PBS and subjected to enzymatic digestion in 0.2% Type I Collagenase (Worthington Biochemical Co., Lakewood, NJ) for 45 min with repeated trituration. After adding the inhibition solution (20% FBS in HBSS) the samples were spun for 5 min at 450 g and filtered through a 70 μm strainer. The cells were then blocked with anti-mouse CD16/CD32 antibody (eBioscience, San Diego, CA) for 10 min on ice to prevent non-specific Fc receptor mediated binding. Following the blocking step, cells were incubated with fluorescent-conjugated antibody cocktail (Anti-Sca-1-PE, 600 ng/106 cells and anti CD45-APC, 300 ng/106 cells, eBioscience, San Diego, CA) diluted in 2% FBS in PBS for 1 h on ice, followed by 2 washes in 2% FBS in PBS. Fluorescence activated cell sorting (FACS) was performed on an iCyt Receptor Detection System (Carle Hospital, Urbana, IL) and Sca-1+ CD45− cells were collected in RLT lysis buffer (Qiagen, Valencia CA) for gene expression analysis or in high glucose Dulbecco’s modified Eagle’s medium (DMEM), 10% FBS, 5 μg/ml gentamycin (growth medium) for in vitro experiments.

2.7. RNA extraction, cDNA synthesis and preamplification

Cell lysates were subjected to RNA extraction using RNeasy Micro kit (Qiagen, Valencia CA), following the manufacturer instructions. Flash frozen epididymal fat pads were lysed in QiAzo lysis reagent and were subjected to RNA extraction using RNeasy Lipid Tissue Mini kit (Qiagen, Valencia CA). RNA quantification was completed in duplicate on a Take-3 application plate using a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT). Reverse transcription was performed using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY) with starting RNA concentrations of 500 ng for tissue or 25 ng for cell lysates. Preamplification of Sca-1+ CD45− cell cDNA was performed using TaqMan PreAmp Master Mix (Life Technologies, Grand Island, NY). This technique allows for unbiased targeted amplification of genes using a primer pool of up to 100 TaqMan primers, resulting in a high quantity of primer-specific amplified cDNA from minimal RNA (10–20 ng) (Mengual et al., 2008). Primer pool consisted of 32 inventoried Taqman primers, diluted to 0.2 μl in Tris-EDTA buffer. After mixing the PreAmp reagent (25 μl per reaction) with the primer pool (12.5 μl per reaction) and sample cDNA (12.5 μl), individual reactions were amplified for 14 cycles in a thin-walled 0.2 ml PCR tube using a thermo-cycler (ABI Geneamp 9700, Life Technologies, Grand Island, NY).

2.8. Quantitative PCR

Quantitative PCR was performed on an Applied Biosystems 7900HT Fast RT PCR machine using inventoried Taqman primers purchased from Applied Biosystems (Life Technologies, Grand Island, NY). Primer information and assay ID numbers are provided in Table 1. Glyceraldahyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene and relative gene expression analysis was presented relative to the reference group (Con-Sed) using the ΔΔCt method. All replicate Ct values were within 0.2 Ct units of each other.

2.9. In vitro sodium-palmitate assay

A single FACS-isolation was used to obtain a pool of adipose-derived Sca-1− CD45− cells from 3 mice. Cells were seeded on 100 mm plastic culture dishes (1 x 10^6 cells per plate) for 8 days. Growth medium was changed every 3–4 days (see Section 2.6 for medium information). Cells were then seeded at equal density on laminin coated 6-well plates. Cells were allowed to adhere for 24 h in growth medium. A previously published in vitro free fatty acid (FFA) assay was used (Linden et al., 2014) with modifications. Cells were incubated with 0.75 mM sodium-palmitate (5:1 FFA to protein molar ratio) in DMEM with 5% BSA (palmitate, n = 3 wells) or 5% BSA in DMEM alone (vehicle, n = 3 wells) for 3 days. Prior to application, palmitate was conjugated to BSA for 1 h at 40 °C with agitation (140 rpm). At the end of 3 days, media was removed, 350 μl buffer RLT (Qiagen, Valencia CA) was added to each well and lysates were stored at −80 °C until used for high throughput microfluidics qPCR. Images were obtained pre- and post-incubation, using a Zeiss AxioCam digital camera and Axiovision software at 20× magnification (Zeiss, Thornwood, NY, USA).

