NORTHWESTERN UNIVERSITY

Sexually dimorphic mechanisms of skeletal muscle adaptation

A DISSERTATION

SUBMITTED TO THE GRADUATE SCHOOL
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

for the degree

DOCTOR OF PHILOSOPHY

Walter S. and Lucienne Driskill Graduate Program in Life Sciences

By

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EVANSTON, ILLINOIS

June 2022

ABSTRACT

Skeletal muscle is a highly sexually dimorphic tissue, with males and females exhibiting differences in muscle size, gene transcription, and metabolism. This thesis describes two models wherein males and females responded to an intervention with the same physiological adaptation but through two distinct mechanisms. In the first model, mice of both sexes treated with glucocorticoid steroids once a week for a month had significantly increased skeletal muscle specific force and ATP concentration. In males, this response was the result of improvements in calcium handling and protein synthesis, while in females it was the result of changes in lipid metabolism. The second model examined patients with peripheral artery disease who were responsive or non-responsive to exercise intervention. Male responders were distinguished from non-responders by decreased expression of extracellular matrix and vasculature genes; female responders had increased expression of mitochondrial genes compared to non-responsive females. Lipid metabolism was a major feature of the skeletal muscle response of females in both models, suggesting that female skeletal muscle may leverage changes in oxidative metabolism to make beneficial adaptations. Overall, this work demonstrates the critical importance of investigating both sexes when determining the mechanism of action behind skeletal muscle adaptations to ergogenic stimuli.

ACKNOWLEDGEMENTS

I was profoundly lucky in my graduate career to have had many exceptional mentors. Dr. Danielle Maatouk took a chance on me when I first began graduate school, even though all of my relevant bench experience came from working with strawberries. I learned so much from Danielle, including but not limited to how to sex a mouse at embryonic day 12.5, use a confocal microscope, start a lab as a young investigator, and be a supportive mentor even when your second-year grad student has accidentally flooded the lab. After Danielle passed away and I needed a new advisor, Dr. Elizabeth McNally agreed to take me on. Beth taught me not only the important nitty-gritty work of our field—experimental design, grant and paper writing, delivering great talks to any audience—but also how to have confidence in myself as a scientist. I'm so grateful to her and the McNally lab for taking me in when I needed a new home and giving me the space to learn and grow. I'm also thankful to have had the opportunity to work with Drs. Joseph Bass, Grant Barish, and Debu Chakravarti, who knew me when I worked with Danielle and agreed to serve as members of my committee when I began my thesis work with Beth. Their expertise and insight steered me in avoiding many would-be disasters.

The Maatouk lab was my first home at Northwestern. I will always be grateful to Chris Futtner, who never failed to be generous with his time, knowledge, or a well-timed dad joke, and Alex García-Moreno, who is a brilliant scientist, friend, conference roommate, and co-author.

I learned so much from so many people in the McNally lab that it would be truly impossible to fit it all into this document. As a stab at it, though, I have to thank Mattia Quattrocelli, Dave Barefield, and Alexis Demonbreun for their expert guidance into the world of striated muscle biology; Eugene Wyatt, Anthony Gacita, Kate Fallon, Chi-Chi Ohiri, and James Warner for their insightful questions during lab meeting and sick moves on the dance floor; Ellis Kim, Andy Vo, and Tess Pottinger for taking me under their wing; Pat Page for his exquisite

histology and equally exquisite knowledge of obscure *Star Wars* trivia; Greg Aubert, Lisa Castillo, Dominic Fullenkamp, Tanner Monroe, Frank Li, Brian Leverson, Konrad Sawicki, Ashlee Long, Joe O'Brien, Julie Fischer, Alex Telenson, Daniel Selgrade, Alex Willis, Garima Tomar, Nora Ibrahim, Michelle Lee, and Aaron Zelikovich for letting me teach and be taught in equal measure; Tharrie Daniels, Olga Garcia, Kiara Meza, Trina West-Shields, Taurean Egan-Henderson, and Michelle Mohney for solving every logistical problem I ever brought them, no matter its size; Lorenzo Pesce, Sam Kearns, and Avery Robinson for their assistance in translating my bioinformatics desires into legible code; Nina Reiser, Lauren Vaught, Michele Hadhazy, Jodi Curtin, Eoin Code, Jason Kwon, Ivana Chychula, Ibrahim Tahtah, Matt Velez, Ryan Hsieh, Cory Holgren, Elena Bogdanovic, Claire Oosterbaan, and Gabby Cowen for always being willing to take five minutes to talk through a problem or grab a beer; and Meg Roy-Puckelwartz, whose office door was always open and was the first person to say, "Hey, maybe you should talk to Beth about joining the lab; I think you'd be a good fit."

Finally, I have to thank all of the friends and family who gave me generous support throughout my time in graduate school. Thank you to Ellie Kline, Teresa Daubenspeck, and Erin Millen for a decade of brunches, picnics, cocktail hours, and listening to me cry over my thesis. Thank you to Alanna Quick, Anahita Sotoohi, and Patricia Chilinski for two decades of weekend trips, movie marathons, *Star Trek* memes, and listening to me cry over my thesis. Thank you to my parents, who taught me to be organized, thoughtful, curious, and not to be ashamed of displays of emotion such as crying over one's thesis. Thank you to Mercè Correa, for showing me from the beginning that it was possible to be a scientist *and* a woman *and* a citizen of the world. And last but absolutely not least, thank you to Ben Thomson, who not only taught me how to do a Western blot but also ensured that I physically survived graduate school by nursing me through pneumonia, shingles, COVID, and a concussion. I probably could have done it without you and Eliza, but it would have been way less fun.

LIST OF COMMON ABBREVIATIONS

ABI: ankle-brachial index

AR: androgen receptor

CLI: critical limb ischemia

DHT: dihydrotestosterone

DMD: Duchenne muscular dystrophy

ECM: extracellular matrix

ER: estrogen receptor

GR: glucocorticoid receptor

GRE: glucocorticoid response element

LGMD: limb girdle muscular dystrophy

MR: mineralocorticoid receptor

PAD: peripheral artery disease

PRE: prednisone-responsive enhancer

WGA: wheat germ agglutinin

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Chapter 1. Introduction

(modified from Quattrocelli et al., Journal of Neuromuscular Diseases, 2021)

1.1 Overview

Glucocorticoid steroids are powerful hormones that normally act to regulate blood pressure, volume status, and metabolism. An estimated one percent of the US population uses glucocorticoid steroids as anti-inflammatory and immunosuppressive aids for acute and chronic disorders including autoimmune disease, muscle disease, and inflammation associated with cancer treatment. The therapeutic benefits of glucocorticoids are hampered by extensive side effects that include diabetes mellitus and muscle weakness. Despite this, and the known clinical complications of muscle atrophy, glucocorticoid steroids are still used because there is no alternative. In this introduction, I will describe how skeletal muscle adapts to certain stimuli, including exercise and atrophic conditions. I will also discuss glucocorticoid steroids, how they modulate target gene transcription, and how variation in dosing regimen alters their effects in skeletal muscle. Due to the highly sexually dimorphic nature of skeletal muscle, I will also discuss how biological sex influences skeletal muscle function as well as its response to glucocorticoids.

This chapter contains excerpts from a review published in 2021, “Mechanisms and clinical applications of glucocorticoid steroids in muscular dystrophy” (Quattrocelli et al. 2021). For the published review, Dr. Aaron Zelikovich, Julie Fischer, and myself contributed to design of the review and wrote individual sections. Dr. Mattia Quattrocelli designed the review and wrote multiple sections; he and Dr. Elizabeth McNally edited the manuscript. For this chapter, I have adapted my section of the review discussing glucocorticoid steroid action and regulation of gene expression, as well as the figure appearing below as Figure 1.1. The remainder of the chapter was written by myself for this dissertation.

1.2 Introduction

1.2.1 Skeletal muscle, force production, and adaptation to exercise

Skeletal muscle makes up approximately 40% of total body weight in humans and is crucial for the maintenance of body posture, movement, and the production of force (Frontera and Ochala 2015). A structurally complex tissue, skeletal muscle is composed of a mix of cell types including myofibers, satellite cells, neurons, and endothelial and smooth muscle cells. Myofibers and satellite cells are organized into fascicles, which are bundled together by connective tissue. Action potentials, oxygen, and nutrients are delivered to muscle cells by nerves and capillaries. Satellite cells are the regenerative stem cells of skeletal muscle; they fuse to form new multinucleated myofibers during growth, repair, and regeneration (Buckingham 2006, Yin et al. 2013). A human myofiber is generally 1cm long and 100μm in diameter and contains what has been approximated as thousands of myofibrils made up of actin and myosin in repeated units called sarcomeres (Frontera and Ochala 2015). The sarcomere is the basic contractile unit of the myofiber and its mechanism of function has been well-characterized. In response to an action potential, depolarization of the cell membrane leads to Ca^{2+} release from where it is stored in the sarcoplasmic reticulum. Ca^{2+} then binds troponin, exposing an active site on actin and allowing a myosin head to bind and form a cross-bridge. As the myosin head bends, it pulls the actin filament towards the center of the sarcomere, until the binding of a new ATP molecule causes the myosin head to detach and release the cross-bridge. This process of contraction and relaxation is accordingly dependent on both ATP and Ca^{2+}, with modulation of either affecting both speed and maximum force produced (Dutka and Lamb 2004, Calderon et al. 2014). Due to the parallel organization of sarcomeres and myofibrils, contraction is repeated and amplified across the entirety of the myofiber. A single contractile response is a twitch, many of which fuse to form a tetanus during repetitive high-frequency stimulation.

Different muscles throughout the body perform a wide array of functions and are specialized for their particular roles by metabolic state, contractile speed, and calcium handling (Bottinelli and Reggiani 2000, Spangenburg and Booth 2003). Myofibers are generally classified by their predominant myosin heavy chain (MHC) isoform, which dictates the rate of cross-bridge cycling. The major MHC isoforms in adults are type I (slow), IIa (intermediate), IIx (fast), and IIb (fast), although the latter is not expressed in humans (Smerdu et al. 1994). Slow MHC isoforms generally consume ATP less rapidly than fast isoforms and accordingly have different metabolic needs; this is often generalized as a reliance on oxidative metabolism by slow fibers and glycolytic metabolism by fast fibers, although there is a large overlap in metabolic enzyme activity across the major fiber types (Hintz et al. 1984). The metabolic requirements of different fiber types are also dependent on the type of sarcoplasmic reticulum Ca^{2+} (SERCA) pump that is expressed. SERCA1, expressed in fast type II fibers, is twice as fast and present at a greater density than SERCA2, which is expressed in slow type I fibers, and may accordingly have a higher demand for ATP (Everts et al. 1989, Wu and Lytton 1993, Periasamy and Kalyanasundaram 2007). Most muscles are composed of a mix of fiber types.

The metabolic requirements of skeletal muscle change with exercise intensity. The ATP required for contraction during submaximal exercise is mostly provided by oxidative metabolism of lipids and carbohydrates (Spriet and Watt 2003). Anaerobic ATP regeneration pathways such as phosphocreatine and lactate only become major contributors at very high exercise intensity (Sahlin 1986, Westerblad et al. 2010). As exercise continues, it leads to decreased contractility and eventual fatigue. The rate at which fatigue progresses depends heavily on the metabolic capacity of the muscle being exercised; due to their high capacity for oxidative metabolism, slow type I fibers are more fatigue-resistant than fast type II fibers (Scott et al. 2001). With training, muscle adapts to exercise. Broadly, exercise can be divided into two major types: resistance training, which consists of few, high-force contractions, and endurance training, consisting of

more, lower-force contractions. Resistance training leads to increased muscle mass and strength (Atha 1981, Schoenfeld et al. 2017, Lixandrao et al. 2018) with relatively few metabolic changes (Chapman et al. 2020). In contrast, the muscle adaptation to endurance exercise is predominantly metabolic in nature. Myofibers exhibit increased mitochondrial biogenesis and oxidative capacity (Holloszy 1967, Gollnick et al. 1973, Holloszy and Booth 1976) and, under some training conditions, undergo a fiber type switch from glycolytic to more oxidative (Andersen and Henriksson 1977, Fitts 2003, Rockl et al. 2007). Endurance training is also angiogenic, improving oxygen delivery to match the increased demands of muscle mitochondria (Hudlicka et al. 1992).

Numerous mediators have been proposed as regulators of skeletal muscle adaptation to exercise. One of the most comprehensively studied is the transcription factor PGC1α, expression of which is upregulated by endurance exercise (Baar et al. 2002, Norrbom et al. 2004, Koves et al. 2005). A master regulator of oxidative metabolism, PGC1α upregulation increases mitochondrial biogenesis, fatty acid oxidation, and angiogenesis (Lin et al. 2002, Gerhart-Hines et al. 2007, Wende et al. 2007, Arany et al. 2008). PGC1α may also mediate the fiber type switch between fast and slow that has been observed in response to endurance exercise; transgenic expression of PGC1α in mice leads to a higher percentage of type I and IIA fibers (Lin et al. 2002), possibly via MEF2 or PPARβ/δ (Wu et al. 2001, Luquet et al. 2003, Wang et al. 2004, Schuler et al. 2006). However, mice with whole-body deletion of PGC1α still undergo some skeletal muscle adaptation after exercise training, suggesting that other factors also mediate this process (Leick et al. 2008). Other transcription factors whose DNA binding activity is altered by endurance exercise include MEF2 (Yu et al. 2001), histone deacetylases (McGee et al. 2009), and NRFs (Baar et al. 2002, Wright et al. 2007).

1.2.2 Skeletal muscle sexual dimorphism

Skeletal muscle is one the most sexually dimorphic tissues in the body, with approximately 3,000 genes differentially expressed between men and women (Welle et al. 2008, Oliva et al. 2020). Men are broadly characterized as having greater muscle size and strength, while women have greater resistance to fatigue (Miller et al. 1993, Lindle et al. 1997, Ivey et al. 2000, Welle et al. 2008, Wust et al. 2008, Ansdell et al. 2019). The most well-described contributors to this paradigm are sex hormones, which have a direct effect on muscle size (Deane et al. 2013) and contractility (Moran et al. 2006, Moran et al. 2007). However, males and females also exhibit differences in fiber type composition and substrate utilization that have yet to be linked to direct actions by sex steroids (Tarnopolsky et al. 1990, Friedlander et al. 1998, Horton et al. 1998, Tarnopolsky 2000, Carter et al. 2001, Riddell et al. 2003, Knechtle et al. 2004, Roepstorff et al. 2005, Roepstorff et al. 2006, Haizlip et al. 2015). In addition to the myofibers themselves, muscle performance—whether that be force output or resistance to fatigue—is the result of concordant action of multiple cell types. Male satellite cells exhibit greater proliferative capacity than female satellite cells in mice and poultry (Neal et al. 2012, Song et al. 2013), which may contribute to hypertrophy and force generation. Vascular function can influence skeletal muscle response to aerobic exercise (Dvoretskiy et al. 2020) and functional metrics such as oxygen uptake kinetics and vasodilatory response to exercise as well as capillary density have been observed as significantly greater in women versus men (Roepstorff et al. 2006, Parker et al. 2007, Beltrame et al. 2017). Some of this may be attributed to sex steroid action, as estrogen has demonstrated angiogenic activity in models of murine hindlimb ischemia (Ruifrok et al. 2009, Matsubara et al. 2012). Inflammatory cytokines, the production of which differs between the sexes (Klein and Flanagan 2016), have been shown to have a dose-dependent effect on skeletal muscle differentiation, homeostasis, and repair (Londhe and Guttridge 2015). The degree to which years or decades of intense athletic activity

influences this paradigm is also unclear. Most of the work discussed in this section was performed in muscle collected from healthy volunteers who were either untrained or acutely trained for a few days or weeks prior to biopsy. Investigations of highly-trained amateur or professional athletes have found that differences in force production between male and female muscle were attributable to size differences (Bishop et al. 1987, Gore 2007), perhaps because female muscle responds to certain types of exercise with changes in fiber type (Esbjornsson Liljedahl et al. 1996). Further work is needed to fully understand the adaptations that male and female skeletal muscle undergo in response to many years of serious exercise.

