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Increased fibrosis: A novel means by which GH influences white adipose tissue function

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ABSTRACT

Objective: White adipose tissue (WAT) fibrosis – the buildup of extracellular matrix (ECM) proteins, primarily collagen – is now a recognized hallmark of tissue dysfunction and is increased with obesity and lipodystrophy. While growth hormone (GH) is known to increase collagen in several tissues, no previous research has addressed its effect on ECM in WAT. Thus, the purpose of this study is to determine if GH influences WAT fibrosis.

Design: This study examined WAT from four distinct strains of GH-altered mice (bGH and GHA transgenic mice as well as two tissue specific GH receptor gene disrupted lines, fat growth hormone receptor knockout or FaGHRKO and liver growth hormone receptor knockout or LiGHRKO mice). Collagen content and adipocyte size were studied in all cohorts and compared to littermate controls. In addition, mRNA expression of fibrosis-associated genes was assessed in one cohort (6 month old male bovine GH transgenic and WT mice) and cultured 3T3-L1 adipocytes treated with GH.

Results: Collagen stained area was increased in WAT from bGH mice, was depot-dependent, and increased with age. Furthermore, increased collagen content was associated with decreased adipocyte size in all depots but more dramatic changes in the subcutaneous fat pad. Notably, the increase in collagen was not associated with an increase in collagen gene expression or other genes known to promote fibrosis in WAT, but collagen gene expression was increased with acute GH administration in 3T3-LI cells. In contrast, evaluation of 6 month old GH antagonist (GHA) male mice showed significantly decreased collagen in the subcutaneous depot. Lastly, to assess if GH induced collagen deposition directly or indirectly (via IGF-1), fat (Fa) and liver (Li) specific GHRKO mice were evaluated. Decreased fibrosis in FaGHRKO and increased fibrosis in LiGHRKO mice suggest GH is primarily responsible for the alterations in collagen.

Conclusions: Our results show that GH action is positively associated with an increase in WAT collagen content as well as a decrease in adipocyte size, particularly in the subcutaneous depot. This effect appears to be due to GH and not IGF-1 and reveals a novel means by which GH regulates WAT accumulation.

1. Introduction

White adipose tissue (WAT) fibrosis is a characteristic feature of obesity and lipodystrophy and associated with inflammation, insulin resistance, and decreased adipocyte size [1]. In addition, it is believed to negatively impact metabolism by limiting the ability of adipocytes to expand [2–4] and by contributing to other WAT dysfunctions such as hypoxia and immune cell infiltration [5–7]. Indeed, mice lacking collagen VI, a collagen preferentially expressed in WAT, display dramatic WAT expansion accompanied by significant improvements in glucose and lipid metabolism as well as reduced WAT immune cell infiltration and inflammation [2]. Though WAT fibrosis is currently associated with the obese state, it is possible that it is a general feature of unhealthy WAT and, thus, a consequence of other disease states as well.

Abbreviations: GH, growth hormone; GHR, growth hormone receptor; GHRKO, growth hormone receptor knockout; FaGHRKO, fat growth hormone receptor knockout; LiGHRKO, liver growth hormone receptor knockout; IGF-1, insulin-like growth factor 1; WAT, white adipose tissue; ECM, extracellular matrix; bGH, bovine growth hormone; GHA, growth hormone receptor antagonist; AT, adipose tissue; Sc, subcutaneous; Mes, mesenteric; Peri, perigonadal; Retro, retroperitoneal; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitors of MMPs

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Growth hormone (GH) altered mice provide a unique perspective on the relationship of WAT and health due to the profound impact that GH has on WAT mass and distribution as well as its negative effect on lifespan and healthspan [8–11]. That is, mice with global excess in GH action are lean but suffer drastic metabolic and lifespan consequences, whereas mice with a reduction in GH action are obese but lack the metabolic dysfunction associated with obese states [12]. These contradictory and counterintuitive phenotypes – unhealthy leanness and healthy obesity – allow us to examine WAT fibrosis independent of the normal adiposity/health relationship and determine if WAT fibrosis is a hallmark of unhealthy WAT irrespective of obesity status.

We focused mainly on bovine GH transgenic (bGH) mice, which have life-long, chronic, high serum levels of GH, insulin-like growth factor 1 (IGF-1), and insulin resulting in a giant, lean phenotype. Important to consider is that their pattern of GH secretion is non-pulsatile, and thus pathologic. Despite their leanness, bGH mice are unhealthy and suffer from insulin resistance [13–15], increased cancer incidence [16], and shortened lifespans [17]. Similar complications are seen in human patients with acromegaly [18–21]. Notably, high levels of GH have been shown to promote fibrosis in numerous tissues such as muscle, bone, heart and kidney [22–27]. However, no results have been reported on the effect of GH on the ECM in WAT. Thus, the primary objective of this study was to examine the effect of chronic excess GH exposure on collagen accumulation in WAT using bGH mice. For comparison, we also assessed a mouse line with decreased GH stimulus, the GH receptor antagonist (GHA) mouse, to determine if the inhibition of GH action decreased collagen content. Lastly, to determine if GH induced increased collagen deposition directly or indirectly (via IGF-1), we studied WAT samples from adipose tissue-specific growth hormone receptor (GHR) knockout animals (FaGHRKO) [28] and liver-specific GHR knockout animals (LiGHRKO) [29], the latter of which have elevated circulating GH but reduced endocrine IGF-1.