2.10. High throughput microfluidics quantitative PCR

RLT (Qiagen, Valencia CA) lysates from the in vitro experiment were subjected to RNA extraction and cDNA synthesis as mentioned above. qPCR was performed using a dynamic array integrated fluidic circuit (IFC) on a Biomark HD (Fluidigm, San Francisco, CA). This high throughput microfluidic qPCR system allows for a more accurate, highly repeatable analysis of multiple genes in multiple samples. Gene expression was assessed using inventoried Taqman primers (primer information and assay ID numbers are provided in Table 1). GAPDH was used as the housekeeping gene and relative gene expression analysis was presented relative to vehicle condition using the ΔΔCt method. All replicate Ct values were within 0.2 Ct units of each other.

2.11. Sirius red staining

Adipose tissue was fixed in 4% formalin and embedded in paraffin. 8 μm sections were mounted on a microscope slide and were prepped for staining. Tissue sections were fixed in Bouin’s solution and blocked in Fast Blue solution (0.1% Fast Blue RR salt, 7 mM magnesium sulfate and 60 mM magnesium borate). Slides were immersed in Pico-Sirius red solution (0.1% Sirius red F3B in picric acid) and then quickly washed with acidified H2O (0.5% acetic acid in dH2O). After dehydration the tissue with ethanol and xylene, cover slips were mounted with histological mounting medium (Permount, Fisher Scientific, Pittsburgh, PA). Collagen staining was visualized under light microscopy (20×) using an inverted microscope (Leica DMRXA2). Representative images were captured using a digital camera – AxioCam, and Axiovision software (Zeiss, Thornwood, NY).

<table>
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<tr>
<th>Gene/alias</th>
<th>Assay ID</th>
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<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
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</tr>
<tr>
<td>CD90 (Thy 1)</td>
<td>Mm00493881_m1</td>
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<td>CD105 (Eng, Endoglin)</td>
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<td>Collagen 1 alpha 1 (Col1a1)</td>
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2.12. Statistical analysis

All data are presented as means ± standard error of the mean. Mixed model (within and between subjects) three-way ANOVA was used to investigate main and combined effects of diet (HFD vs. Con), exercise (Ex vs. Sed) and tissue (adipose vs. muscle), on Sca-1⁺CD45⁻ stromal cell gene expression, followed by Tukey post hoc analysis. Two-way ANOVA followed by LSD post hoc analysis was used to investigate diet (HFD vs. Con) and exercise (Ex vs. Sed) main effects and diet by exercise interaction on adipose weight, skeletal muscle weight, ADSC and MDSC relative quantity, fasting insulin, fasting glucose, HOMA-IR, and cytokine gene expression in adipose tissue. Changes in body weight were analyzed using a three-way repeated measures ANOVA followed by LSD post hoc analysis to investigate main and combined effects of diet (HFD vs. Con) and exercise (Ex vs. Sed) over time (Pre, 8, 13, 21 and 24 week). Student’s *t*-test analysis was used to investigate the effect of palmitate compared to vehicle on ADSC gene expression. Before each analysis, distribution curve normality was verified (Shapiro-Wilk test, \( p < 0.05 \)) and log transformation was used to correct non-normal distribution when needed. Statistical analysis (Two-way ANOVA, three-way repeated measures ANOVA and *t*-test) was performed using SPSS Ver. 20 (IBM, Chicago, IL) or (Mixed model three-way ANOVA) using SAS Ver. 9.3 (SAS Institute, Cary, NC). Differences were considered significant at \( p \leq 0.05 \).

