The effect of diet-induced obesity on extracellular matrix remodeling during skeletal muscle regeneration

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ABSTRACT

Diet-induced obesity has previously been shown to occur with the concomitant rise in the expression of proinflammatory cytokines and increases in collagen deposition. While it has been known that the regenerative process of skeletal muscle is altered in obese mice following an acute muscle injury, we sought to examine differences in the expression of various markers of extracellular matrix remodeling and repair. Our laboratory has previously reported an impaired inflammatory and protein synthetic signaling in these mice that may contribute negatively to the muscle regenerative process. To expand upon this previous investigation, tissues from these animals underwent further analysis to determine the extent of changes to the regenerative response within the extracellular matrix, including transcriptional changes in Collagen I, Collagen III, and Fibronectin. Here, we show that the expression of Collagen III:I is significantly increased at 3-days post-injury in obese injured animals compared to lean injured animals (p = 0.0338), and by 28-days the obese injured animals exhibit a significantly lower Collagen III:I than their lean injured counterparts (p = 0.0035). We demonstrate an impaired response to an acute muscle injury in obese mice when compared with lean counterparts. However, further studies are required to elucidate translational consequences of these changes, as well as to determine any causative mechanisms that may be driving this effect.

Introduction

Obesity is an increasingly prevalent disease in the United States with approximately 33% of Americans being classified as obese.1 This disease is associated with numerous pathophysiological conditions including diabetes mellitus and cardiovascular disease. The condition has also been associated with an increased risk for the development of certain cancers and overall mortality.2 However, obesity is more commonly associated with the substantial metabolic and endocrine disruption that are commonly driven by changes in insulin signaling and chronic inflammation.3 Interestingly, as shown by our laboratory and others, there are some indications that obesity may be associated with dysregulations in the wound healing process of skeletal muscle.3,4

Obesity is commonly associated with the increased expression of proinflammatory cytokines.3–7 Increased TGF-β expression has been shown to correspond with the reduced cross-sectional area of muscle fibers, diminished force transduction, and an accumulation of Collagen I in the extracellular matrix (ECM) of muscle.8 Collagen tends to accumulate in response to skeletal muscle damage, and increased expression of collagen markers are commonly associated with increased ECM remodeling.9 The ECM is essential for skeletal muscle growth, repair, contraction, and force transmission of skeletal muscle.10 It has been shown that the interaction between myoblasts, differentiated muscle fibers, and ECM components is of central importance for the repair of muscle tissue.11

Abbreviations: ECM, Extracellular Matrix; MMPs, Matrix Metalloproteinases; TIMPs, Tissue Inhibitors of Metalloproteinases; PBS, Phosphate Buffered Saline; TA, Tibialis Anterior; Ct, Cycle Threshold; MRF, Myogenic Regulatory Factor.

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When skeletal muscle is damaged, fiber regeneration is stimulated to occur.12 Two main components of the ECM that play a prominent role in remodeling and repair are fibronectin and collagen types I and III.13 An inflammatory response is stimulated as part of the repair process when the muscle is damaged. With this inflammation, the biosynthesis of Collagen I and III occurs.14 The matrix metalloproteinases (MMPs) are responsible for the degradation of collagens, and in skeletal muscle MMP2 and MMP9 are typically the most abundant.15 In turn, the MMPs are regulated by the aptly named tissue inhibitors of metalloproteinases (TIMPs). Both MMPs and TIMPs have been implicated in the dysregulation of muscle remodeling following acute injury.16 While the MMPs and TIMPs are regulators of ECM degradation, TGF-β acts to modulate the deposition of ECM proteins.17

It is known the extracellular matrix is a key component of the muscle regeneration process. However, little is known about the effect obesity has on ECM remodeling and repair following muscle damage. The purpose of this study was to examine the effects of obesity on the remodeling of the ECM, and to examine various gene expression markers as part of the remodeling process following acute muscle injury. Our laboratory has previously reported the regenerative process of skeletal muscle was altered following an episode of acute muscle damage between lean and obese mice.7 To build on this, we have expanded our initial investigation of the same animals, and hypothesized that the remodeling process of the extracellular matrix would be negatively altered following bupivacaine-induced muscle damage in obese animals.