2. Materials and methods

2.1. Animals

All mice were housed in the facility at the Edison Biotechnology Institute where they were kept on a 14-hour light/10-hour dark cycle and had ad libitum access to water and normal chow unless otherwise noted. Development and breeding of bGH and GHA transgenic mice, both on a C57BL/6J background, have been described previously [17,30–32]. Generation of adipose tissue-specific (FaGHRKO) and liver-specific (LiGHRKO) GHR mice, both in the C57BL/6 background, have also been described previously [28,29]. All animal procedures were approved by the Ohio University Institutional Animal Care and Use Committee.

Four separate cohorts of mice were utilized in this study and will be referred to as cohort 1 through 4 for clarity. Cohort 1 consisted of two male and female bGH and wild type (WT) control mice from three different age groups (26 weeks, 42 weeks, and 64 weeks). Cohort 2 was comprised of 6-month old male bGH mice and WT littermate control mice. Males were solely used in this cohort due to availability of samples. Since bGH mice have very little WAT, it was not possible to perform all of the experiments for this group on the same mice. Thus, separate mice from this cohort were necessary to complete the immunohistochemistry (n = 8), hydroxyproline (n = 10), qPCR expression (n = 8) and RNA-seq (n = 3) analyses. Cohort 3 consisted of 6-month old male GHA mice and WT littermate controls (n = 7). Cohort 4 included a subset of the adipose tissue samples (n = 8) from FaGHRKO and LiGHRKO male mice along with floxed littermate controls collected and described previously [28,29].

2.2. Body composition

Body composition and body weight were measured one day prior to dissection (cohorts 2–4). Body weight was measured using a Mettler Toledo PL 202-S balance, and body composition was measured using the Minispec mq Benchtop Nuclear Magnetic Resonance analyzer (Bruker Instruments, Minispec ND2506) as previously described [33].

2.3. Tissue weights

Mice were fasted for 12 h prior to being sacrificed by cervical dislocation. For all cohorts, four distinct WAT depots [inguinal subcutaneous (sc), perigonadal (peri), mesenteric (mes), and retroperitoneal (retro)] were collected and weighed. WAT used for histological measures was fixed in a 10% formalin solution, then rinsed and stored in a 70% ethanol solution. For gene expression (RNA) analysis, the harvested WAT depot was flash frozen in liquid nitrogen and stored at − 80 °C until further processing.

2.4. Immunohistochemistry

Formalin-fixed WAT samples from cohorts 1–4 were sent to AML Labs (Baltimore, MD) for paraffin embedding, sectioning and staining with picrosirius red, a general collagen stain. Slides were analyzed using a Nikon Eclipse E600 microscope. Images for both collagen staining and cell sizing were obtained using a Spot RT digital camera at 200 × magnification. For collagen staining quantification, pictures of 20 non-overlapping fields were taken per WAT depot per mouse and then analyzed using ImageJ software [34]. Collagen staining [35] and cell size [28] were quantified as described previously.

2.5. Cell culture

Adipogenic differentiation was induced by treating confluent 3T3-L1 preadipocyte cells with 1 μM dexamethasone, 0.5 μM isobutylmethylxanthine, 100 nM insulin, and 1 μM rosiglitazone in growth media (DMEM- high glucose with 10% FBS). After 2 days, the medium was replaced with growth medium containing 100 nM insulin and 1 μM rosiglitazone 2 more days, then for 2–3 days with growth medium alone to allow for differentiation. Mature 3T3-L1 adipocytes were starved for 4 h in DMEM-high glucose alone and treated with or without 500 ng/ml of recombinant bGH (ProSpec) for 24 h.

2.6. Quantitative PCR and RNAseq

Total RNA was isolated using a QIAzol™ Lysis Reagent (Qiagen). 1 μg of total RNA was reverse transcribed in 20 μl using the High Capacity cDNA Reverse Transcription (RT) Kit (Applied Biosystems). A portion (5 μl) of diluted (1/10) RT reaction was amplified with specific primers (300 nM each) in a 20 μl PCR reaction using an iQ™ SYBR® Green Supermix (BioRad). Analysis of gene expression was carried out in a BioRad MyiQ™ sequence detector with initial denaturation at 95 °C for 3 min, followed by 40 PCR cycles, each cycle consisting of 95 °C for 10 s and 58 °C for 1 min. SYBR green fluorescence emissions were monitored after each cycle. For each gene, mRNA expression was calculated relative to acidic ribosomal protein 36B4 (36B4) expression. Amplification of specific transcripts was confirmed by the melting-curve profiles (cooling the sample to 68 °C and heating slowly to 95 °C with measurement of fluorescence) at the end of each PCR. Primer sequences used included: Col1a1 forward 5′-GGGTCTAGACATGTTCAGC TTT-3′; Col1a1 reverse 5′-ACCCTTAGGCCATTGTGTATG-3′; Col3a1 forward 5′-CCCTTCTTCATCCCACTCTTATT-3′; Col3a1 reverse 5′-GAT CCTGAGTCACAGACACATATT-3′; Col4a1 forward 5′-TGGCTTCTGCTG CTCTTC-3′; Col4a1 reverse 5′-ACGCCATGACAGTCACATT-3′; Col5a1 forward 5′-GCCCTGCTGCTGTCTTC-3′; Col5a1 reverse 5′-GCACAGAA ACCTGTGGT-3′; Col6a1 forward 5′-GGCGACCCTGGGTATGA-3′; Col6a1 reverse 5′-TACCCGACTGGTCCAAGAT-3′.

For RNA-Seq studies, mRNA from frozen WAT samples (cohort 2) was isolated and sequenced at the Ohio University Genomics Facility