![Fig. 2.](image-url) Long-term HFD results in obesity, insulin resistance and low-grade inflammation. Body weight (A), hindlimb grip strength (B, C), and spleen weight (K) were measured in grams. Fasting plasma insulin (D) and serum amyloid A (J) were measured using an ELISA kit, while fasting plasma glucose (E) was measured using a colorimetric assay. HOMA-IR (F) was calculated from fasting insulin and glucose values. Cytokine gene expression in adipose tissue (G, H, I) was examined using qPCR. Con-Sed, Con-Ex and HFD-Sed, \( n = 5 \) and HFD-Ex, \( n = 3 \). † \( p < 0.05 \) vs. pre-intervention (Pre); ‡ \( p < 0.05 \) vs. Con diet; ‡‡ \( p < 0.05 \) vs. Sed; †† \( p < 0.05 \) vs. Con-Sed. All values are mean ± SEM.
3. Results

3.1. Long-term HFD results in obesity, low-grade inflammation and insulin resistance

Body weight increased steadily in all groups (time effect, \( p < 0.05 \)) throughout the intervention. However, compared to control diet, 8 weeks of HFD resulted in a robust increase in body weight (diet effect, \( p < 0.05 \)) and this difference was observed throughout the duration of the study (time by diet interaction, \( p < 0.05 \), Fig. 2A). Similarly, compared to control diet, 24 weeks of HFD resulted in increased fasting insulin, fasting glucose and HOMA-IR (diet effect, \( p > 0.05 \), Fig. 2D, E, and F, respectively). Finally, HFD resulted in adipose tissue inflammation and systemic inflammation, demonstrated by increased gene expression of macrophage marker and classic cytokines: F4/80, TNFα and IL-10 (diet effect, \( p < 0.05 \), Fig. 2G, H and I, respectively), and increased serum amyloid A (SAA) and spleen weight (diet effect, \( p < 0.05 \), Fig. 2J and K, respectively).

3.2. Exercise restores grip strength, but does not attenuate insulin resistance and adipose tissue inflammation

All mice adhered to the exercise program and ran a total of 3600 min over 16 week exercise intervention. A significant time by exercise interaction (\( p < 0.05 \)) suggested that exercise contributed to reduced weight gain in HFD-Ex, but post hoc analysis of this interaction was not statistically significant (\( p = 0.039 \) and 0.094 for HFD-Ex vs. HFD-Sed in week 21 and 24, respectively). HFD decreased grip strength compared to control diet (diet effect, \( p < 0.05 \)), whereas progressive aerobic exercise improved grip strength in the HFD-Ex group (exercise effect, \( p < 0.05 \), Fig. 2B and C). Systemic insulin resistance, inflammatory gene expression, and other markers of inflammation did not decrease in adipose tissue in response to training (Fig. 2D-J). Interestingly, although exercise did not attenuate adipose inflammation or SAA, spleen weight (a proxy for systemic inflammation) was significantly different in the HFD-Con group compared to all other groups, including HFD-Ex (\( p < 0.05 \), Fig. 2K). HFD-Ex spleen weight was similar to that of the control diet groups, suggesting that exercise had a beneficial outcome on systemic inflammation. The changes in tissue inflammatory gene expression with HFD prompted us to investigate stromal cell relative quantity and gene expression in response to HFD, exercise or both in combination.