**Methods**

**Animals and housing**

Forty-eight 3-week-old male C57BL/6 mice were purchased from Jackson Laboratories. Animals were housed in the University of Arkansas...
Central Laboratory Animal Facility. Animals were housed under a 12 h dark, 12 h light cycle, with ad libitum food and water. The overall study consisted of 2 separate animal experiments. Experiment 1 examined gene expression 3 days post-bupivacaine injection. Experiment 2 examined gene expression 28 days post-bupivacaine injection. For experiments 1 and 2, mice were randomly assigned to one of four groups: 1) lean uninjured (n = 5–9); 2) lean injured (n = 5); 3) obese uninjured (n = 4–6); 4) obese injured (n = 6–8). Mice were fed a lean diet (lemon; 10% kcals fat Research Diets, New Brunswick, New Jersey) or a high-fat diet (HFD; 60% kcals fat, Research Diets, New Brunswick, New Jersey) for 12 weeks. Independent data collected from these animals have been previously published. All procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (Protocol 12030).

Bupivacaine injection

At fifteen weeks of age, bupivacaine (Hospira, Lake Forest, IL) injections were performed as previously described. Mice were anesthetized with a subcutaneous injection of a cocktail containing ketamine hydrochloride (45 mg/kg body weight), xylazine (3 mg/kg body weight), and acepromazine (1 mg/kg body weight). Muscle damage was induced by injecting 0.03 ml of 0.75% bupivacaine (Marcaine) in the left and tibias were extracted to examine the early and late regenerative response within skeletal muscle. Mice were anesthetized with a subcutaneous injection of a cocktail containing ketamine hydrochloride (90 mg/kg body weight), xylazine (3 mg/kg body weight), and acepromazine (1 mg/kg body weight). The left TA was snap-frozen in liquid nitrogen and stored at −80 °C for gene expression analysis. The right TA was cut at the midbelly, mounted in optimum cutting temperature compound (OCT), and then dropped in liquid nitrogen-cooled isopentane. After the TA was dissected out, the tibia was removed and measured with a plastic caliper (VWR, Radnor, PA, USA). Tibia measurement—a stable indicator of total body size—was used to normalize muscle weights.

RNA isolation, cDNA synthesis, and quantitative RT-PCR

RNA isolation, cDNA synthesis, and Real-Time PCR were completed as previously described. RNA was extracted with Trizol reagent (Life Technologies, Grand Island, NY, USA). TA muscles were homogenized in Trizol. Total RNA was isolated, DNase treated, and concentration and purity were determined by fluorometry using the Qubit 2.0 (Life Technologies, Grand Island, NY, USA). TA muscles were homogenized in Trizol. Total RNA was isolated, DNase treated, and concentration and purity were determined by fluorometry using the Qubit 2.0 (Life Technologies, Grand Island, NY, USA). cDNA was reverse transcribed from 1 μg of total RNA using the Superscript Vilo cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA). Real-time PCR was performed, and results were analyzed using the StepOne Real-Time PCR system (Life Technologies, Applied Biosystems, Grand Island, NY). cDNA was amplified in a 25 μl reaction containing appropriate primer pairs and TaqMan Universal Master Mix (Applied Biosystems). Samples were incubated at 95 °C for 4 min, followed by 40 cycles of denaturation, annealing, and extension at 95 °C, 55 °C, and 72 °C respectively. TaqMan fluorescence was measured at the end of the extension step of each cycle. Fluorescence labeled probes for TGFB (FAM dye), Collagen-I (FAM dye), Collagen-III (FAM dye), and MMP-2 (FAM dye), MMP-9 (FAM dye), Fibronectin-I (FAM dye), TIMP-1 (FAM-dye), and 18S (VIC dye) was purchased from Applied Biosystems and quantified with TaqMan Universal Master Mix. Cycle threshold (Ct) was determined, and the ΔCt value was calculated as the difference between the Ct value and the 18s Ct value. Final quantification of gene expression was calculated using the ΔΔCt method 