1.2.3 Glucocorticoid steroid molecules

The adrenal cortex produces two classes of corticosteroids that are responsible for modulating an organism's response to stress: mineralocorticoids, which increase water retention to maintain blood pressure, and glucocorticoids, which suppress the immune system and increase circulating glucose to provide nutrients for the brain. Endogenous glucocorticoids (cortisol in humans, corticosterone in mice) are signaling molecules that respond to both stress and circadian rhythm (Spencer et al. 2018). Circulating glucocorticoid levels peak at the beginning of the daily active phase. Through a feedback loop that can be hijacked by stress, adrenocorticotropic hormone (ACTH) stimulates the adrenal gland to produce cortisol (Figure 1.1). Secreted cortisol is then circulated throughout the body, binding to the glucocorticoid receptor (GR) in target tissues as well as suppressing the production of more ACTH by the pituitary. Synthetic glucocorticoids are classified as short- or long-acting dependent upon the duration of ACTH suppression they elicit (Axelrod 1976), although most have a serum half-life of approximately 1-3 hours (Disanto and Desante 1975, Mollmann et al. 1995, Queckenberg et al. 2011). The most commonly prescribed synthetic glucocorticoids—dexamethasone, deflazacort, and prednisone—are used to treat many acute and chronic disorders, including myopathies like

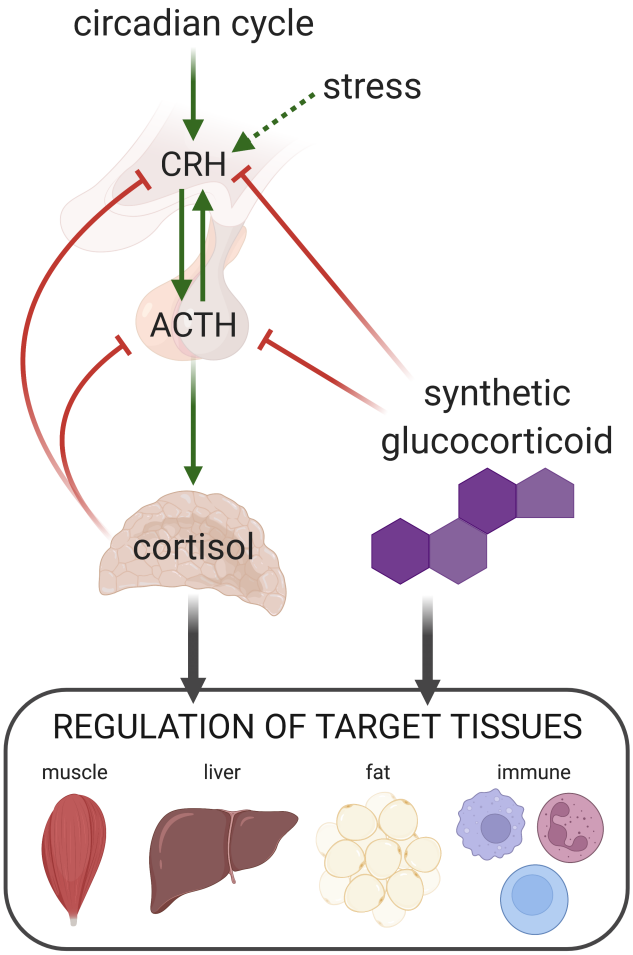

Figure 1.1 Glucocorticoids act on the hypothalamic-pituitary-adrenal axis. During conditions of stress or circadian stimuli, corticotropin releasing hormone (CRH) stimulates adrenocorticotropic hormone (ACTH) production from the anterior pituitary which in turn triggers the adrenal gland to release cortisol. Cortisol acts in a negative feedback loop, repressing ACTH and CRH, while also regulating gene expression in various target tissues. Synthetic glucocorticoids act similarly. Adapted from Quattrocelli et al. (2021).

Duchenne muscular dystrophy (DMD), but their efficacy is hampered by extensive side effects (Desmet and De Bosscher 2017). Novel synthetic glucocorticoid development is now geared towards reducing these side effects. CpdX is a glucocorticoid receptor agonist that is anti-inflammatory (Hua et al. 2019) without inducing skin atrophy or growth inhibition (Hua et al. 2019), but it has not been evaluated for efficacy in treating myopathy. The most promising is the novel glucocorticoid derivative vamorolone, which was found to increase specific force in the

mdx mouse model of DMD (Heier et al. 2013) and improve membrane stability in boys with DMD (Conklin et al. 2018). Because relatively small alterations to glucocorticoid molecular structure can result in large changes in biological activity (Bromberg and Carter 2004), development of novel synthetic glucocorticoids is an ongoing process that aims to either reduce side effects or improve certain aspects of glucocorticoid function, such as activation of GR.

1.2.4 Glucocorticoid receptor regulation and modulation of target gene expression

Glucocorticoids are signaling molecules that coordinate the body's response to stress by propagating tissue-specific transcriptional programs. GR can precisely control target gene activation or repression due to its own extensive regulatory features. There are four domains present in all isoforms of GR: a C-terminal steroid-binding domain, a short DNA-binding domain, a flexible hinge, and an N-terminal transactivation domain that binds co-factors and transcriptional machinery (Giguere et al. 1986, Kumar and Thompson 2005). The two most predominant isoforms, GRα and GRβ, are splice variants that differ only in the length of their steroid-binding domain. Due to the truncation of GRβ's steroid-binding domain, it cannot bind glucocorticoids, enter the nucleus, or modulate transcription on its own. However, GRβ can dimerize with GRα and serve as its dominant-negative inhibitor (Oakley et al. 1996, Oakley et al. 1999, Hinds et al. 2010). Although overactive GRβ can result in clinical glucocorticoid resistance (Rodriguez et al. 2016), it can also be protective against steroid-induced atrophy (Hinds et al. 2016). Other isoforms are produced from the GRα transcript via alternative translation initiation sites, resulting in transactivation domains of varying size (Lu and Cidlowski 2005). These isoforms have similar ligand and DNA affinity to GRα but regulate different genes (Lu et al. 2007), likely because their more truncated transactivation domains cannot bind as well to co-factors and transcriptional machinery. Polymorphisms in *NR3C1*, the gene that encodes GR, have been associated with body mass index (Huizenga et al. 1998, Cellini et al. 2010), obesity

(Lin et al. 2003), coronary artery disease (Lin et al. 2003), and grip strength (Muller et al. 2020). Interestingly, some of these variants have sex-specific associations (van Raalte et al. 2012, Ash et al. 2016). GR is further regulated by post-translational modifications such as phosphorylation, ubiquitinylation, and SUMOylation that can alter its ligand- and DNA-binding affinity (Wallace and Cidlowski 2001, Le Drean et al. 2002, Blind and Garabedian 2008, Chen et al. 2008, Galliher-Beckley and Cidlowski 2009, Galliher-Beckley et al. 2011).

In the absence of its ligand, GR is sequestered in the cytoplasm in a chaperone-bound complex with Hsp70, which assists in folding of the nascent GR protein, and Hsp90, which helps open the steroid-binding pocket (Grad and Picard 2007). Following ligand binding, GRα is transported into the nucleus where it recognizes glucocorticoid response elements (GREs) that it binds either by itself or in concert with other transcription factors, termed co-factors. As a member of the nuclear receptor superfamily, GR interacts with a diverse group of coactivators and co-repressors that enhance or inhibit nuclear receptor-mediated transcription (Lonard and O'Malley 2005, Lonard and O'Malley 2007). In addition to these nearly ubiquitous co-factors, GR can interact with lineage-specific co-factors to accomplish tissue-specific gene regulation (Lim et al. 2015). Few skeletal muscle-specific GR co-factors have been identified; the most well-studied is FOXO1, which is required for steroid-induced atrophy (Waddell et al. 2008). Although the classical paradigm of GR binding is that it binds DNA either as a monomer in conjunction with co-factor(s) or as a homodimer, recent work has suggested that the oligomeric state of GR might be more complicated. An imaging-based study found that GR formed a tetramer—a dimer of dimers—upon binding DNA (Presman et al. 2016). As of yet, it is unclear how frequently GR binds DNA in this tetrameric form and whether there are specific transcriptional processes that are mediated by different oligomeric forms of GR. Interestingly, GR binding is not a strong predictor of GR-dependent gene regulation, although the likelihood of an occupied GRE being responsible for transcriptional regulation of a gene increases the closer that the GRE is to the

gene's transcriptional start site (Thormann et al. 2018, Thormann et al. 2019). GR binding sites can also work concordantly, with clusters of GREs mediating GR-dependent transcription (Thormann et al. 2018). GR binding can further control gene expression by modulating the epigenetic landscape around its target genes (Wu et al. 2015, Guo et al. 2017, Clark et al. 2019). This epigenetic remodeling is likely a crucial component of GR-induced gene regulation, although there have been few studies investigating how loss of chromatin-modifying co-factors impacts expression of GR target genes.

Although the majority of glucocorticoid signaling occurs through direct modulation of gene transcription, rapid non-genomic effects have also been observed and remain poorly understood. Until very recently, it was not clear that these non-genomic effects occurred in skeletal muscle or, if they did, what their mechanism of action might be. It was recently shown that membrane-bound GR can rapidly activate p38-MAPK through Ras/Raf signaling, leading to fiber type-specific increased force production (Perez et al. 2013, Boncompagni et al. 2015). Skeletal muscle expresses membrane-bound GR throughout development, suggesting that non-genomic GR signaling might play a role in myogenesis, but the specifics of such a mechanism remain unclear.

1.2.5 Glucocorticoid receptor interactions with other nuclear receptors

Nuclear receptors can share ligands, co-factors, or DNA response elements depending on the receptors in question. There are two receptors that interact with GR meaningfully in the context of glucocorticoid signaling and skeletal muscle. These are described below.

Mineralocorticoid receptor. The other major corticosteroid receptor is the mineralocorticoid receptor (MR). MR and GR are structurally similar (Arriza et al. 1987) and can form heterodimers (Trapp et al. 1994, Liu et al. 1995). Endogenous corticosteroids bind MR with 5- to 10-fold higher affinity than GR (Reul et al. 2000), so it is likely that basal circulating cortisol

binds MR preferentially and GR is not occupied until ligand levels rise due to stressful stimuli or the circadian cycle (Reul and de Kloet 1985, Spencer et al. 1990). Synthetic glucocorticoids such as deflazacort (Heier et al. 2019), prednisone (Grossmann et al. 2004), and dexamethasone (Rupprecht et al. 1993) have less affinity for MR as a result of subtle differences in their chemical structure (Brookes et al. 2012). The role of MR in cardiac and skeletal muscle is an area of ongoing interest. A recent investigation of cardiomyocyte-specific deletion of GR and MR found that mice lacking both were resistant to the cardiac remodeling and left ventricular dysfunction characteristic of loss of GR in the heart (Oakley et al. 2019). This suggests that, despite their similarities, GR and MR signaling are not compensatory mechanisms and inhibition of MR can provide unique benefit to striated muscle. Application of a combinatorial angiotensin-converting enzyme inhibitor/MR antagonist treatment to *mdx* mice heterozygous for utrophin resulted in improved heart function and skeletal muscle force and membrane stability (Rafael-Fortney et al. 2011, Chadwick et al. 2017). *mdx* mice treated with vamorolone, an MR antagonist and GR agonist, had improved cardiac function (Heier et al. 2019). Overall, it appears that MR inhibition can improve the phenotype of DMD-related cardiomyopathy by preventing aldosterone-mediated fibrosis and dysfunction in the heart. However, how MR and GR might interact in the context of glucocorticoid treatment in skeletal muscle is not fully understood.

Androgen receptor. Like MR, the androgen receptor (AR) shares some homology with GR and the two proteins can form heterodimers (Chen et al. 1997). They have substantial overlap in their agonist-dependent interactomes, indicating that they share many regulatory features (Lempiainen et al. 2017). AR is a crucial regulator of skeletal muscle development; its ligand, dihydrotestosterone (DHT), is known to promote skeletal muscle hypertrophy (Deane et al. 2013) and is accordingly banned as an anabolic agent by the World Anti-Doping Agency. Androgen deficiency in men is associated with loss of skeletal muscle mass (Basaria et al. 2006,

Maggio and Basaria 2009); castrated male mice begin to exhibit skeletal muscle atrophy as quickly as seven days after androgen loss (Serra et al. 2013). Reciprocally, treatment with testosterone can improve regeneration of skeletal muscle following cardiotoxin injury (Serra et al. 2013). In addition to hypertrophy, DHT also regulates the activity of enzymes in the electron transport chain (Georgieva et al. 2017). Although AR and GR are known to interact in non-muscle tissues and AR plays a crucial role in skeletal muscle development and function, how interactions between AR and GR might influence the skeletal muscle response to glucocorticoids remains poorly understood.

1.2.6 Sexual dimorphism and glucocorticoids

Glucocorticoid responsiveness is a sexually dimorphic biological process. GR interacts with the sex steroid hormone receptors either through direct heterodimerization or shared co-factors (Chen et al. 1997, Bolt et al. 2013). In mice and rats, females exhibit higher basal and stress-induced corticosterone levels (Babb et al. 2013, Spencer and Deak 2017). Adult women have higher salivary cortisol (Larsson et al. 2009) and lower GR expression in leukocytes than men (Lu et al. 2017), which may be an adaptation to higher circulating levels of ligand. Males and females respond very differently to synthetic glucocorticoid treatment whether delivered systemically (Duma et al. 2010) or topically (Baida et al. 2020). In the liver, dexamethasone modulates its anti-inflammatory effect through sex-specific signaling pathways; as a result, female mice require a much higher dose of dexamethasone to survive a lipopolysaccharide challenge than males (Duma et al. 2010). Although both sexes are equally sensitive to the anti-inflammatory effect of the topical glucocorticoid fluocinolone acetonide, females are more sensitive to glucocorticoid-induced atrophy (Baida et al. 2020).

Although there is compelling evidence that sexual dimorphism plays a crucial role in both glucocorticoid signaling and striated muscle function, there has been little investigation of how

the two intersect. Cardiomyocyte-specific deletion of GR leads to heart failure and death in both males and females, but females are more resistant and live almost twice as long as males (Cruz-Topete et al. 2019). Pregnant *mdx* female mice have improved membrane stability that is attributable to their increased levels of circulating corticosterone (Shimizu-Motohashi et al. 2015). Future investigations of myopathies affecting patients of both sexes should take into account the diverse signaling environments of male versus female skeletal muscle when evaluating the impact of glucocorticoids.

1.2.7 Glucocorticoid-induced atrophy in skeletal muscle

In response to a stress stimulus, endogenous glucocorticoids trigger a coordinated whole-body response designed to release useful metabolites such as amino acids, fatty acids, and glucose into circulation for use by the central nervous system (Figure 1.2) (Olefsky and Kimmerling 1976, Christiansen et al. 2007, Macfarlane et al. 2008). Hepatic gluconeogenesis, skeletal muscle proteolysis, and adipose tissue lipolysis are all upregulated in response to increased glucocorticoids (Sistare and Haynes 1985, Smith et al. 1990, Xu et al. 2009, Wang et al. 2016) while pancreatic β-cell activity is suppressed to prevent peripheral tissues from diverting circulating glucose (Delaunay et al. 1997). When exposed to high or chronic doses of glucocorticoids, skeletal muscle catabolism leads to atrophy.