3.3. Long-term HFD decreases ADSC relative quantity

Despite the significant increase in body weight with HFD, average epididymal fat weight was not different between the groups (Fig. 3A). Subcutaneous fat was visibly increased with HFD and may account for the increase in body weight, but not assessed in the current study. Whole tissue (epididymal fat) adipogenic gene expression (ZFPI23, Fabp4, DLK-1) was not altered with HFD, confirming lack of adipogenesis in this depot (data not shown). Representative gating strategy plots are presented for epididymal ADSCs and MDSCs (Fig. 3B and E, respectively). ADSC relative quantity decreased by 10% with HFD (diet effect, \( p < 0.05 \), Fig. 3C). Exercise training did not result in a significant change in ADSC relative quantity. Gastrocnemius–soleus complex weight was not different between the groups (Fig. 3D) and MDSC relative quantity (6–7% of mononuclear cells) did not change with HFD, exercise training or the combination of the two (Fig. 3F). We next investigated the mRNA expression of two common MSC cell surface markers: CD90 and CD105. In ADSCs, CD90 gene expression was not influenced by HFD or exercise (Fig. 3G). ADSC CD105 gene expression, on the other hand, was significantly lower compared to MDSCs (tissue effect, \( p < 0.05 \), Fig. 3H). HFD
decreased, while exercise markedly increased CD105 gene expression in ADSCs (diet by exercise by tissue interaction, \( p < 0.05 \), Fig. 3H). In MDSCs, cell surface marker gene expression was not changed by diet or exercise intervention.

3.4. ADSC ECM-related gene expression is altered following long-term HFD, but is not responsive to exercise

Stromal cells regulate tissue matrix remodeling via synthesis and secretion of structural proteins and enzymes that regulate the presence of ECM proteins. MDSC ECM gene expression was not significantly altered in response to HFD, exercise or both combined (Fig. 4A–F). In contrast, significant changes in ECM gene expression were readily apparent in ADSCs. Compared to MDSCs, connective tissue growth factor (CTGF) gene expression was reduced, and collagen 1α1 (Col1α1), matrix metalloproteinase 2 (MMP2), MMP9, tissue inhibitor of matrix metalloproteinase 1 (Timp1) and Timp2 gene expression were all increased in ADSCs (tissue effect, \( p < 0.05 \), Fig. 4). Significant diet effects and tissue by diet interactions were noted for Col1α1, MMP2, MMP9 and Timp1 gene expression (\( p < 0.01 \)). Post hoc analyses revealed Col1α1, MMP2

![Diagrams](image_url)

**Fig. 4.** ADSC ECM-related gene expression is altered following long-term HFD, but is not responsive to exercise. CTGF, Col1α1, MMP2, MMP9, Timp1, Timp2, VEGFa and PGC1α (A–H) gene expression was examined in freshly collected ADSCs and MDSCs using qPCR. Con-Sed, Con-Ex and HFD-Sed, \( n = 5 \) and HFD-Ex, \( n = 3 \). \* \( p \leq 0.05 \) vs. Muscle all groups; \# \( p \leq 0.05 \) vs. Muscle Con-Sed; \# \( p \leq 0.05 \) vs. Adipose Con-Sed. All values are mean ± SEM.
and Timp1 mRNA was increased in response to HFD ($p < 0.05$, Fig. 4B, C and E, respectively). HFD decreased MMP9 gene expression ($p < 0.05$, Fig. 4D).

In contrast to diet, exercise training did not significantly alter ADSC ECM-associated gene expression. The loss of two mice at the end of our study was unfortunate and must be taken into consideration when interpreting the results. To verify changes in gene expression in this group ($n = 3$), adipose tissue collagen was directly visualized using Sirius red staining. Adipose tissue collagen accumulation was apparent in response to HFD (Fig. 5A). In addition, adipose tissue TGFβ and collagen 1α1 mRNA were significantly increased with HFD (diet effect, $p < 0.05$, Fig. 5B–C). However, exercise training did not reduce collagen accumulation or whole tissue gene expression.

Finally, vascular growth factor (VEGFα) and the transcriptional factor PGC-1α promote angiogenesis and vessel remodeling necessary to support tissue growth. VEGFα mRNA levels decreased in MDSCs and

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**Fig. 5.** HFD results in adipose tissue fibrosis. Adipose tissue fibrosis was visualized in representative samples using histology (Sirius red, A) and assessed in all samples via gene expression (qPCR, B–C). $^d p \leq 0.05$ vs. Con diet. All values are mean ± SEM. A, scale bar = 250 μm.