**Sirisus red stain**

Sirisus Red Collagen staining was performed as described previously using a 0.1% Sirus Red solution (Spectrum, Gardenia, Cali, USA; cat. no. S1066) into 500 mL picric acid (EM Science, Hatfield, Pa.; cat. no. px1001-1) on tissues collected at 28-days. Frozen TA muscle was stained in picro-sirus red for 1 h. Slides were washed with acidic water and then dehydrated with 100% ethanol. The sections were then cleared with xylene and mounted. For Sirus red staining, approximately 3-distinct digital images from stained muscle sections (10 μm) from the mid-belly of the TA at a 20x objective magnification were taken and analyzed. The intensity of Sirus red was measured using Nikon Nis-Element BR software. Slides were quantified for percent area stained with Sirus Red per image and averaged within each animal for analysis.

**Statistical analysis**

All data were analyzed using GraphPad Prism Version 9.1. A two-way ANOVA was performed to analyze the main effects of Injury and Diet and to determine if there are any interactions between the dependent variables at 3-days and 28-days post-bupivacaine injection. When a significant interaction was detected, differences among the individual means were assessed with Fisher's LSD post-hoc analysis. Statistical significance was set at p < 0.05. All figures are presented as the mean ± the standard error of the mean.

**Results**

**Body and muscle wet weight**

Representative muscle and body weights for this research design have been published previously. We found a main effect of diet to increase body weight in the obese animals when compared to the lean mice at both the 3-day (~20%) and 28-day (~43%) time points (p < 0.05). In addition, we found a 16% and 36% reduction in the TA weight normalized to body weight in the obese mice at both the 3-day and 28-day time points post-bupivacaine injection, respectively. There was no difference in TA muscle mass to body weight ratio in the lean or obese group 3 days post-bupivacaine injection.

**Sirisus red staining, collagen, and fibronectin gene expression**

Sirisus Red staining was found to have a main effect of diet to increase the intensity of Sirus red staining (F(1, 15) = 8.837, p = 0.0095, Fig. 1). There was a main effect of injury to increase Fibronectin expression at both timepoints (3-day, F(1, 16) = 33.13, p < 0.0001; 28-day, F(1, 19) = 11.06, p = 0.0036; Fig. 2A and E). The same relationship was found with Collagen 1 expression (3-day, F(1, 16) = 33.91, p < 0.0001; 28-day, F(1, 18) = 4.578, p = 0.0463; Fig. 2B and F). However, an interaction effect was found with Collagen III expression at 3-days and 28-days post-bupivacaine injection (F(1, 16) = 5.064, p = 0.0389; Fig. 2C) with significant differences between Obese Injured and Lean Injured (p = 0.0172), Obese Uninjured and Obese Injured (p = 0.0001), Obese Uninjured and Obese Injured (p = 0.0299), and Lean Uninjured Obese Injured (p = 0.0003; Fig. 2C).

There was a main effect of diet to decrease Collagen III expression by 28 days (F(1, 17) = 5.008, p = 0.0389). Taking collagen expression as the ratio of Collagen III:1, an interaction effect was found for both the 3-Day (F(1, 16) = 20.30, p = 0.0004) and 28-Day (F(1, 13) = 5.975, p = 0.0295; Fig. 2D and H). Within the 3-Day timepoint, Lean Injured was significantly lower than Lean Uninjured (p = 0.0392), Obese Uninjured
was significantly lower than Lean Uninjured ($p = 0.0011$), Obese Uninjured was significantly lower than Obese Injured ($p = 0.001$), and Obese Injured was significantly higher than Lean Injured ($p = 0.0035$, Fig. 2H).

**MMP2, MMP9, TIMP1, TGF-β gene expression**

A main effect of Injury was found at the 3-Day timepoint to increase
Following acute skeletal muscle injury, the migration of inflammatory cells and muscle satellite cells along with the extracellular matrix to the site of injury is imperative to an efficient regenerative response, and it has been demonstrated previously that the integrity of the extracellular matrix is crucial in facilitating this response. While the deposition of Collagen I can be beneficial in wound repair, it’s spatial orientation and coherent cross-linking with other Collagen I fibrils contribute to a very high tensile strength and is associated with stiffer areas of scarring. Increased overall collagen deposition is associated with increased stiffness of surrounding skeletal muscle and has been shown to impair muscle progenitor cell proliferative activity. This is consistent with our previous findings where we demonstrated altered myogenic proliferative markers consistent with reduced regenerative ability. In contrast, Collagen III forms a looser network and is better suited for the maintenance of structural integrity and dispensability of the region.