Glucocorticoid-induced atrophy in skeletal muscle is a relatively swift process; a single, very large bolus of methylprednisolone can stimulate muscle atrophy in the rat within 24 hours (Wu et al. 2011), while three months of low daily glucocorticoids is sufficient to increase the likelihood of muscle wasting in human patients (Zikan et al. 2012). Glucocorticoids induce atrophy in skeletal muscle by increasing protein degradation while concordantly repressing protein synthesis. Upon glucocorticoid stimulus, GR coordinates with PPARβ/δ to upregulate FOXO1 via a GRE in the *Foxo1* promoter (Qin et al. 2014). FOXO1 in turn activates the muscle-

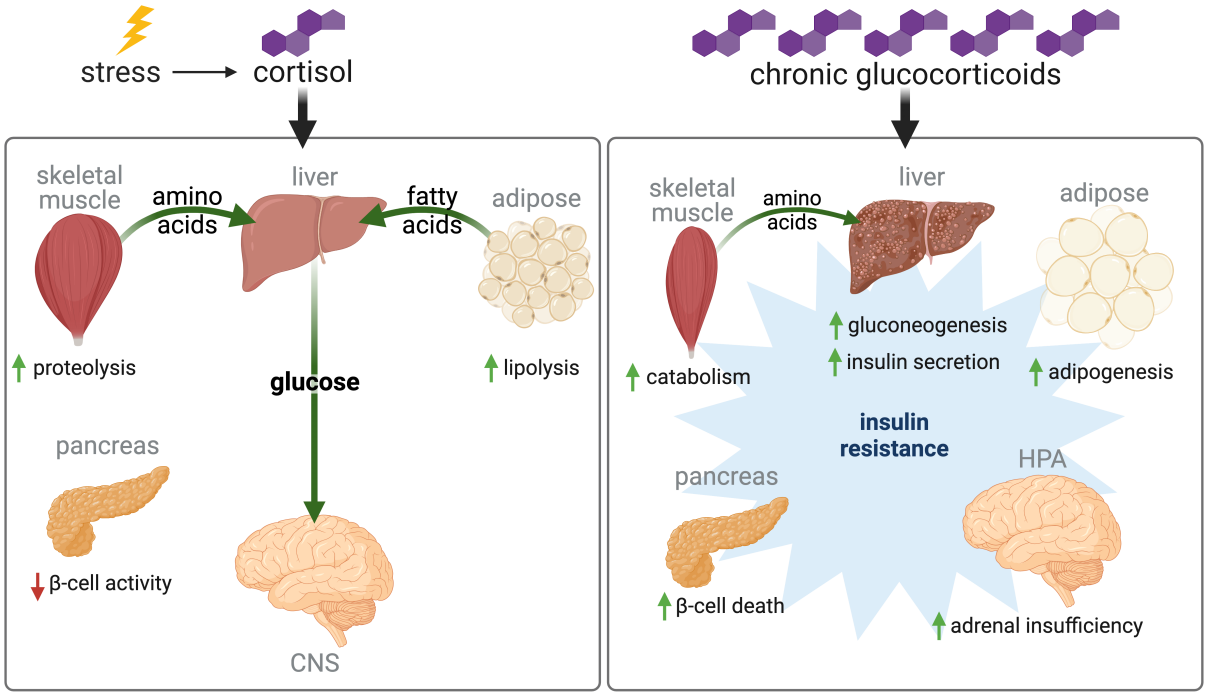

Figure 1.2 Glucocorticoid excess through chronic dosing has adverse effects throughout the body. In response to a stress stimulus, cortisol acts to release metabolites into circulation for use by the central nervous system. Skeletal muscle proteolysis and adipose tissue lipolysis supply metabolites to the liver for gluconeogenesis, while reduced β-cell activity prevents peripheral tissues from siphoning off circulating glucose. Under chronic exposure to glucocorticoids, skeletal muscle proteolysis leads to atrophy and increased gluconeogenesis and insulin secretion from the liver, along with increased β-cell death, results in insulin resistance. Adipose tissue increases adipogenesis, expanding fat depots. Dysfunction of the hypothalamic-pituitary-adrenal axis leads to adrenal insufficiency.

specific E3 ubiquitin ligases MuRF-1 and atrogin-1, also known as the atrogenes, which initiate proteasomal degradation and eventually lead to atrophy (Castillero et al. 2013, Sato et al. 2017). GR also upregulates expression of the gene encoding myostatin (Ma et al. 2001), a negative regulator of myogenic differentiation that can inhibit the AKT/mTOR pathway (Amirouche et al. 2009). Another mTOR inhibitor, the transcription factor REDD1, is also upregulated by glucocorticoid stimulus (Wang et al. 2006). Repression of mTOR by glucocorticoids reduces the activity of its phosphorylation target, p70S6K, a ribosomal protein whose phosphorylation is required for protein synthesis (Schakman et al. 2008). Thus, under chronic or high doses of

glucocorticoids, atrogenes increase proteasomal degradation while mTOR repression reduces protein synthesis, resulting in skeletal muscle atrophy. It is unclear the extent to which sex influences this process, as the topic remains poorly investigated. One group found that testosterone mitigates glucocorticoid-induced atrophy in rats by preventing atrogin-1 upregulation (Zhao et al. 2008). Others have observed that hindlimb unloading, an atrophy model that also upregulates MuRF-1 and atrogin-1 (Bodine et al. 2001), results in more profound loss of mass in female rats than males, possibly via FOXO3 (Sandri et al. 2004, Yoshihara et al. 2019).

Skeletal muscle is not the only organ that responds to chronic glucocorticoids maladaptively. Metabolic changes in liver, pancreas, and adipose can lead to diabetes mellitus, non-alcoholic fatty liver disease, and hepatic steatosis (Figure 1.2) (Woods et al. 2015, Marino et al. 2016, Suh and Park 2017). The liver responds to increased glucocorticoids by continuing to upregulate gluconeogenesis, leading to hyperglycemia and insulin resistance (Vegiopoulos and Herzig 2007). Although insulin production is initially suppressed by acute exposure to glucocorticoids, insulin resistance after chronic glucocorticoids leads to compensatory hyperinsulinemia (Delaunay et al. 1997). In adipose, chronic glucocorticoid treatment suppresses lipolysis and increases adipogenesis (Xu et al. 2009, Lee et al. 2014). It is important to think of these changes as occurring not in isolation but rather in an interconnected network. Skeletal muscle, liver, and adipose provide the majority of the body's fuel storage and they communicate amongst one another as well as with the central nervous system to respond to the body's metabolic needs (Viscarra and Ortiz 2013, Hong et al. 2014, Shimizu et al. 2015). Mice with skeletal muscle-specific GR knockout exhibit significant changes not only in skeletal muscle but also metabolic gene expression in liver and adipose (Shimizu et al. 2015). Thus, the metabolic perturbations that occur in response to chronic glucocorticoids are the result of both tissue-specific changes and alterations in the muscle-liver-adipose signaling axis.

1.2.8 Acute or low doses of glucocorticoids can improve muscle function

Glucocorticoids are a particularly striking example of the influence of dose on drug responsiveness. Although high or chronic treatment results in profound skeletal muscle atrophy, glucocorticoids in low doses are thought of as performance-enhancing and are banned by anti-doping agencies. Investigations into the effects of lower doses and short-term exposure to glucocorticoids have reported inconsistent findings, likely due to variation in dose, time frame, and metrics used to evaluate muscle performance (Table 1.1).

Single dose. Only a handful of studies have investigated the impact of a single dose of glucocorticoids on exercise performance in humans, all of which were performed exclusively in men and the majority of which did not observe any change in performance. Two investigations by the same group administered 4mg dexamethasone four hours prior to high intensity interval training and evaluated speed and aerobic capacity as VO_2 max (Petrides et al. 1994, Petrides et al. 1997). This group initially distinguished their participants as suppressors or non-suppressors based on ACTH levels following dexamethasone administration and they observed no change in exercise performance between suppressors and non-suppressors regardless of treatment with placebo or dexamethasone. Another group administered 20mg prednisolone two hours prior to a cycling test at 80-85% VO_2 max and observed no difference in distance to exhaustion between placebo and prednisolone groups (Arlettaz et al. 2006). This same group also reported no change in distance to exhaustion between vehicle and a group treated with 30mg prednisolone two hours before a cycling test at 70-75% VO_2 max, although they did observe in a parallel investigation that carbohydrate oxidation significantly decreased and fat oxidation significantly increased in men treated with 20mg prednisolone two hours prior to a cycle test performed at 60% VO_2 max (Arlettaz et al. 2008, Arlettaz et al. 2008). A single 0.25mg dose of the synthetic ACTH Synacthen® was sufficient to elevate plasma cortisol but had no effect on a 20-km cycling time trial (Baume et al. 2008). A fourth group administered 20mg prednisolone

Table 1.1 Previous studies investigating the impact of glucocorticoid dosing regimen on skeletal muscle performance

Study	Drug	Dose*	Duration	Sex	Evaluation	
					Metric	Result
Petrides et al. (1994)	DEX	4mg	Single dose	Male	HIIT, treadmill to 90% VO_2 max	No change
Petrides et al. (1997)	DEX	4mg	Single dose	Male	HIIT, treadmill to 90% VO_2 max	No change
Arlettaz et al. (2006)	PREDL	20mg	Single dose	Male	Cycling to exhaustion, 80-85% VO_2 max	No change
Arlettaz et al. (2008)	PREDL	20mg	Single dose	Male	Cycling to exhaustion, 70-75% VO_2 max	No change
Baume et al. (2008)	SYN	0.25mg i.m.	Single dose	Male	Cycling, 20-km time trial	No change
Tacey et al. (2019)	PREDL	20mg	Single dose	Male	HIIT, cycling at 90-95% peak heart rate	5% decrease in total work
Marquet et al. (1999)	DEX	0.5mg 1.5 mg	Twice daily, 5 days/week for 3 weeks	Male	Cycling to exhaustion	No change
Nordsborg et al. (2008)	DEX	2mg	Twice daily for 5 days	Male	One-legged kicking	Time to exhaustion increased
Casuso et al. (2014)	DEX	2mg	Twice daily for 5 days	Male	One-legged kicking	Time to exhaustion increased
					20min shuttle run	Distance run increased
Arlettaz et al. (2007)	PREDL	60mg	Daily for 7 days	Male	Cycling to exhaustion, 70-75% VO_2 max	Time to exhaustion increased
Collomp et al. (2008)	PREDL	60mg	Daily for 7 days	Male	Cycling to exhaustion, 70-75% VO_2 max	Time to exhaustion increased
Le Panse et al. (2009)	PRED	50mg	Daily for 7 days	Female	Cycling to exhaustion, 70-75% VO_2 max	Time to exhaustion increased
Zorgati et al. (2014)	PRED	60mg	Daily for 7 days	Male	Hopped on dominant leg to exhaustion	Peak force increased

DEX = dexamethasone
HIIT = high intensity interval training
PREDL = prednisolone
PRED = prednisone
SYN = Synacthen®
i.m. = intramuscular
*unless otherwise specified, drugs were orally administered

twelve hours prior to high intensity internal training at 90-95% VO_2 max and found that men given prednisolone had a 5% reduction in total work performed compared to men given a placebo (Tacey et al. 2019). This small body of work suggests that a single low dose of glucocorticoids is insufficient to improve muscle performance and may in fact negatively impact it under conditions of near-maximal aerobic capacity, but the question has not been comprehensively assessed.

Short-term dosing. There have been slightly more studies investigating exercise performance after short-term dosing with glucocorticoids; these studies were also predominantly performed in men. One investigation, which assessed cycling to exhaustion in individuals administered either 0.5 or 1.5mg dexamethasone twice a day, five days a week, for three weeks, observed no change between men given placebo or either dose of glucocorticoid (Marquet et al. 1999). Two studies that investigated men given 2mg dexamethasone twice a day for five days found that kicking time to exhaustion but not intensity increased with dexamethasone treatment compared to controls (Nordsborg et al. 2008, Casuso et al. 2014). This same dexamethasone treatment also improved distance achieved in a 20-minute shuttle run (Casuso et al. 2014). A trio of studies that investigated both men and women found that 50-60mg of prednisone taken daily for seven days significantly increased cycling time to exhaustion at 70-75% VO_2 max (Arlettaz et al. 2007, Collomp et al. 2008, Le Panse et al. 2009). Men taking 60mg prednisone daily for seven days also had significantly increased force but no change in time to exhaustion when they hopped on their dominant leg (Zorgati et al. 2014). None of these short-term dosing studies observed diminished performance after glucocorticoid treatment, suggesting that these doses of oral glucocorticoids administered for a few weeks are insufficient to induce atrophy in young, healthy, and active individuals. Indeed, despite the range of dose and duration of treatment, many of these short-term dosing studies observed improvements in muscle performance. Specifically, short-term glucocorticoids improved resistance to fatigue (Arlettaz et

al. 2007, Collomp et al. 2008, Nordsborg et al. 2008, Le Panse et al. 2009, Casuso et al. 2014), with only one study also reporting a modest improvement in force production (Zorgati et al. 2014). As resistance to fatigue is reliant upon fatty acid metabolism (Hurley et al. 1986, Mendenhall et al. 1994, Phillips et al. 1996), it is tempting to speculate that the improvements in lipid oxidation observed by Arlettaz et al. (2008) after a single dose of glucocorticoid might mediate resistance to fatigue after short-term treatment. However, further investigation is needed to test this hypothesis.

Animal models. Some of the earliest published investigations of glucocorticoids and muscle performance were a pair of studies from 1932 that evaluated working capacity and treadmill run-to-exhaustion in male and female dogs treated acutely with cortico-adrenal extract (Eagle and Britton 1932, Eagle et al. 1932). The investigators found that cortico-adrenal extract increased distance run to exhaustion after a single dose. Two years later, Dwight Ingle found that, one hour after adrenalectomy, male rats had a significant deficit in gastrocnemius work capacity that could be mostly rescued by cortin administration (Ingle 1934). Increased running time to exhaustion was also observed by a group that administered a single dose of 100mg/kg cortisol acetate to female rats (Gorostiaga et al. 1988). A single dose of 2mg/kg dexamethasone was also found sufficient to increase treadmill running time to exhaustion in male mice (Morrison-Nozik et al. 2015). More recently, a group investigating running motivation in male mice found that neither 1mg/kg nor 3mg/kg prednisolone administered orally ad libitum for ten days had a significant impact on free wheel running or grip hanging (Redon et al. 2020). Although this dose was reported as ergogenic, 1mg/kg administered daily is sufficient to induce skeletal muscle atrophy after four weeks in wild type mice (see Figure 2.1) as well as mouse models of DMD and limb girdle muscular dystrophy (LGMD) types 2B and 2C (Quattrocelli et al. 2017, Quattrocelli et al. 2017). Similar to investigations in humans, the variability in dose, duration of treatment, and performance evaluation makes it difficult to draw strong conclusions

from these animal models. However, they do suggest that a modest dose of glucocorticoids is sufficient to improve performance in normal, uninjured muscle.

Injury models. Glucocorticoids can be administered for acute or chronic injury to suppress inflammation and promote healing. As such, studies investigating the role of glucocorticoids in muscle repair following injury are complicated by additional variables such as disease or injury model and the difficulty of ascertaining the extent to which beneficial effects are the result of direct improvements in the myofiber contractile ability versus suppressed inflammation. For example, two days after contusion injury, rats treated intramuscularly with a single dose of 25mg/kg methylprednisolone acetate showed significant improvements in tetanic force (Beiner et al. 1999). However, this improvement over controls was lost by seven days, suggesting that the trade-off between the beneficial effects of immune suppression and the risk of atrophy in this model occurred somewhere between two and seven days. Two once-weekly doses of 1mg/kg prednisone administered to male animals following cardiotoxin injury were sufficient to significantly reduce injury area, decrease the number of infiltrating macrophages, and increase treadmill run to exhaustion and tetanic force (Quattrocelli et al. 2017). Mouse models of chronic muscle injury such as the *mdx* model of DMD respond to chronic glucocorticoid treatment with atrophy after a month of treatment; however, reducing treatment to 5mg/kg three days a week or 1mg/kg once a week was sufficient to prevent atrophy and in fact lead to improvements in treadmill run-to-exhaustion, grip strength, and tetanic force without any trade-off in immune suppression as measured by number of infiltrating macrophages (Morrison-Nozik et al. 2015, Quattrocelli et al. 2017). Improvements in grip strength were maintained out to 40 weeks of treatment (Quattrocelli et al. 2019). This 1mg/kg once-weekly treatment course was also found to provide benefit to two other models of muscular dystrophy, LGMD2B (*Dysf-null* mice) and LGMD2C (*Sgcg-null* mice) (Quattrocelli et al. 2017). Both male and female mice responded to four weeks of weekly prednisone with improvements in treadmill run to exhaustion,

tetanic force, and grip strength compared to vehicle-treated animals. Both sexes continued to exhibit improvements in all three metrics up to 32 weeks of treatment (Quattrocelli et al. 2019). The precise mechanism by which glucocorticoids exert this beneficial impact on injured muscle is unclear. However, there appears to be a role for KLF15, which acts in concert with GR and MEF2C to regulate nutrient utilization in response to weekly or short-term treatment with glucocorticoids (Morrison-Nozik et al. 2015, Quattrocelli et al. 2019).