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**Fig. 6.** ADSC cytokine gene expression is enhanced compared to MDSC, but is not affected by diet or exercise. TNFα, IL-1β, IL-10, IL1-ra, IL-6, TGFβ1 (A–F) gene expression was examined in freshly obtained ADSC and MDSC using qPCR. Con-Sed, Con-Ex and HFD-Sed, $n = 5$ animals and HFD-Ex, $n = 3$ animals. $^m p \leq 0.05$ vs. Muscle all groups. All values are mean ± SEM.
increased in ADSCs in response to HFD (tissue by diet interaction, \(p < 0.05\), Fig. 4G–H). Exercise did not impact VEGFa or PGC-1α gene expression in stromal cells from either tissue.

3.5. ADSC cytokine gene expression is enhanced relative to MDSCs, but is not affected by diet or exercise

Sca-1\(^+\)CD45\(^−\) stromal cells can modulate the activity of immune cells (Aggarwal & Pittenger, 2005) and are an established source of cytokine production (Aggarwal & Pittenger, 2005; Kilroy et al., 2007). Pro-inflammatory cytokine (tumor necrosis factor α (TNFα), interleukin-1β (IL-1β)), anti-inflammatory (IL-10, IL-1 receptor antagonist (IL-1ra)), and IL-6 gene expression was significantly higher in ADSCs compared to MDSCs (tissue effect, \(p < 0.05\), Fig. 6A–E). Transforming growth factor β1 (TGFβ1) gene expression was not altered. ADSC and MDSC cytokine gene expression (TNFα, IL-1β, IL-10, IL-1ra, and TGFβ1) was not significantly influenced by HFD or exercise (Fig. 6A–D, F). ADSC IL-6 gene expression was decreased as a result of exercise (exercise by tissue interaction, \(p < 0.05\), Fig. 6E).

3.6. Palmitic acid alters ADSC gene expression in vitro

In vivo experiments demonstrated that HFD-induced obesity significantly increased ADSC ECM gene expression. We tested the hypothesis that direct exposure to the long chain FFA, palmitate, in the adipose tissue microenvironment in that direct exposure to the long chain FFA, palmitate, in the adipose tissue microenvironment significantly increased ADSC ECM gene expression. We tested the hypothesis that direct exposure to the long chain FFA, palmitate, in the adipose tissue microenvironment significantly increased ADSC ECM gene expression. ADSCs maintained their fibroblast-like triangular morphology in culture in response to long-term incubation with palmitate (Fig. 7A), but gene expression was significantly affected. Specifically, in response to palmitate, MMP9 and TIMP1, as well as TGFβ1 and IL-6, gene expression significantly increased in response to palmitate (\(p < 0.05\), Fig. 7B and C, respectively).

4. Discussion

The purpose of this study was to investigate adipose- and skeletal muscle-resident Sca-1\(^+\)CD45\(^−\) stromal cell responses to long-term HFD-induced obesity, alone or in combination with a 16 week endurance exercise program. Adult mice (3 month old at onset and 9 month old at the end of the study) were exposed to prolonged 60% HFD (total of 24 weeks) to mimic an obesogenic lifestyle. Accordingly, HFD resulted in a doubling of average body weight (60 g HFD versus 30 g Con), as well as insulin resistance and fasting hyperglycemia (over 200 mg/dL). Long-term HFD-induced obesity did not affect stromal cell quantity or function in skeletal muscle, but significantly influenced stromal cell relative quantity and gene expression in adipose tissue. Most importantly, obesity resulted in striking and consistent changes in ADSC gene expression related to ECM remodeling, yet endurance training did not alter this profile.