There have been previous reports of increased collagen deposition with obesity and our results are consistent with that finding. By 3 days following acute muscle injury, lean and obese animals exhibited large increases in the expression of Collagen I, but obese injured animals exhibited greater expression of Collagen III when compared to all other groups. Increases in Collagen III gene expression are a common finding in models of multiple injuries. However, 28 days following acute injury, there is a marked reversal of the Collagen III:I ratio in all groups. While the lean injured animals have a significantly lower Collagen III:I ratio compared to the lean uninjured animals, the obese injured and obese uninjured animals were not statistically different from each other. This could be due in part to an impaired expression of Collagen III leading up to 28 days in the obese injured group, although there was no interaction effect nor main effects detected due to high variability in samples. There have been reports of increases in the Collagen III to I ratio during later timepoints of regeneration, and our data support these findings.

Remodeling of the ECM directly affects the ability of the skeletal muscle to regenerate following injury. The amount and type of collagen deposition, as well as the composition of the entire ECM play a crucial role in the ECM remodeling response. Our laboratory has previously reported altered skeletal muscle regenerative responses following an episode of acute muscle damage between lean and obese mice. While the primary goal of that study was to examine how obesity alters the regenerative capacity of muscle, this investigation directly builds upon that work by specifically exploring changes in the remodeling of the ECM of skeletal muscle. The initial hypothesis of these studies was that obese mice would exhibit reduced myogenic regulatory factor (MRF) expression, as well as reduced markers of inflammation and protein synthesis following acute muscle damage. To that end, we previously demonstrated a blunted inflammatory response via TNF-α in obese mice following an acute injury, with a concomitant effect of impaired IL-6 signaling and decreases in markers of protein synthetic signaling.

Building upon that work and expanding the scope of the initial investigation to include markers of skeletal muscle ECM remodeling, we hypothesized that these same animals would exhibit markers consistent with an impaired remodeling of the extracellular matrix. Here, we demonstrate an impaired regenerative response in the ECM following an acute skeletal muscle injury, possibly through impaired extracellular matrix remodeling. However, the current findings only extend to an altered transcriptional response, and further investigations will be required to ascertain the extent of translational outcomes beyond Sirus Red staining.

Following an acute skeletal muscle injury, the migration of inflammatory cells and muscle satellite cells along with the extracellular matrix to the site of injury is imperative to an efficient regenerative response, and it has been demonstrated previously that the integrity of the extracellular matrix is crucial in facilitating this response. While the deposition of Collagen I can be beneficial in wound repair, it’s spatial orientation and coherent cross-linking with other Collagen I fibrils contribute to a very high tensile strength and is associated with stiffer areas of scarring. Increased overall collagen deposition is associated with increased stiffness of surrounding skeletal muscle and has been shown to impair muscle progenitor cell proliferative activity. This is consistent with our previous findings where we demonstrated altered myogenic proliferative markers consistent with reduced regenerative ability. In contrast, Collagen III forms a looser network and is better suited for the maintenance of structural integrity and dispensability of the region.

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**Ethical approval statement**

All procedures are approved by the University of Arkansas Institutional Animal Care and Use Committee (Protocol 12030). All animals were housed at the University of Arkansas Central Laboratory Animal Facility according to the guidelines set forth by the Institutional Animal Care and Use Committee.

**Authors’ contributions**

TAW and NPG synthesized the experimental design. TAW, LAB, and RAP assisted with animal experiments. JWD, LAB, WAH, RAP, MER, and MAT collected and analyzed data. JWD, ERS, LAB, WAH, RAP, MER, MAT, NPG and TAW edited and revised the manuscript. TAW acquired funding.

**Conflict of interest**

The authors have no conflicts of interest to report.

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