1.2.9 Atrophy in the context of peripheral artery disease

Peripheral artery disease (PAD) is a progressive arterial disease of the lower extremities. Arterial thickening, most commonly from atherosclerotic plaques, reduces blood flow in the legs, resulting in movement-associated ischemia and pain. This can eventually lead to claudication, which is characterized as reproducible fatigue and discomfort in leg muscles that is stimulated by exertion and alleviated by rest. Diagnosis commonly occurs on the basis of pain upon walking, ankle-brachial index (ABI), and imaging (Treat-Jacobson et al. 2019). A severe form of PAD known as critical limb ischemia (CLI) is defined by ischemic pain even at rest and the presence of non-healing ulcers or gangrene that put patients at high risk for amputation (Kinlay 2016). Treatment of PAD and CLI predominantly aims to improve quality of life by reducing functional impairment and loss of mobility. For patients with severe claudication and CLI, arterial revascularization is a surgical option that can preserve limb function through interventions such as balloon angioplasty, stenting, or vascular grafting, which can increase blood flow (Kinlay 2016). Although therapeutic angiogenesis has been proposed as a method to grow new vessels and expand existing vasculature, it is has had limited success when transitioned into the clinic (Iyer and Annex 2017). For patients with asymptomatic PAD or with intermittent claudication, supervised exercise has been employed as therapy.

Patients with symptomatic PAD exhibit significant skeletal muscle atrophy and impaired lower limb function. Biopsies taken from patients with PAD show skeletal muscle atrophy, fiber type changes, abnormal morphology such as myofibrillar and sarcomeric disorganization, increased fibrosis, fat, and connective tissue, and makers of injury such as central nuclei (Makitie and Teravainen 1977, Hedberg et al. 1988, Weiss et al. 2013, Koutakis et al. 2014, Koutakis et al. 2015). Patients with PAD not only exhibit decreased walking time and speed compared to non-PAD controls but also reductions in lower limb muscle strength and peak torque (McDermott et al. 2002, Dziubek et al. 2015, Schieber et al. 2017). Unlike glucocorticoids, PAD-induced atrophy is likely not the result of impaired protein synthesis, as the fractional synthetic rate of protein has been shown to be the same in both PAD and non-PAD groups, both of whom were responsive to oral amino acid supplementation (Killewich et al. 2007). One candidate mechanism is oxidative stress, which contributes to muscle wasting in the development of sarcopenia, cachexia, and disuse atrophy (McClung et al. 2007, Powers et al. 2012, Kim et al. 2015, Matsui 2017, Abrigo et al. 2018). PAD patients have greater amounts of oxidative stress markers such as superoxide anion and hydrogen peroxide, the levels of which have been shown to be inversely correlated with walking distance (Dopheide et al. 2013, Weiss et al. 2013, Koutakis et al. 2018). Skeletal muscle mitochondria from patients with PAD and mouse models of hindlimb ischemia have been found to exhibit defects in the mitochondrial respiratory chain complexes I, III and IV, which may be the source of increased reactive oxygen species (Pipinos et al. 2003, Pipinos et al. 2006, Pipinos et al. 2008, White et al. 2016). However, not all PAD patients demonstrate impaired mitochondrial function, and it may be that mitochondrial defects are a particular hallmark of more severe PAD and CLI (Hou et al. 2002, Hart et al. 2018, Ryan et al. 2018). Another candidate mechanism for PAD-induced muscle wasting is denervation atrophy, which has been shown in various models to lead to reductions in fiber size and contractility (Castro et al. 1999, Giangregorio and McCartney 2006, Rowan et al.

2012, Carlson 2014). Denervation of calf muscle has been observed in ischemic legs of PAD patients and poorer peroneal nerve function is associated with reduced walking distance (England et al. 1992, Regensteiner et al. 1993, Garg et al. 2011). Patients with all degrees of PAD severity have demonstrated neuropathy, although some metrics such as reduction in action potential amplitude and sensory deficits are stronger in patients with more severe PAD compared to those with intermittent claudication (Weber and Ziegler 2002, Lang et al. 2006).

Regular exercise can substantially improve quality of life for patients with PAD. Supervised physical therapy programs associate with reduced circulating inflammatory markers and improved endothelial function, capillary density, and oxygen uptake after twelve weeks (Hiatt et al. 1996, Tisi et al. 1997, Andreozzi et al. 2007, Duscha et al. 2011). Twelve weeks of treadmill-based supervised exercise therapy also improves walking ability in patients with asymptomatic PAD as well as those with intermittent claudication (Parmenter et al. 2015). Endurance-type exercise such as treadmill walking appears to offer a more consistent benefit than resistance training, which has shown to be either no different from or less effective than endurance training at improving walking distance (Hiatt et al. 1994, McGuigan et al. 2001, McDermott et al. 2009, Ritti-Dias et al. 2010, Parmenter et al. 2020, McDermott et al. 2021). It is possible that, due to the mitochondrial dysfunction exhibited by skeletal muscle in individuals with PAD, the metabolic changes associated with endurance exercise offer greater therapeutic value to patients. Despite the significant benefits reported by randomized trials, the extent to which walking ability improves after exercise therapy is highly variable and a subset of patients do not respond to exercise therapy at all (Parmenter et al. 2015, Zakari et al. 2018). There is currently no way to distinguish exercise-responsive patients from non-responders prior to exercise intervention (Treat-Jacobson et al. 2019).

1.3 Hypothesis and research design

Skeletal muscle is a highly adaptable tissue that can modulate its contractile ability and metabolism based on external stimuli. In response to chronic glucocorticoid treatment or ischemia-reperfusion, skeletal muscle experiences substantial wasting that leads to weakness; however, when exposed to endurance exercise or acute glucocorticoids, skeletal muscle undergoes ergogenic adaptations that improve strength and resistance to fatigue. The mechanisms responsible for these beneficial adaptations are not fully understood, nor is the extent to which the highly sexually dimorphic nature of skeletal muscle influences these processes.

Investigations of acute glucocorticoid treatment in humans and animals have shown that low doses can improve muscle performance without inducing atrophy. In the work discussed in Chapter 2, I tested the hypothesis that the ergogenic skeletal muscle response to weekly glucocorticoid steroids is modulated by sex. I treated both male and female mice with 1mg/kg prednisone once a week for four weeks and found that this dosing regimen was able to significantly improve specific force and increase skeletal muscle ATP and NAD+. Using RNA-sequence and ChIP-sequence analysis, I was able to profile a sexually dimorphic response to weekly prednisone. Male muscle responded to weekly prednisone with improvements in calcium-handling and IGF1/PI3K signaling, while female muscle exhibited changes in lipid metabolism that lead to increased endurance. Both male and female muscle experienced alterations to chromatin architecture in response to weekly prednisone, but these changes were maintained longer in females than in males.

In Chapter 3, I describe exercise responsiveness to PAD as another model of ergogenic muscle adaptation. I tested the hypothesis that PAD patients who are responsive to exercise have baseline differences in skeletal muscle prior to exercise intervention that mediate their responsiveness and distinguish them from non-responders. Using RNA-sequence analysis and

histopathology, I found that responders and non-responders had divergent transcriptomes prior to exercise therapy. I also found these transcriptomic differences to be sex-specific. While male responders expressed fewer extracellular matrix and fibrosis genes than male non-responders, female responders expressed more mitochondrial genes than female non-responders. With these data, I was able to propose candidate biomarkers that might be used to distinguish responders and non-responders prior to intervention.

In Chapter 4, I summarize and interpret the findings from chapters 2 and 3. I also discuss the relationship between exercise adaptation and ergogenic remodeling of skeletal muscle and the role that sexually dimorphic pathways play in both of these biological processes. These findings have broad implications for how interventions aiming to improve skeletal muscle health and performance should take into account sex of participants.

Chapter 2. Intermittent glucocorticoid treatment enhances skeletal muscle performance through sexually dimorphic mechanisms

(modified from Salamone et al., Journal of Clinical Investigation, 2022)

2.1 Overview

Glucocorticoid steroids are commonly prescribed for many inflammatory conditions, but chronic daily use produces adverse effects including muscle wasting and weakness. In contrast, shorter glucocorticoid pulses may improve athletic performance, although the mechanisms remain unclear. Muscle is sexually dimorphic and comparatively little is known about how male and female muscles respond to glucocorticoid steroids. We investigated the impact of once-weekly glucocorticoid exposure on skeletal muscle performance comparing male and female mice. One month of once-weekly glucocorticoid dosing improved muscle specific force in both males and females. Transcriptomic profiling of isolated myofibers identified a striking sexually dimorphic response to weekly glucocorticoids. Male myofibers had increased expression of genes in the IGF1/PI3K pathway and calcium handling, while female myofibers had profound upregulation of lipid metabolism genes. Muscles from weekly prednisone-treated males had improved calcium handling, while comparably treated female muscles had reduced intramuscular triglycerides. Consistent with altered lipid metabolism, weekly prednisone-treated female mice had greater endurance relative to controls. Using chromatin immunoprecipitation, we defined a sexually dimorphic chromatin landscape after weekly prednisone. These results demonstrate that weekly glucocorticoid exposure elicits distinct pathways in males versus females resulting in enhanced performance.

This chapter has been published under the same title (Salamone et al. 2022). I conceived experiments, carried out analyses, and drafted the manuscript. Patrick Page, Garima Tomar, and Michele Hadhazy assisted with the animal experiments. Dr. Mattia Quattrocelli

carried out muscle force analyses and provided editorial comments on the manuscript. Drs. David Barefield and Ibrahim Tahtah assisted with measurements of calcium transients, and David Barefield provided critical edits to the manuscript. Dr. Elizabeth McNally was responsible for data integrity, study design, analyses, and editing the manuscript.

2.2 Introduction

Glucocorticoids are powerful stress hormones that modulate the body's carbohydrate, lipid, and protein metabolism in response to external or circadian stimuli. Synthetic glucocorticoids such as prednisone are used as anti-inflammatory agents for conditions ranging from rheumatoid arthritis to muscular dystrophy to asthma. The therapeutic benefits of glucocorticoids are hampered by extensive side effects especially when taken chronically, and these side effects include insulin resistance and muscle weakness (Strehl et al. 2016). Chronic glucocorticoid treatment is known to induce skeletal muscle atrophy by stimulating atrogene expression (Bodine et al. 2001, Braun et al. 2013), and despite this, glucocorticoids are still widely used because of their effectiveness as anti-inflammatory agents.

Glucocorticoids exert their effects through the glucocorticoid receptor (GR). Upon ligand binding, GR is released from a chaperone complex in the cytoplasm and transported into the nucleus, where it binds glucocorticoid response elements (GREs) near target genes. GR interacts with a diverse group of co-activators and co-repressors in order to regulate target gene expression in a tissue- and stimulus-specific manner (Lonard and O'Malley 2005, Lonard and O'Malley 2007). The role of GR as an anti-inflammatory transcription factor is well delineated (Barnes 1998, Coutinho and Chapman 2011). In muscle, glucocorticoids and GR have been well-characterized in the promotion of skeletal muscle atrophy (Waddell et al. 2008, Watson et al. 2012, Braun et al. 2013, Braun et al. 2014). In high or chronic dosing schemes, glucocorticoids activate the ubiquitin proteasome pathway, upregulating the muscle-specific

ubiquitin ligases MuRF-1 and atrogin-1, which directly increase polyubiquitination-mediated degradation of proteins and initiate muscle wasting (Castillero et al. 2013, Sato et al. 2017). Concurrently, these high doses of glucocorticoids inhibit mammalian target of rapamycin (mTOR) (Shah et al. 2000, Shah et al. 2000, Shimizu et al. 2011), resulting in decreased protein synthesis.

Glucocorticoids have been used to enhance muscle performance and/or improve recovery from injury, often in short dosing regimens thought to act through anti-inflammatory properties (Collomp et al. 2016). Studies examining lower dose and short-term exposure to glucocorticoids have found inconsistent results on skeletal muscle performance, likely related to varying dosing schemes. Single dose or short-term dosing, for less than one week, has been found to result in either no change or modest improvements in athletic performance in both humans (Collomp et al. 2016) and mice (Morrison-Nozik et al. 2015, Redon et al. 2020). In mice, a chronic once-weekly dosing regimen improved muscle strength and endurance in models of Duchenne Muscular Dystrophy (DMD) and Limb Girdle Muscular Dystrophies 2B and 2C (Quattrocelli et al. 2017, Quattrocelli et al. 2019). The molecular mechanisms responsible for this benefit to muscle are likely multifold, and studies in mouse models of DMD indicate that the transcription factor KLF15 is relevant in the diseased muscle setting (Morrison-Nozik et al. 2015, Quattrocelli et al. 2019). Few of the studies examining glucocorticoid dosing and response have considered sex-specific responses, despite skeletal muscle being a highly sexually dimorphic tissue and the observations of sex-specific transcriptional programs driven by glucocorticoids in other tissues (Duma et al. 2010).

To investigate how prednisone dosing impacts atrogene expression and muscle performance, we treated a cohort of male and female adult mice with once-weekly or daily doses of prednisone for one month and then evaluated skeletal muscle function and gene expression. We found that daily prednisone treatment resulted in decreased specific force and

increased atrogene expression in both males and females. In contrast, once-weekly prednisone-treated mice of both sexes exhibited increased specific force, increased skeletal muscle ATP, and no increase in atrogene expression. When we investigated the skeletal muscle transcriptome of these animals, we found that male and female mice had almost no common genes responsive to weekly prednisone. Instead, male skeletal muscle appeared to respond to weekly prednisone with genes implicated in hypertrophy and improved calcium handling, while females expressed genes underscoring a metabolic shift towards increased lipid utilization. We found evidence of a sex-specific GR DNA-binding pattern, consistent with weekly prednisone improving muscle performance through independent mechanisms in male and female mice.

2.3 Materials and methods

2.3.1 Animals

Male and female C57BL/6J mice were obtained from The Jackson Laboratory at eight weeks of age. Mice were acclimatized for two weeks prior to treatment onset. Mice were randomly assigned to treatment cohorts. Unless otherwise specified, all animals were ten weeks of age at treatment onset. For long term experiments, mice were assigned to treatment group based on initial weight and percent fat mass such that each treatment group began with the same mean body mass and mean percent fat mass. Mice were housed in a specific pathogen-free facility and maintained on a 14-hour/10-hour light/dark cycle with light cycle beginning at 6AM.

2.3.2 Drug treatments

Prednisone (P6254, Sigma-Aldrich) was resuspended in DMSO (D4540, Sigma-Aldrich) at 5mg/mL each week. Daily treatment was 1mg/kg body weight prednisone in 50µL PBS given

every day via i.p. injection. Vehicle treatment was the equivalent amount of DMSO proportional to body weight in 50µL PBS. Weekly treatment was one day of prednisone treatment (1mg/kg prednisone in 50µL PBS) and six subsequent days of vehicle treatment (DMSO in 50µL PBS). Unanesthetized mice were injected daily at 7AM with Sterile BD Micro-Fine IV Insulin Syringes (14-829-1A, Fisher Scientific). Mice were weighed weekly and body weight was used for dosing calculations. Short-term (4-week) treatments consisted of five weekly prednisone injections total, with the final injection given two days prior to sacrifice. Long-term (38 week) treatments consisted of 39 weekly prednisone injections total, with the final injection given two days prior to sacrifice. Androgen receptor activity was suppressed via the competitive antagonist flutamide (F9397, Sigma-Aldrich), which was administered at 15mg/kg in 90% corn oil/10% ethanol. Estrogen receptor activity was suppressed via the competitive antagonist fulvestrant (I4409, Sigma-Aldrich), which was administered at 5mg/kg in 95% corn oil/5% DMSO. Sex steroid receptor antagonists were administered daily as 100µL i.p. injections.