Despite the significant increase in body weight and diabetic state with long-term HFD, average epididymal fat weight was not different between the groups. Subcutaneous fat pads were visibly increased in response to HFD, but we intentionally targeted epididymal fat due to the amount of readily accessible tissue in mice and the established association of visceral fat with metabolic disease. These results are similar to seminal work previously published by Surwit and colleagues almost 30 years ago (Surwit et al., 1988) and more recently by Strissel et al. (2007). In the latter study, epididymal fat pad weight decreased while subcutaneous fat pad mass increased with sustained HFD during weeks 12 through 20 (Strissel et al., 2007). Hepatosteatosis was also observed, which together with subcutaneous fat accumulation, may account for the increase in body weight (Strissel et al., 2007). It is interesting to speculate that differences in ADSC quantity or function may underlie the divergent growth responses in depot-specific growth. ADSC relative quantity was reduced in epididymal adipose with HFD in the current study, and several factors may account for the decrease, including 1) SVF enrichment (fibroblasts, endothelial cells, and immune cells) (Church et al., 2014) and 2) cell death due to inflammation. Our preliminary examination of early and late adipogenic marker gene expression (ZFP423, Ebf1, DLK1, and PPARG) in both whole tissue and ADSCs suggests minimal capacity for adipogenesis, an event that may force existing adipocytes to hypertrophy with long-term HFD. Hyperplasia is, in fact, lower in omental fat compared to subcutaneous fat in women (Drolet et al., 2008). Thus, resistance to adipogenesis and subsequent adipocyte hypertrophy may prompt changes in the microenvironment to accommodate growth.

Sca-1\(^−\)\(^−\) mice demonstrate deficiency in MMP activity and enhanced fibrosis in muscle (Kafadar et al., 2009). In addition, these...
mice develop insulin resistance and glucose intolerance when exposed to a HFD (Staszkiewicz et al., 2012). Recent studies suggest that Sca-1+ cells in the adipose SVF are MSCs that secrete proteins necessary for ECM and vascular remodeling, including membrane-associated and secreted collagenases, or MMPs (Qin et al., 2014; Tokunaga et al., 2014). In the current study, we demonstrate that Sca-1+ CD45− stromal cells display a gene signature that depicts strong capacity for secretion of ECM modulatory factors in response to long-term HFD. ADSC-mediated secretion of MMP2, Timp1, Timp2 and Col1+ cells in the adipose SVF are MSCs that secrete proteins necessary to a HFD (Staszkiewicz et al., 2012). Recent studies suggest that Sca-1+ cells in the adipose SVF are MSCs that secrete proteins necessary to a HFD (Staszkiewicz et al., 2012). Recent studies suggest that Sca-1+ cells in the adipose SVF are MSCs that secrete proteins necessary to a HFD (Staszkiewicz et al., 2012).

The marked decrease in MMP9 gene expression with HFD concur with published reports of decreased MMP9 levels in adipose tissue of insulin resistant rodents (Miksztowicz et al., 2014). This may reflect failure of ADSCs to regulate immune cell activity (Ding et al., 2009) and angiogenesis (van Hinsbergh & Koolwijk, 2008) in the pathologically expanded adipose tissue. Similarly, the increases in VEGFa and PGC-1α gene expression suggest an attempt to initiate vascularization necessary to prevent adipocyte hypoxia. The lack of change in cytokine gene expression (TNFx, IL-1β, IL-10, IL-1ra, IL-6, and TGFβ1) in response to HFD provides evidence that ADSCs are not a primary cellular source of systemic inflammation. Overall, our gene expression analyses suggest that ADSCs serve as interstitial stromal cells that modify the extracellular environment to accommodate adipocyte growth.

A moderate intensity exercise training program did not appear to reverse the ADSC gene expression profile elicited by HFD, and this was in agreement with results for SAA, insulin resistance, plasma glucose, and adipose tissue TNFα mRNA levels. Exercise did decrease spleen weight, which is a proxy for systemic inflammation. This suggests some involvement of exercise in the modification of systemic inflammation. Other studies have reported suppression of systemic and adipose inflammation with exercise in both animal and human studies (Kawanishi et al., 2013a, 2013b; Linden et al., 2014; Ryan et al., 2014; Trachta et al., 2014; Vieira et al., 2009), yet in our review of animal studies, including the aforementioned, we noted that the mice were significantly younger at study completion (2–4 month old versus 9 month old in the current study). We purposefully used older mice to mimic adult-onset weight gain and susceptibility to the diabetic condition. Although the exercise protocol we used was longer and more intense than commonly used in similar studies (Kawanishi et al., 2013a, 2013b; Linden et al., 2014; Vieira et al., 2009), it is possible that our paradigm simply could not overcome tissue dysfunction as a result of the combination of advanced age at the initiation of the study and consumption of a diet that was exceptionally high in fat (60% kcal).