2.3.3 Muscle mechanics, treadmill, and body composition

In situ tetanic force and contraction and relaxation time from the tibialis anterior muscle were evaluated at 9AM just prior to sacrifice using a Whole Mouse Test System with a 1N dual-action level arm force transducer (300C-LR, Aurora Scientific). Mice were anesthetized with 3% isoflurane in 100% O_2. Tetanic isometric contraction was induced with the following specifications using 100mA stimulation: initial delay, 0.1s; frequency, 200Hz; pulse width, 0.5ms; duration, 0.5s. Length was adjusted to a fixed baseline of 30mN resting tension. Response to repetitive force was evaluated by repeating tetanic contractions every 10s for 50 cycles. Contraction time was assessed as time to maximal tetanic value within the 0.0-0.5s range of every tetanus; relaxation time was assessed as time to 90% minimum tetanic value within the 0.5-0.8s range of every tetanus. Specific force was calculated using myofiber cross-

sectional area, described below, after sacrifice. Mice were run on a treadmill (Exer3/6 without electrical stimulation grills, Columbus Instruments) at a 15° incline with a start speed of 1m/min. Speed was increased by units of 3m/min in 2min increments to a final speed of 15m/min. The assay was terminated when mice stopped for longer than 15s on the rest pad and could not be prodded to return to running. The number of times a mouse had to be prodded to run was recorded and reported as stimuli per kilometer run. Treadmill experiments were performed two days after the most recent weekly injection beginning at 8.30AM. Body composition was evaluated prior to and during treatment, occurring monthly at one day after the most recent weekly injection at 1PM. Percent fat mass, percent lean mass, and hydration ratio were determined in unanesthetized mice using MRI (EchoMRI). For all muscle mechanics, treadmill, and body composition assays, the operator was blinded to treatment group.

2.3.4 FDB isolation, calcium indicator dye loading, and calcium transient and sarcomere shortening measurements

The flexor digitorum brevis (FDB) muscle was dissected from mouse hind-limb foot pads and incubated in DMEM (SH30022.01, HyClone) with 2mg/mL bovine serum albumin (SLBT0167, Sigma Aldrich) and 40mg/mL collagenase II (17101-015, Invitrogen) and was incubated at 37°C with 10% CO_2 for 90 minutes (Demonbreun and McNally 2015). The FDB muscles were moved to Ringer's solution (123mM NaCl, 2mM $CaCl_2$, 5mM KCl pH 7.4) and triturated to isolate individual myofibers. MatTek 10mm glass bottom dishes were coated with 20μg/mL laminin for one hour (23017-015, Gibco), after which the FBDs were adhered to the surface for 30 to 45 minutes at 37°C with 10% CO_2. After 1 hour, the media was changed with 300μL of the Ringer's solution with 6 g/L glucose and 1% penicillin/streptomycin for overnight incubation at 37°C maintained in an incubator with 5% CO_2. Leak-resistant Indo-1-AM (145,

TEF Labs) cytosolic calcium indicator dye was resuspended in DMSO and 10% Pluronic. FDB muscles were loaded with 5 μM Indo-1 for 45 minutes in a 37°C incubator with 10% CO_2.

Isolated FDB muscles loaded with Indo-1 dye were imaged on a Nikon Diaphot inverted microscope equipped with a high-speed camera and photomultiplier tubes integrated with the FluoroDaq system (IonOptix) to measure cytosolic calcium transients and cell shortening as previously described (Kim et al. 2019). FDB muscles were paced with platinum electrodes designed for 35mm MatTek dishes and connected to a high-voltage follow stimulator (701C, Aurora Scientific). Cells were paced at 40 Hz for 100ms using a 0.2ms pulse width at 18–20 V. A video sarcomere length system (900B, Aurora Scientific) was used to measure sarcomere spacing from bright-field images using a fast-Fourier transform. Aurora Scientific's 950A calcium fluorescence analysis module was used to record the calcium transients and sarcomere length shortening data and analyze the parameters of average transients. FDB isolation and calcium transient measurements were performed blinded to treatment group.

2.3.5 High-performance liquid chromatography

Gastrocnemius muscle was flash-frozen immediately after sacrifice. Prior to extraction, approximately a quarter of each gastrocnemius was isolated on dry ice using a razor blade and then weighed. Briefly, muscles were pulverized to powder using a mortar and pestle chilled on dry ice and then homogenized in 10% perchloric acid. After incubating on ice, samples were neutralized in 3M potassium chloride, vortexed, and then diluted in phosphate buffer (30.8mM K_2HPO_4, 19.2mM KH_2PO_4). NAD+ and ATP were measured by high-performance liquid chromatography with a Shimadzu LC-20A pump (Shimadzu Scientific Instruments Incorporated) and UV-VIS detector, using a Supelco LC-18-T column (58970-U, Millipore-Sigma). HPLC was run at a flow rate of 1mL/min as 100% phosphate buffer (0-5min), a linear gradient to 95%

phosphate buffer in methanol (5-6min), 95% phosphate buffer (6-11min), a linear gradient to 85% phosphate buffer in methanol (11-13min), 85% phosphate buffer (13-23min), and then a linear gradient to 100% phosphate buffer (23-30min). ATP and NAD+ peak values were normalized to tissue weight. Extraction of nucleotides, HPLC, and analysis were performed by an operator blinded to treatment group.

2.3.6 Histology and immunofluorescence microscopy

Cross-sectional area for white adipose tissue was evaluated from hematoxylin and eosin staining of 10μm paraffin sections imaged with a Zeiss Axio Observer.Z1 (Zeiss) at 10X (LD A-Plan 10x/0.25 Ph1, Zeiss) for quantification and 20X (Plan-Apochromat 20x/0.8, Zeiss) for representative images. Adipocyte cross-sectional area was measured by hand in Fiji (Schindelin et al. 2012) from three images per animal. The percent of fibers positive for succinate dehydrogenase was evaluated from 10μm cryosections of tibialis anterior incubated with sodium succinate and nitroblue tetrazolium at 37°C for 90 min. Two whole sections from each animal were imaged as 10X tiles (EC Plan-Neofluar 20x/0.3, Zeiss) using a Zeiss Axio Imager.M2 (Zeiss); representative images were taken at 20X (Plan-Apochromat 20x/0.8, Zeiss). Fiber type was evaluated by immunofluorescence; briefly, 10μm serial cryosections of tibialis anterior were fixed in acetone and then incubated overnight with primary antibodies against Myh7 (1:10), Myh2 (1:30), Myh4 (1:10), and Myh1 (1:30) (BA-F8, SC-71, BF-F3, and 6-H1, Developmental Studies Hybridoma Bank) at 4°C. Sections were then incubated with secondary antibodies Alexa Fluor 488 goat anti-mouse IgG1 and Alexa Fluor 594 goat anti-mouse IgM (A21121 and A21044, Life Technologies). Two whole sections from each animal were imaged as 10X tiles with a Zeiss Axio Imager.M2. The number of fibers stained each color was counted by hand in Fiji. Cross-sectional area for skeletal muscle was evaluated from 10μm cryosections of tibialis anterior fixed in 4% PFA and incubated overnight with an antibody against dystrophin

(1:1000, PA1-37587, Invitrogen) at 4°C. Sections were then incubated with AlexaFluor 488 donkey anti-rabbit IgG (A32790, Life Technologies). Two whole sections from each animal were imaged as 10X tiles with a Zeiss Axios Imager.M2 and then cross-sectional area was evaluated using the MATLAB program SMASH (Smith and Barton 2014). All stains were performed, imaged, and analyzed with operator blind to treatment.

2.3.7 Serum collection and analysis

At the end of treatment, mice were fasted for four hours starting at 5AM and blood was collected by heparinized microhematocrit capillary tube (22-362-566, Fisher Scientific). Blood was allowed to clot for 30min at RT and then centrifuged for 5min at 12,000 x g at 4°C. Plasma was collected as supernatant and used to determine circulating insulin (NC9440604, Fisher Scientific), corticosterone (ADI-900-097, Enzo Life Sciences), estradiol (Calbiotech; conducted by University of Virginia Center for Research in Reproduction), and testosterone (IBL; conducted by University of Virginia Center for Research in Reproduction). Blood glucose was measured from a drop of blood collected from the tip of the tail with an AimStrip Plus glucometer (Germaine Laboratories). Insulin, corticosterone, estradiol, and testosterone levels were evaluated by an operator blinded to treatment group.

2.3.8 Immunoblot analysis

Relative protein abundance and phosphorylation was assessed by immunoblot. Briefly, snap-frozen muscles were ground to powder using a dry ice-chilled mortar and pestle and then resuspended in 500µL ice-cold 1x PBS + 1mM $CaCl_2$ + 1mM $MgCl_2$ or 1x RIPA (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, 0.5mM EGTA pH 8.0, 0.1% sodium deoxycholate, 140mM NaCl) with EDTA-free protease inhibitor cocktail (11836170001, Sigma Aldrich) and phosphatase inhibitor (4906837001, Sigma Aldrich). Tissue was homogenized using the TissueLyzer II

(Qiagen) for 6min at $30s^{-1}$, resting every 2min. Samples homogenized in RIPA were then incubated with 1% Triton-X100 (9002-93-1, Sigma Aldrich) and 0.1% SDS (L3771-500G, Sigma Aldrich) for 1hr at 4°C. Samples were centrifuged to pellet insoluble material and then supernatant total protein was quantified using Bradford reagent (5000205, BioRad). Protein was separated using 4-15% gradient or 7.5% TGX gels (4561086 and 4561026, BioRad) and then transferred to PVDF membrane (1620177, BioRad) for 3hrs at 300mA at 4°C. Blots were blocked for 90min at RT in StartingBlock T20 (37543, Thermo Fisher) and incubated with primary antibody diluted in T20 overnight at 4°C. Blots were washed four times in 1x TBS + 0.1% Tween-20 (BP337-500, Thermo Fisher), incubated for one hour at RT with secondary antibody conjugated to HRP, and washed again in TBST. Blots were imaged with SuperSignal West Pico Chemiluminescent Substrate (34080, Thermo Fisher) using an iBright CL1000 (Thermo Fisher). Total protein was assessed using the Pierce Reversible Protein Stain Kit (24585, Thermo Fisher). Densitometry was performed using Fiji and protein was normalized either to the total protein band at 42kDa or as phospho- to total protein. Antibodies used for immunoblots are listed in Table 2.1.

Table 2.1 Antibodies used for immunoblot

Antibody	Dilution	Catalog #	Source
4EBP1	1:1,000	A300-501A	Bethyl
phospho-4EBP1	1:1,000	2855	Cell Signaling Technology
S6K	1:1,000	A300-510A	Bethyl
CASQ1/2	1:1,000	ab3516	Abcam
SERCA2	1:10,000	MA3-919	Thermo Fisher
Goat anti-mouse HRP	1:2,500	115-035-003	Jackson ImmunoResearch Laboratories
Goat anti-rabbit HRP	1:2,500	111-035-003	Jackson ImmunoResearch Laboratories

2.3.9 RNA sequence analysis and quantitative RT-PCR

Gene expression analysis was performed on RNA extracted from myofibers isolated from quadriceps with TRIzol (15596018, Life Technologies). Briefly, directly following harvest, the right quadriceps was cut in half and then sliced following the natural myofiber orientation before being incubated in collagenase II for 1hr at RT. The muscle was manually dissociated and then filtered through a 40μm cell strainer (22-363-547, Fisher Scientific). The unfiltered fraction was collected and processed with TRIzol. RNA was then isolated using the PureLink RNA Mini Kit (Thermo Fisher) per manufacturer's instructions and resuspended in 30μL of RNase-free water. RNA was quantitated with a Qubit fluorometer (G2943, Agilent Technologies) and then libraries were prepared from 1μg RNA per sample using the TruSeq Stranded Total RNA Library Prep Kit (RS-122-2203, Illumina). Libraries were sequenced on the NextSeq 500 System as 150bp paired-end fragments (SY-415-1001, Illumina). Raw reads are available on GEO as accession GSE168964. Raw reads were aligned to mm10 with Bowtie2 (Langmead et al. 2009, Trapnell et al. 2009) and then read counts per gene were quantified with HTseq. Counts per million and fold change were calculated using the Bioconductor package EdgeR. Heatmaps were visualized from z-score with median in RStudio (Team 2020). Gene ontology (GO) analysis was conducted via the Wiki Pathways Mouse 2019 module in Enrichr (Chen et al. 2013, Kuleshov et al. 2016). Results from RNASeq were validated by quantitative RT-PCR with primers listed in Table 2.2 using iTaq Universal SYBRGreen Supermix (1725124, BioRad). Correlation between gene expression and phenotypic data was assessed using Pheno-RNA (Darwiche and Struhl 2020). Genes with log counts per million > 2.5 and absolute log fold change > 0.5 (females: 721; males: 470) were used for the analysis along with specific force, relaxation time, and contraction time. The Pearson correlation coefficient was calculated for each gene and phenotype in three animals per sex per treatment group. A control group

Table 2.2 qPCR primers for gene expression

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
Acad11	TAACGGCAAGAAGTGGTGGA	TGTGCTGTCTGTGTCTGGAT
Acsl1	GACTTGTTGAAACTTGGGAA	CATCTATCTGCGACCTGAAA
Acsm3	CCCAGCAGTAGATGCCGTG	TTCGTCGTGTTTTGTGTCCA
Acss2	GCAACTACAAACATCTGCTACA	ATCTTGGTGGTCTCCCCTG
Acss3	AAGTCTTCCGAGTTCCCGTT	CCTGGTGGAGGTGTTTTGG
Agpat2	CATCATCCCCGTGGTGTA	GAAATCTGTAGAAAGGTGGC
Akt1	GCCTGCCCTTCTACAACCA	CATACACATCCTGCCACACG
Ar	ACTATTACTTTCCACCCCA	CAGAGTCATCCCTGCTTC
Atp2b2	AATGCCCGCTGTTTTGCT	ATCTGCCAGGACCATCTCA
Cacna1h	CCCATCAACCCCACCAT	AGCATAGATAAAAAACAGGAG
Casq2	TGCGGAGAAGAGTGACCC	AGCAACAAGCAGTGGAAAGT
Chpt1	GCTCATTGGCAGACTTACG	GTCCCACATTGTTGCTCCT
Cidea	TTCCTCGGCTGTCTCAA	CAGATTCCTTAACACGGC
Cidec	ATCGGAAGGTTCGCAAAGG	CCAGCACCAGGGAGAAGG
Ehhadh	CTTGGGCTGTCACTATCG	TTGGGACTGGCTTGTTTA
Esr1	AGCATTCAAGGACACAA	CTTCCAAGTCATCTCTCTG
Fabp4	GTGGGAGTGGGCTTTGC	GCTCTTCACCTTCCTGTCGT
Fbxo32	GGCTACTGTGGAAGAGACT	CAGGAGAGAATGTGGCA
Hadha	TGAAGTGTTGCTGGGGAT	CACGAATGTTCCTGCCA
Igf1	TCACACCTCTTCTACCTGGC	GTGCCCTCCGAATGCTG
Irs2	ATCAGGTATCTGGGGTGGAG	GACGGTGGTGGTAGAGGAAA
Itga3	TCATCTGTCTTCCACGGCTT	CTGGTTGAGGACTGGGTAGG
Itga5	AAGGGAGAGGAGCCTGTGG	CGGGTGAAGTTTTCTGTGGA
Jun	GCCCCTGTCCCCTATCG	TGAGTTGGCACCCACTGTTA
Junb	TCACGACGACTCTTACGCAG	GACCCTTGAGACCCCGATAG
Lipe	TGAGATTGAGGTGCTGTCGT	GGTAACTGTGAGCCTGGGAT
Pik3c2a	AGCCCACCATTCGTTTCC	GCTTCAGCATCTGTAGTTTG
Pik3ca	CCTGGGGAAACATAAACTT	AAACTTCACCACACTGCTG
Slc27a1	GGAGTCGTGGAGGTCTGAAG	GATGATTGATGGTTGCCGC
Tnnc1	AGGTGATGAGGATGCTGG	ACTTCCCTTTGCTGTCGTC
Tnni1	TGTCTCTCAGTGCCCTTCA	ATCTCTCTGGTGTTGTGGA
Tnnt1	GCACTAAAAGACCGCATTG	AGTTTCATCTCCCGACCAG
Trim63	TAGCCTGATTCCTGATG	GGTCCAGTAGGGATTCG

was generated by shuffling counts per million between samples as described in the original publication (Darwiche and Struhl 2020).

2.3.10 ChIP-qPCR and ChIP sequence analysis

Chromatin immunoprecipitation was performed on chromatin isolated from snap-frozen whole left quadriceps. Muscle was ground to powder on dry ice and then dounced six times in 2mM disuccinimidyl glutarate (20593, Thermo Fisher) on ice. The muscle homogenate was filtered through first a 100µm and then 40µm cell strainer (22-363-549 and 22-363-547, Fisher Scientific) and the filtrate was incubated for 20min at RT. The cells were pelleted and then resuspended in 1% PFA for a 5min fixation at RT. Pellets were resuspended in Fast IP Buffer (5mM EDTA pH 7.5, 50mM Tris pH 7.5, 0.05% NP-40, 150mM NaCl) and lysed by passing through a Sterile BD Micro-Fine IV Insulin Syringe (14-829-1A, Fisher Scientific) twice. Chromatin was sheared via Bioruptor Ultrasonicator (Diagenode), 6 cycles, 30s on/off, and then spun down to remove impurities. The supernatant was split in half and incubated with 6µg of either anti-GR antibody (sc-393232, Santa Cruz) or an IgG2B isotype control (MAB004, R&D Systems) overnight at 4°C, with 5% reserved as input. Protein A/G Magnetic Beads (88803, Thermo Fisher) were pre-blocked with 0.5% BSA in PBS overnight at 4°C. After overnight incubation, the antibody-chromatin mix was added to the magnetic beads and incubated for 6hrs at 4°C. Beads were washed six times with ice-cold Fast IP Buffer, twice with ice-cold TE pH 8.0, and then eluted at RT (105mM sodium bicarbonate, 1.05% SDS). Following an overnight incubation at 65°C to reverse crosslinks, DNA was purified with a QIAquick PCR Purification Kit (28106, Qiagen) and eluted with 30µL EB. GR enrichment was determined as percent input by quantitative PCR. Positive and negative control loci as well as putative GREs identified are listed in Table 2.3.

Table 2.3 qPCR primers used to identify GR enrichment at putative binding sites

Category	Gene of Interest	Location	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
negative control	n/a	chr15:15,752,358-15,752,522	GCCGAAATGTATGAGTAGCCA	AATGAATGAGCCCTTCCCCA
positive control	*Fkbp5*	chr17:28,420,152-28,420,344	GCCACATTCAGAACAGG	TACTCCAACAAACCCCAC
calcium-handling	*Cacna1h*	chr17:25,434,130-25,434,246	CACGCCTGCTGAGCCCCG	TCCTTCCCCACCCCCACTGC
calcium-handling	*Tnnc1*	chr14:31,206,433-31,206,508	GCAGAACCTTCCACGCACT	CGACCCAGGGGCTTTGA
calcium-handling	*Tnnt1*	chr7:4,522,438-4,522,594	TAGAGTCAAAGGAGGAGGGG	GACACTGAGATAAGGGGCGA
IGF1 pathway	*Akt1*	chr12:112,666,788-112,666,871	CCTTTACCCTCTAAGCCATCT	TTACCCATCCTCCCTCTCC
IGF1 pathway	*Ar*	chrX:98,148,575-98,148,652	CAACCATACTACGCCAGCAC	TTTCCTTTTCTCCCCTCCC
IGF1 pathway	*Pik3c2a*	chr7:116,444,010-116,444,188	CCTCTCCTCCGACAGTTAC	GCCAGACATCACACCCAG
IGF1 pathway	*Pik3ca*	chr3:32,454,532-32,454,665	GCACGCTGCTGTCTTTGT	ATAATACCCCAGTTCCCCA
lipid metabolism	*Cidea*	chr18:67,511,497-67,511,622	TTACTCTTCCCCACTTATGAT	CTGTCTGTGTCTGCTGATGT
lipid metabolism	*Cidec*	chr6:113,385,708-113,385,789	TGGGTTCTGGAATGTGGT	TAGGGTGAAGTCTCTGGC
lipid metabolism	*Esr1*	chr10:5,674,439-5,674,518	GGAACACTGGTGAAGGCT	ATGCTCTCTTTTAGTATTATTTTA
lipid metabolism	*Slc27a1*	chr8:71,561,278-71,561,409	ATTACTCTTTGAGGGGACAT	AAGGGAGTAGTGGGGGAA
lipid metabolism	*Slc27a1*	chr8:71,575,870-71,575,940	CAGGAGGCAGAGACAGGC	TAGAACTTGCTACATAGACCAGG

Chromatin immunoprecipitation for sequencing was performed on two biological replicates per treatment group on isolated myofibers from the left quadriceps. Briefly, myofibers were isolated as described above and then snap-frozen after filtering. Snap-frozen myofibers were ground to powder on dry ice and then fixed in 1% PFA for 5min at RT. Chromatin was isolated and sheared as described above before being incubated with 5µg of anti-H3K27ac antibody (39133, Active Motif) overnight at 4°C, with 5% reserved as input. Immunoprecipitation was performed as described above with a high salt wash buffer (50mM Tris pH 7.5, 500mM NaCl, 0.1% SDS, 1% Triton X-100). Chromatin was quantitated with a Qubit fluorometer

(G2943, Agilent Technologies) and then libraries were prepared from 5ng chromatin per sample using the TruSeq DNA Library Prep Kit (FC-121-2003, Illumina). Libraries were sequenced on the HiSeq 4000 System as 50bp single-end fragments. Reads were trimmed to remove TruSeq adaptor sequences with homerTools (Heinz et al. 2010) and then aligned to mm10 with Bowtie2 (Langmead et al. 2009, Trapnell et al. 2009). Peaks were called with HOMER using command findPeaks with parameters -style histone -F 2 with input tag directories used as controls. Peaks unique to weekly prednisone-treated skeletal muscle were identified by comparing vehicle- and weekly-treated peak files using mergePeaks with parameter -d given. Significantly enriched motifs were identified using findMotifsGenome with parameters -size given -mask. Raw reads and peak files are available on GEO as accession 188302. Regions called as peaks or not peaks were validated by qPCR using primers listed in Table 2.3.

2.3.11 Liquid chromatography with tandem mass spectrometry and analysis

Untargeted lipidomics was performed on muscle and serum from female mice treated for four weeks of vehicle or weekly prednisone. Quadriceps muscle were snap-frozen and then ground to powder with dry ice-chilled mortar and pestle. Frozen samples were sent on dry ice to the Mass Spectrometry Core at the University of Illinois Chicago, where LC/MS/MS and data analysis were performed. Briefly, 10µL of SPLASH Lipidomix was added as an internal standard prior to extraction. The lipid extracts were dried and resuspended in 100µL methanol:chloroform (9:1, v/v) prior to analysis. MS analysis of the crude lipid extracts was performed using an Agilent 6545 Q-TOF LC–MS system controlled by the Agilent Mass Hunter acquisition software. The mass spectrometer was operated in 2 GHz extended dynamic range mode employing precursor ion analysis for relative quantification experiments in positive ion mode. In positive ion mode, internal reference mass calibration ions m/z 121.0509 and 922.0098 were used. Mobile phase A was H_2O:methanol (90:10, v/v) with 10mM ammonium

acetate and 0.5mM ammonium fluoride. Mobile phase B was isopropanol:methanol:acetonitrile (5:3:2, v/v/v) with 10mM ammonium acetate and 0.5mM ammonium fluoride. The resuspended lipid extracts (2µL) were loaded onto a 2.1 × 100 mm Agilent Poroshell C18, 2.7µm column (Agilent Technologies Inc., Santa Clara, CA, USA) and separation performed using an Agilent 1290 UPLC system using the following gradient: 70% B at 0–1 min, 86% B at 3.5–10 min, 100% B at 11–17 at a flow rate of 400µL/ min. A post column equilibration time of 5 min was used for all runs. Source parameters were as follows: gas temp (200°C), drying gas (11 L/min), nebulizer (35 psi), sheath gas temp (350°C), sheath gas flow (12 L/min), VCap (3000 V), and fragmentor (145 V). Data was collected for relative quantification using a scan speed of 4 MS spectra per second. Pooled samples were prepared by combining 5µL of each of the samples and an iterative MS/MS workflow was performed in the Mass Hunter acquisition software across six injections of the pooled samples with a scan speed of 10 MS and 3 MS/MS spectra per second.

Raw LC–MS/MS data was used to create a fragmentation-based (MS/MS) library containing m/z precursors and retention times for all MS/MS identified lipids using the Lipid Annotator software (Agilent Technologies Inc., Santa Clara, CA, USA) and the following settings: lipid species for positive ([M + H]+, [M + Na]+, [M + NH_4]+, [M + H – H_2O]+, [M + Na – H_2O]+ , [M + NH_4 – H_2O]+), Q-Score ≥ 60, and mass deviation ≤ 10ppm. Raw LC–MS data files were processed using the Profinder software (vB.10.00, Agilent Technologies Inc., Santa Clara, CA, USA). Here, molecular features were extracted for peaks ≥ 5000 counts in the positive mode ([M + H]+, [M + Na]+, [M + NH_4]+, [M + H – H_2O]+, [M + Na – H_2O]+, [M + NH_4 – H_2O]+) using an isotope model of common organic molecules (no halogens). The resulting compound list was further filtered for those having an absolute height of ≥10,000 counts, quality score of ≥60, and compounds having two or more isotopes present. Additionally, retention time for each compound was aligned to ±0.1 min using a mass accuracy window of ≤5.0ppm and peaks integrated using the Agile integrator in the Profinder software. Each of the integrated peaks was

manually reviewed for retention time and fragmentation matching. The processed data file was then exported as a .cef and imported into the Mass Profiler Professional software (v15.1, Agilent Technologies Inc., Santa Clara, CA, USA) where each data set was analyzed separately. All compound abundance values were baseline corrected to the median abundance and normalized by the closest lipid standard. Compounds that were not present in all biological replicates of either treatment group were further filtered. Outliers were identified by Dixon's Q test and outliers falling outside the 95% confidence interval were removed. Compounds of interest were identified by Welch's *t* test as having a p-value less than or equal to 0.05 and are listed in Tables 2.4 (quadriceps) and 2.5 (serum).

2.3.12 Statistical analyses

Statistical analyses were performed with Prism (Graphpad). When comparing three groups, analyses were performed as either one-way ANOVA (for treatment as the only variable) or two-way ANOVA (for time and treatment as variables). When comparing two groups, Mann-Whitney-Wilcoxon was used. For analyses with small sample number, a Welch's *t* test with was used. For ANOVA and *t* test analyses, a p-value less than or equal to 0.05 was considered significant, although all p-values less than 0.1 are reported in figures. Dot plots and marked line plots depict mean ± SD.

2.4 Results

2.4.1 Weekly glucocorticoid treatment improves force in both sexes

Male and female ten-week-old C57BL/6J mice were treated with either weekly or daily injections of prednisone (1 mg/kg i.p.) for four weeks (Figure 2.1A). At the end of treatment, we analyzed muscle force, relaxation and contraction time, and response to repetitive force in the tibialis anterior muscles of each group. Daily prednisone treatment induced comparable atrophy

Table 2.4 Abundance of compounds of interest from untargeted lipidomics in quadriceps

Compound	Compound Class	Vehicle					Weekly					Fold Change	P-value
CL 78:12	cardiolipin	2.14	2.88	2.59	0.86	3.03	5.53	2.44	3.23	4.17	5.27	1.79	0.0324
DG 12:0/18:2	diglyceride	9.79	7.98	8.99	8.33	10.11	7.28	7.99	6.98	8.40	7.99	0.85	0.0267
DG 14:0/16:1	diglyceride		16.53	16.44	15.46	16.97	15.87	0.12	12.66	1.45	0.03	0.37	0.0323
DG 18:0/18:1	diglyceride	2.48	1.86	2.08	1.68	2.59	1.25	1.68	1.73		1.78	0.75	0.0520
DG 18:1/18:1	diglyceride	3.06	1.96	3.36	2.24	3.17	2.08	1.62	1.84		1.89	0.67	0.0270
DG 18:1/22:6	diglyceride	2.43	1.70	2.08	1.15	2.27	1.16	1.35	1.29		1.45	0.68	0.0540
LPC 19:1	lyso-phosphatidylcholine	3.23	0.24	-0.51	-0.24	2.37	-0.43	-0.83	-18.6	-5.44	-22.1	-9.33	0.0528
PC 13:0/20:4	phosphatidylcholine	0.72	0.06	-0.37	-2.20	0.52	-0.06	-0.11	4.81	4.95	3.72	-10.50	0.0487
PC 14:0/16:2	phosphatidylcholine	9.79	8.83	9.07	7.10	9.55	9.50	9.25	11.31	10.42	12.12	1.19	0.0507
PC 16:0/16:1 RT: 6.592	phosphatidylcholine	1.43	-0.52	-0.81	0.52	2.21	2.62	2.33	4.24	3.42	2.30	5.25	0.0076
PC 16:0/16:1 RT: 7.634	phosphatidylcholine	3.23	-13.1	-13.3	-0.10	-12.40	2.63	2.03	2.82	3.53	2.30	-0.37	0.0263
PC 16:0/18:0	phosphatidylcholine	-10.0	-10.6	-10.9		-9.95	5.93	5.01	5.29	4.96		-0.51	<0.000 001
PC 17:0/22:6	phosphatidylcholine	-17.9	1.20	0.79	-20.7	-17.8		1.16	2.96	2.20	0.87	-0.16	0.0561
PC 18:0/20:4	phosphatidylcholine	6.22	13.87	6.14	4.94	-4.94	13.85		14.34	14.92	13.22	2.69	0.0360
PC 18:1/20:5	phosphatidylcholine	0.34	-0.16	-1.22	-0.32	0.16	-0.52	1.41	0.92	1.52	1.74	-4.23	0.0335
PC 19:1/18:2	phosphatidylcholine	0.09	0.29	-0.25	-0.80	-0.09	0.14	1.48	1.80	1.35	0.46	-6.84	0.0114
PE 38:7	phosphatidylethanolamine	6.55	6.04	6.14		6.54	7.77	7.36	7.26	6.08	7.45	1.14	0.0411
PE 38:8	phosphatidylethanolamine	-0.72	-0.33	-0.70		-0.92	0.77	-0.09	0.15	-0.46	-0.23	-0.04	0.0323
PEtOH 16:0/18:3	phosphatidylethanol	0.56	0.01	11.43	12.82	16.95	-0.01	-0.20	0.64	0.86	-0.33	0.02	0.0443
TG 16:0/18:2/22:6	triglyceride		0.59	0.54	0.44	0.58	0.53	0.27	0.30	0.30	0.39	0.67	0.0225
TG 18:0/18:1/18:2 RT: 5.751	triglyceride	16.14	17.66	14.68	14.81	13.88		0.42	0.42	0.36	0.40	0.03	<0.000 001
TG 18:0/18:1/18:2 RT: 5.752	triglyceride	16.26	17.66	14.67	14.81	15.09	0.48	0.42	0.42	0.36		0.03	<0.000 001
TG 18:1/18:1/22:4	triglyceride	6.31	2.81		4.59	5.13	1.81	2.23	1.29	4.62	2.96	0.55	0.0531
TG 18:2/22:6/22:6	triglyceride	-0.52	-1.54	-2.97		-3.78	-3.42	-14.4	-14.4	-14.5	-2.84	4.51	0.0471
TG 56:8	triglyceride		0.59	0.54	0.44	0.58	0.51	0.27	0.30	0.30	0.39	0.66	0.0156
TG 62:14	triglyceride	-0.51	-1.85	-2.98		-3.78	-3.43	-14.4	-14.4	-14.5	-2.85	4.36	0.0484