Lipotoxicity in adipose tissue is a complex process that is triggered by hypercaloric nutrition. As a result of the excess lipid flux into adipose tissue during the progression of obesity, adipocytes hypertrophy and die. This is accompanied by local macrophage infiltration, systemic inflammation and spillover of lipids into the circulatory system with subsequent deposition in distal organs such as liver and skeletal muscle (Frayn, 2002). Wu et al. (2012) demonstrated that FFA treatment can reduce MSC multipotency and initiate adipogenesis when presented in culture. Thus, we hypothesized that the change in ADSC ECM gene expression in response to HFD was triggered directly by the exposure to FFA. While the increase in Timp1 gene expression observed with HFD-conducive gene expression with HFD-induced obesity are not initiated as a result of direct exposure to FFA, but rather alternative cues provided by adipose growth.

Muscle-resident Sca-1+ CD45− cells were minimally responsive to HFD and/or endurance exercise. A significant reduction in VEGF gene expression was observed in MDSCs in response to HFD, yet no other changes were noted in MDSC relative quantity or the expression of genes related to ECM remodeling or inflammation. It is possible that transient changes in MDSC gene expression occurred with the onset of HFD and exercise training, yet these responses were no longer present post-training. A more extensive evaluation of the MDSC gene signature in response to HFD and endurance exercise is necessary to elucidate a novel role for MDSCs in events other than remodeling and growth, such as metabolic flexibility and fatigue resistance. Similarly, the MDSC transcriptome should be evaluated to validate a role for MDSCs in ECM remodeling and myofiber growth in response to strength training.

5. Conclusion

This study provides the first investigation of the epididymal adipose-resident Sca-1+ CD45− stromal cell response to long-term HFD, alone and in combination with progressive endurance exercise training in adult mice. As hypothesized, 24 weeks of HFD result in obesity, adipose inflammation, systemic inflammation and insulin resistance. HFD-induced obesity decreased ADSC relative quantity and substantially altered gene expression of ECM proteins, including MMPs, Timp3, and collagen. Contrary to our hypothesis, exercise did not alter any of the changes observed. With a few exceptions (CTGF, VEGFα, TGFβ1), mRNAs specific to ECM remodeling proteins and inflammatory cytokines were notably lower in Sca-1+ CD45− stromal cells isolated from skeletal muscle (MDSCs) compared to adipose, and gene expression was predominantly unaffected by HFD diet and/or endurance exercise. The mechanistic basis for changes in ADSCs remains unknown, but FFA exposure appears unlikely based on studies conducted in vitro. Overall, this study highlights a role for ADSCs in ECM remodeling as a result of high fat diet-induced obesity. We speculate that ADSCs remodel the connective tissue in a manner to allow for maximal adipocyte filling/expansion and prevent necrosis that can lead to systemic inflammation and Type 2 diabetes, yet this protective countermeasure becomes ineffective without an appropriate reduction in energy intake.

Acknowledgements

The authors would like to acknowledge undergraduate research assistants Paul Jung and Adam Joseph for their invaluable help with data collection. We would also like to thank Dr. Justin S. Rhodes for help and guidance with mixed-model statistical analysis. Finally, we would like to thank Dr. Mark R. Band and the lab team from the Functional Genomics Unit of the W.M. Keck Center, Roy Carver Biotechnology Center, UIUC, for their excellent guidance on preamplification and their help with high throughput microfluidics qPCR. This work was partially funded by the ISEF Doctoral Fellowship and an American College of Sports Medicine Foundation Doctoral Student Research Grant (ACSM 2014-03896) (Y.P.).

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