RT: retention time

Table 2.5 Abundance of compounds of interest from untargeted lipidomics in serum

Compound	Compound Class	Vehicle					Weekly					Fold Change	P-value
ACar 19:1	acyl carnitine	-1.85	-0.44	-1.10	-2.23	0.00	0.01	-0.15	-0.12	-0.30	-0.18	0.13	0.0491
ACar 20:0	acyl carnitine	-0.04	-0.40		-0.36	-0.59	-0.67	-0.70	-0.85		-0.70	2.08	0.0207
CE 18:3	chloresterol ester	7.95	8.22		8.10	7.92	7.79	7.68	7.78	7.54	7.94	0.96	0.0158
DG 14:0_18:2	diglyceride		-15.26	-15.79	-15.66	-15.85	-15.85	-16.11	-15.95	-16.00	-15.98	1.02	0.0313
DG 16:0_16:1	diglyceride		-18.32	-18.86	-18.72	-18.92	-6.52	-5.22	-6.98	-1.31	-19.04	0.42	0.0146
DG 16:1_18:2	diglyceride	-1.58	-16.09	-16.62	-2.35	-16.68	-3.36	-3.77	-2.32	-2.76	-2.01	0.27	0.0599
DG 18:0_18:1	diglyceride		-17.20	-17.74	-17.60	-17.80	-4.03		-4.40	-1.25	-3.43	0.19	0.000001
HexCer_NS d18:1_16:0	hexosylceramide	-0.51	-0.01		-0.11	-0.24	-0.46	-0.59	-0.55		-0.50	2.40	0.0344
LPC 0:0_22:5 RT: 2.029	lysophosphatidylcholine	-3.38	-2.99	-1.01	-0.20	-2.10	-0.35	-0.94	-0.06	0.06	-0.06	0.14	0.0279
LPC 0:0_22:5 RT: 2.029:1	lysophosphatidylcholine	-3.55	-2.99	-4.47	-0.20	-2.09	-0.41	-0.94	-0.06	0.06	-2.16	0.26	0.0458
LPC 17:1	lysophosphatidylcholine	-0.73	-0.79		-0.64	-0.71	-1.41	-1.00	-0.83	-1.43	-0.85	1.55	0.0371
LPC 22:5	lysophosphatidylcholine	-3.38	-2.99	-4.47	-0.20	-2.09	-0.35	-0.94	-0.06	0.06	-2.16	0.26	0.0462
LPE 18:0 RT: 2.645	lysophosphatidylethanolamine	-0.05	-0.27		-0.25	0.05	-0.47	-0.74	-0.19	-0.34	-0.39	3.27	0.0472
LPE 18:0 RT: 2.645:1	lysophosphatidylethanolamine	-0.05	-0.26		-0.25	0.05	-0.46	-0.74	-0.19	-0.34	-0.39	3.30	0.0479
PC 13:0_20:4	phosphatidylcholine	3.23	3.72		3.69	1.37	-7.72	-8.07	-7.30	-1.52	-5.05	-1.97	0.0005
PC 15:0_18:2	phosphatidylcholine	-0.04	-0.20		0.04	-0.15	-0.20	-0.61	-0.35	-0.51	-0.16	4.05	0.0410
PC 16:0_18:1	phosphatidylcholine	0.35	2.78	1.22	2.93	-1.14	-1.52		-0.87	-2.56	-0.50	-1.11	0.0299
PC 16:0_18:2	phosphatidylcholine	-1.02	-1.89	-4.15	-0.65	-1.17	0.03	-0.03	0.65	-0.35	-0.78	0.06	0.0364
PC 16:0_20:4 RT:7.583	phosphatidylcholine		-13.70	-15.46	-14.11	-14.25	1.71		-0.80	3.04	1.99	-0.10	0.000002
PC 17:0_20:4	phosphatidylcholine	0.25	0.28		0.54	0.18	0.12	-0.11	-0.02	0.02	0.07	0.05	0.0090
PC 18:0_22:4	phosphatidylcholine	0.17	0.44		0.34	0.68	0.11	-0.20	0.03	-0.03	0.30	0.10	0.0284
PC 18:1_22:6 RT:6.877	phosphatidylcholine	-1.99	-2.32		-1.69	-2.36	-2.38	-2.82	-2.20	-2.86	-2.81	1.25	0.0396
PC 38:8	phosphatidylcholine	-0.18	-0.77		0.18	-2.86	-2.00	-17.27	-17.21	-2.24	-17.07	12.30	0.0458
PC 42:6	phosphatidylcholine	-2.09	-3.01	-1.40	-1.44	0.45	0.03	-0.35	0.22	0.06	-0.03	0.01	0.0328
PE 16:0_18:2 RT: 6.947	phosphatidylethanolamine	-14.45	-14.55	-20.00	-1.61	-14.76	-2.28	-0.65	-2.51	1.94		0.07	0.0112
PE 16:1_18:2 RT: 5.490	phosphatidylethanolamine	-3.33	-2.52		-2.69	-3.12	-0.56	-1.95	-2.35	0.56	-2.00	0.43	0.0368
PEtOH 16:0_22:5	phosphatidylethanol	-0.01	-0.37		-0.03	-0.02	-0.26	-0.70	-0.51	-0.60	-0.29	4.47	0.0212
PEtOH 18:1_20:4	phosphatidylethanol	-0.03	-0.20		-0.02	0.01	-0.26	-0.69	-0.53	-0.60	-0.28	7.66	0.0063
PG 16:0_20:4	phosphatidylglycerol	-2.52	-14.40	-4.71	-14.81	-14.94		-3.61	-2.15	-2.79	-1.61	0.25	0.0430
PS 20:4_22:6	phosphatidylserine	-1.26	-0.58	-1.37	-1.68	-0.12	-2.73	-1.24		-1.99	-2.01	1.98	0.0504
SM d40:2	sphingomyelin	-1.88	-1.72	-3.76	-2.70	-1.89	-0.06	-0.28		0.06	0.31	0.00	0.0011
TG 12:0_12:0_16:0	triglyceride	-1.39	-0.84	-1.77	-1.41	-1.66	-2.01	-2.57	-2.54	-2.14	-2.02	1.59	0.0033
TG 12:0_12:2_16:0	triglyceride	-10.14	-9.29	-9.72	-9.69	-9.94	-10.13	-10.28	-10.04	-10.01	-10.10	1.04	0.0442
TG 12:0_12:2_18:2	triglyceride	-9.32	-8.47	-8.90	-8.87	-9.12	-9.32	-9.47	-9.22	-9.19	-9.28	1.04	0.0442

Compound	Compound Class	Vehicle					Weekly					Fold Change	P-value
TG 12:0_16:1_18:2	triglyceride	-3.25	-3.13	-3.58	-2.71	-3.02	-4.03	-4.28	-16.97	-16.94	-17.04	3.78	0.0243
TG 14:0_15:0_16:0	triglyceride	-1.21	-0.36	-0.79	-0.76	-1.01	-1.21	-1.36	-1.11	-1.08	-1.17	1.43	0.0442
TG 14:0_15:0_18:2	triglyceride	-1.57	-0.72	-1.15	-1.12	-1.37	-1.56	-1.71	-1.47	-1.44	-1.53	1.30	0.0442
TG 15:0_16:0_18:2	triglyceride	-1.80	-1.62	-1.70	-1.79	-1.74	-1.83	-1.79	-1.87	-1.80	-1.93	1.07	0.0278
TG 15:0_18:2_22:6	triglyceride	-1.21	-0.36	-0.79	-0.76	-1.01	-1.21	-1.36	-1.11	-1.08	-1.17	1.43	0.0442
TG 16:0_16:0_18:0	triglyceride	-1.21	-0.36	-0.79	-0.76	-1.01	-1.21	-1.36	-1.11	-1.08	-1.17	1.43	0.0442
TG 16:0_18:0_20:4 RT: 13.224	triglyceride	-0.80	-0.61	-0.66	-0.99	-0.56	-17.45	-1.93	-17.35	-1.52	-17.42	15.40	0.0267
TG 18:0_18:1_18:1	triglyceride	-18.19	-17.34	-17.77	-17.74	-17.99	-3.02	-18.33	-18.09	-1.74	-3.93	0.51	0.0482
TG 18:0_18:1_18:2 RT: 5.751	triglyceride	-1.21	-0.36	-0.79	-0.76	-1.01	-1.21	-1.36	-1.11	-1.08	-1.17	1.43	0.0442
TG 18:0_18:1_18:2 RT: 5.752	triglyceride	-1.21	-0.36	-0.79	-0.76	-1.01	-1.21	-1.36	-1.11	-1.08	-1.17	1.43	0.0442
TG 18:1_18:2_19:1	triglyceride	-1.21	-0.36	-0.79	-0.76	-1.01	-1.21	-1.36	-1.11	-1.08	-1.17	1.43	0.0442
TG 18:1_18:2_20:1	triglyceride	-1.21	-0.36	-0.79	-0.76	-1.01	-1.21	-1.36	-1.11	-1.08	-1.17	1.43	0.0442
TG 18:2_18:2_22:1	triglyceride	-1.21	-0.36	-0.79	-0.76	-1.01	-1.21	-1.36	-1.11	-1.08	-1.17	1.43	0.0442
TG 46:4	triglyceride	-1.24	-0.61	-0.79	-0.47	-0.70	-0.85	-1.05	-1.26	-1.30	-1.46	1.56	0.0357
TG 47:1	triglyceride	-9.27	-8.42	-8.86	-8.83	-9.07	-9.27	-9.42	-9.17	-9.14	-9.24	1.04	0.0442
TG 49:2	triglyceride	-1.80	-1.62	-1.70	-1.79	-1.74	-1.83	-1.79	-1.87	-1.80	-1.93	1.07	0.0278
TG 55:4	triglyceride	-1.21	-0.36	-0.79	-0.76	-1.01	-1.21	-1.36	-1.11	-1.08	-1.17	1.43	0.0442
TG 56:2 RT: 11.784	triglyceride	-3.85	-5.99	-6.80	-3.51	-5.60	-0.24	-1.28	0.41	-4.42	-4.40	0.39	0.0304

RT: retention time

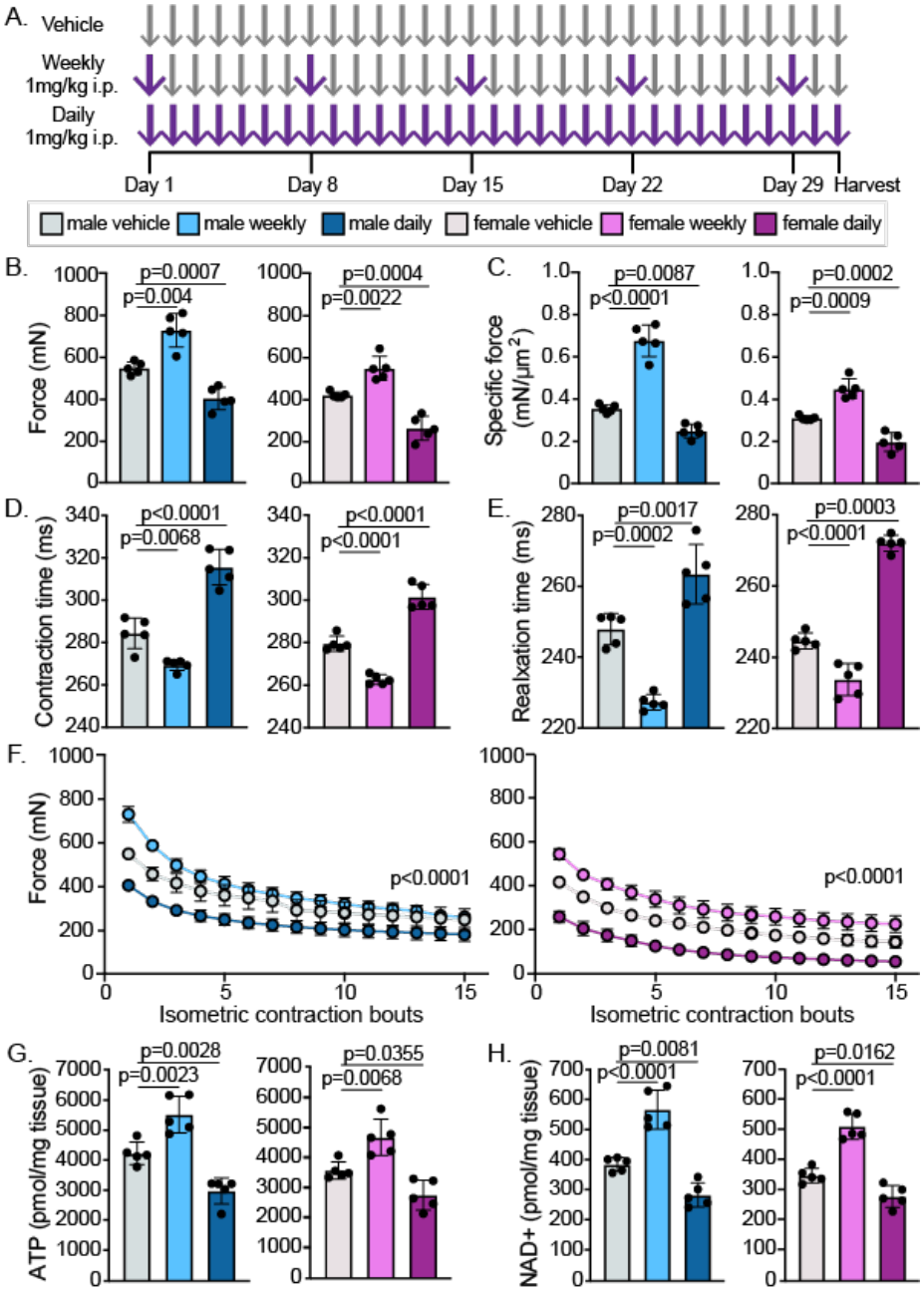

Figure 2.1 Weekly prednisone improved muscle performance in both male and female mice. **(A)** C57BL/6 mice were treated for four weeks with vehicle (DMSO), weekly, or daily injections of prednisone and then analyzed. **(B-F)** Weekly-treated mice exhibited significantly increased maximal tetanic force (B) and specific force (C) and reduced contraction (D) and relaxation (E) time compared to vehicle-treated mice. Once-weekly prednisone-treated mice also exhibited increased response to repetitive force (F) compared to vehicle-treated mice. **(G-H)** Weekly-treated mice had increased concentrations of ATP (G) and NAD+ (H) compared to vehicle-treated animals, while daily-treated mice had significantly reduced concentrations. (A-E, G-H) One-way ANOVA; (F) Two-way ANOVA.

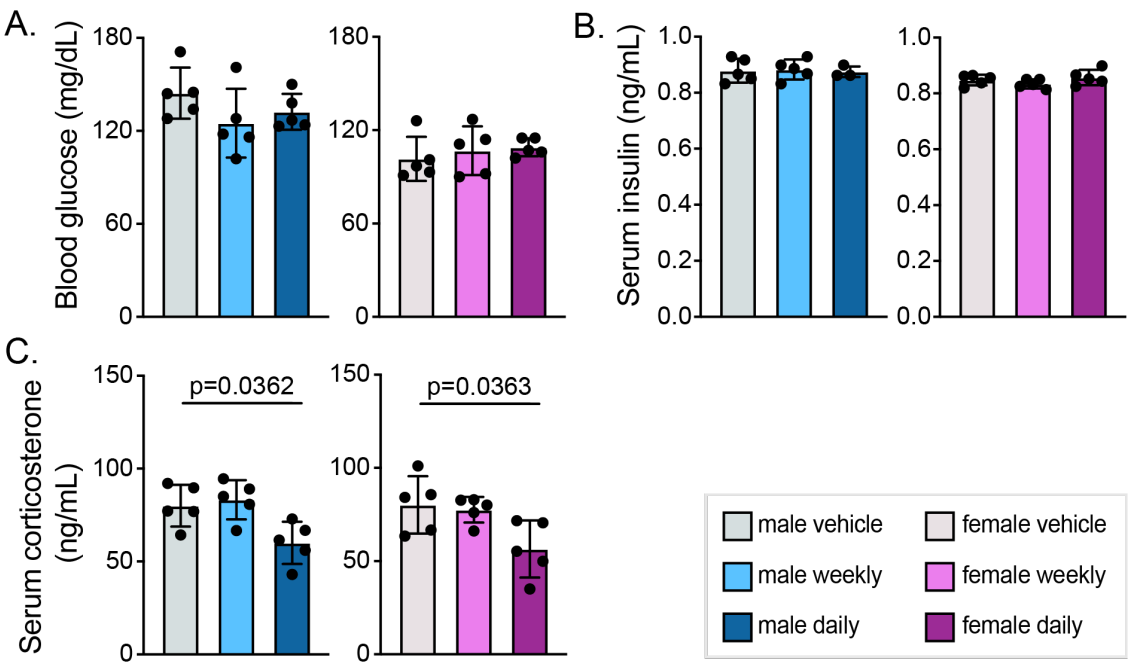

Figure 2.2 Circulating glucose, insulin, and corticosterone were not changed by weekly prednisone. (A-B) Blood glucose (A) and insulin (B) did not change in response to weekly or daily treatment in male or female mice. **(C)** Daily-treated animals of both sexes have reduced circulating corticosterone, but weekly-treated animals did not in comparison to vehicle-treated. (C) One-way ANOVA.

in both male and female mice, leading to reduced max tetanic and specific force, increased contraction and relaxation time, and reduced force over consecutive isometric contraction bouts (Figure 2.1B-F). In contrast, weekly prednisone treatment improved muscle performance in all metrics, suggesting that, unlike daily prednisone, weekly exposure to prednisone did not induce atrophy. Weekly prednisone-treated males and females had significantly more ATP and NAD+ per milligram of tissue compared to vehicle-treated animals (Figure 2.1G-H). Daily-treated animals had significantly less ATP and NAD+ than vehicle-treated animals, reflecting their atrophic state. Neither daily nor weekly prednisone treatment influenced blood glucose or serum insulin levels (Figure 2.2A-B). Chronic treatment with exogenous glucocorticoids is known to suppress endogenous glucocorticoids, and we indeed found that daily treatment resulted in a significant reduction in circulating corticosterone (Figure 2.2C). Weekly prednisone

did not suppress circulating corticosterone. To evaluate the effect of weekly prednisone on fully mature animals, we separately treated a cohort of 18-week-old mice for four weeks and found that they responded to weekly prednisone similarly (Figure 2.3).

Glucocorticoids work primarily through their receptor, GR, a nuclear receptor transcription factor. We hypothesized that the differences we observed between daily and weekly prednisone treated animals was the result of distinct transcriptional profiles. To investigate this, we performed RNA sequencing on myofibers isolated from quadriceps muscle of male and female animals from each treatment group. After filtering for baseline expression, variability, and log2 fold change, in daily prednisone-treated muscle 1777 genes were changed in males and 1224 genes in females compared to vehicle-treated animals of each sex (Figure 2.4A). Less than half the genes were shared between the sexes (Figure 2.4B). In contrast, in weekly prednisone-treated muscles, 407 and 406 genes were changed in males and females, respectively (Figure 2.6A), and the majority were unique to one sex (Figure 2.6B). We asked how many genes had a divergent response to the two treatments, i.e. upregulated by one and downregulated by the other, and the vast majority of genes had the same directionality of transcriptional response after daily and weekly prednisone (quadrants 2 and 4, Fig 4C), suggesting that the transcriptional profiles were overall similar between daily and weekly treatment. We did find that daily versus weekly prednisone had distinct effects on the expression of the canonical mediators of glucocorticoid-induced atrophy. Expression of the genes encoding MuRF-1 (*Trim63*) and atrogin-1 (*Fbxo32*) was unchanged in weekly-treated muscle compared to vehicle-treated, but these atrophy genes were upregulated in daily-treated muscle (Figure 2.4D). In daily prednisone-treated muscle, we observed strong downregulation of the genes encoding the mitochondrial respiratory complex (Figure 2.4E). Taken together, these results suggest that daily and weekly prednisone treatment elicit some similar

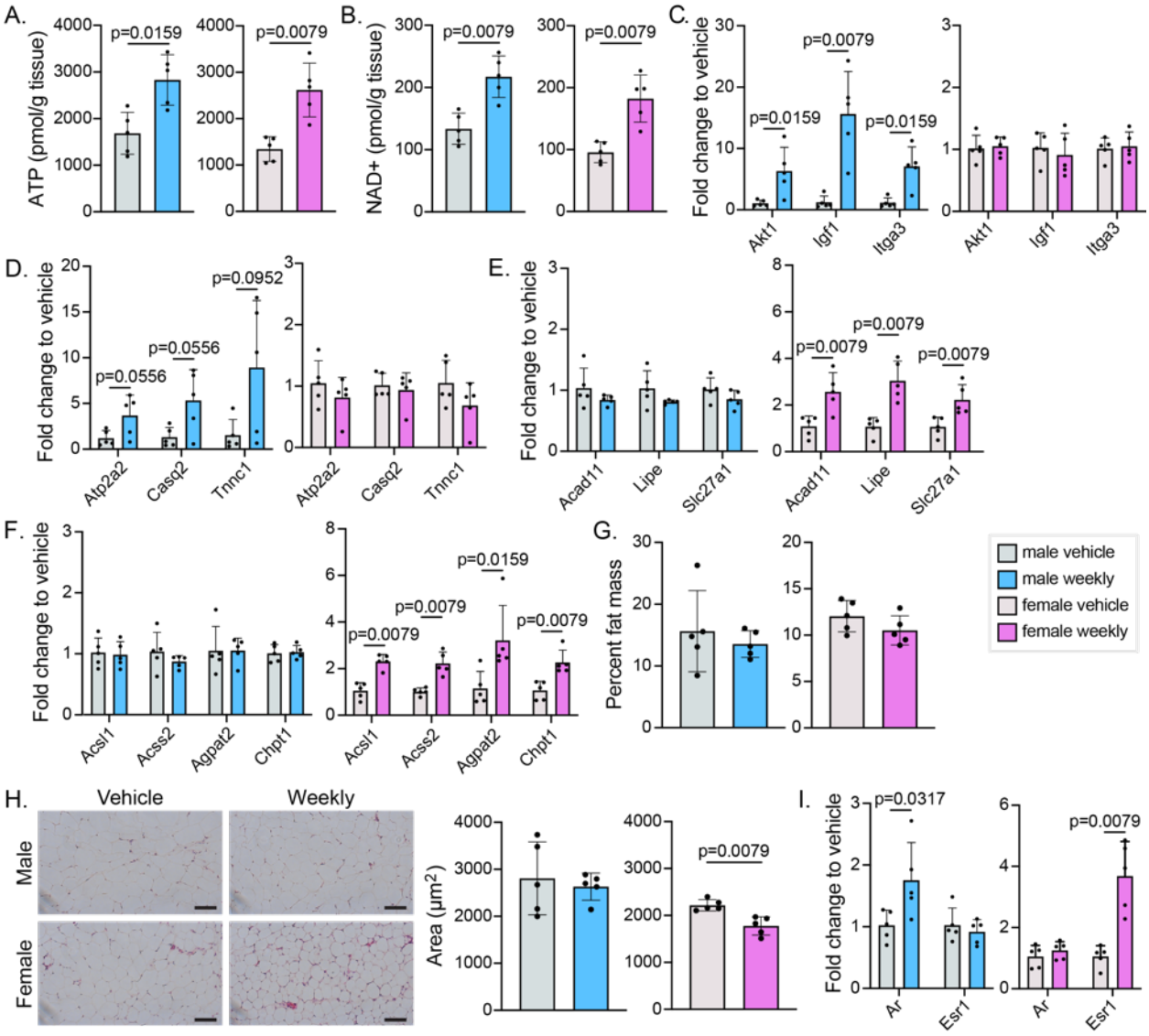

Figure 2.3 Weekly prednisone exerted the same effect in 18-week-old mice as 10-week-old. (A-B) Weekly-treated mice had increased concentrations of ATP (A) and NAD+ (B) compared to vehicle-treated animals. **(C-F)** Animals administered weekly prednisone starting at 18 weeks had sex-specific upregulation of IGF1 pathway (C), calcium-handling (D), and lipid metabolism (E-F) genes. **(G-H)** Although whole body percent fat mass did not change after four weeks of treatment (G), visceral fat pad adipocytes had significantly reduced cross-sectional area in females (H). **(I)** 18-week-old mice had sex-specific upregulation of the genes encoding the sex steroid receptors. (A-I) Mann-Whitney; black bar = 100μm.

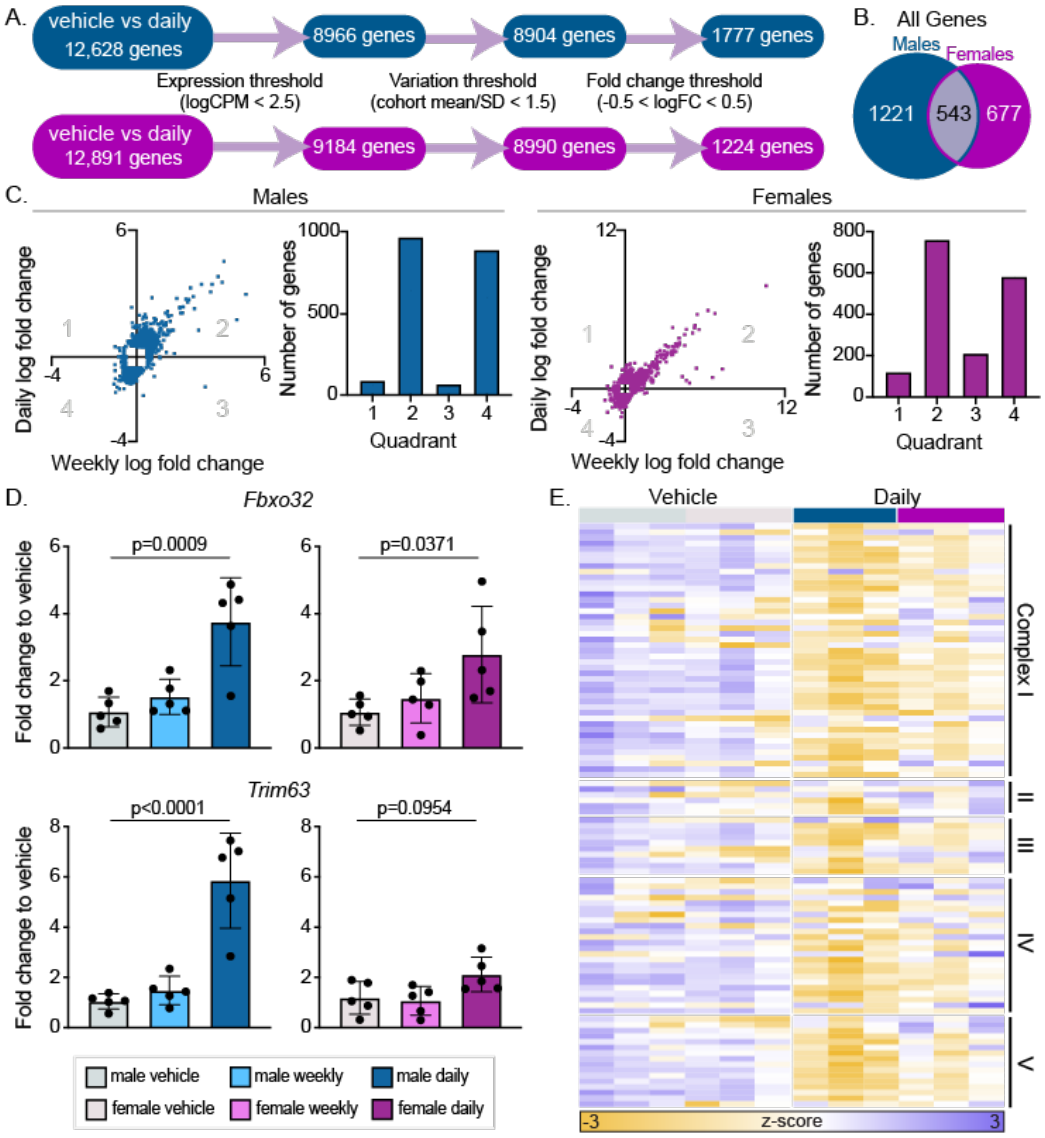

Figure 2.4 Daily and weekly prednisone treatment elicited similar transcriptional profiles with differential atrogene activation. **(A)** RNA sequencing analysis of daily-prednisone treated muscles (quadriceps) compared to vehicle-treated for both sexes; prednisone-responsive genes were identified as being above expression and fold change thresholds and below a variation threshold; n=3 animals per group. **(B)** Less than half of all prednisone-responsive genes were shared among daily prednisone-treated males and females. **(C)** The majority of prednisone-responsive genes above a log2 fold change threshold had the same response to both daily and weekly treatment, i.e. increased (quadrant 2) or decreased (quadrant 4) expression. **(D)** Expression of atrogenes *Fbxo32* and *Trim63* was significantly increased in daily-treated muscle fibers compared to vehicle as evaluated by qPCR and one-way ANOVA, while weekly-treated muscle fibers had no change in expression of these atrogenes. **(E)** Expression of genes encoding the mitochondrial respiratory chain was decreased in response to daily treatment in both sexes compared to vehicle treatment.

transcriptional effects in skeletal muscle but daily prednisone exposure is distinguished by its atrophic gene expression pattern.

Having observed similarities in the transcriptional response between daily and weekly prednisone, we were curious if administering prednisone twice a week would improve upon weekly prednisone or produce an effect more similar to daily exposure. We treated male and female mice with prednisone twice a week for three weeks (Figure 2.5A). With twice-weekly treatment, both sexes had significantly more skeletal muscle NAD+ compared to vehicle, while only female muscle had significantly increased ATP (Figure 2.5B-C). Genes transcriptionally responsive to once-weekly prednisone were similarly responsive to twice-weekly (Figure 2.5D-G), suggesting that while twice-weekly prednisone did not improve upon once-weekly, it also did not induce atrophy. This was further confirmed by qPCR for *Fbxo32* and *Trim63*, which were not upregulated after twice-weekly prednisone treatment (Figure 2.5H).

2.4.2 Males and females have differing transcriptional responses to weekly glucocorticoid treatment

Most of the transcriptional response to weekly prednisone treatment was through increased gene expression, suggesting weekly glucocorticoids can be considered primarily as an activating stimulus. Of those upregulated genes, the majority (87.2% in females, 88.4% in males) were unique to one sex (Figure 2.6B). Genes upregulated in weekly prednisone-treated females were predominantly involved in lipid metabolism pathways (dark purple, Fig 2.6C); however, genes upregulated in weekly-treated males were involved in muscle function and growth pathways such as PI3K/AKT signaling and calcium regulation (orange, Fig 2.6C). To identify any associations between the transcriptional and physiological responses to weekly prednisone, we applied Pheno-RNA, a method that links genes to phenotypic outcomes (Darwiche and Struhl 2020). Compared to a group of control genes, genes responsive to

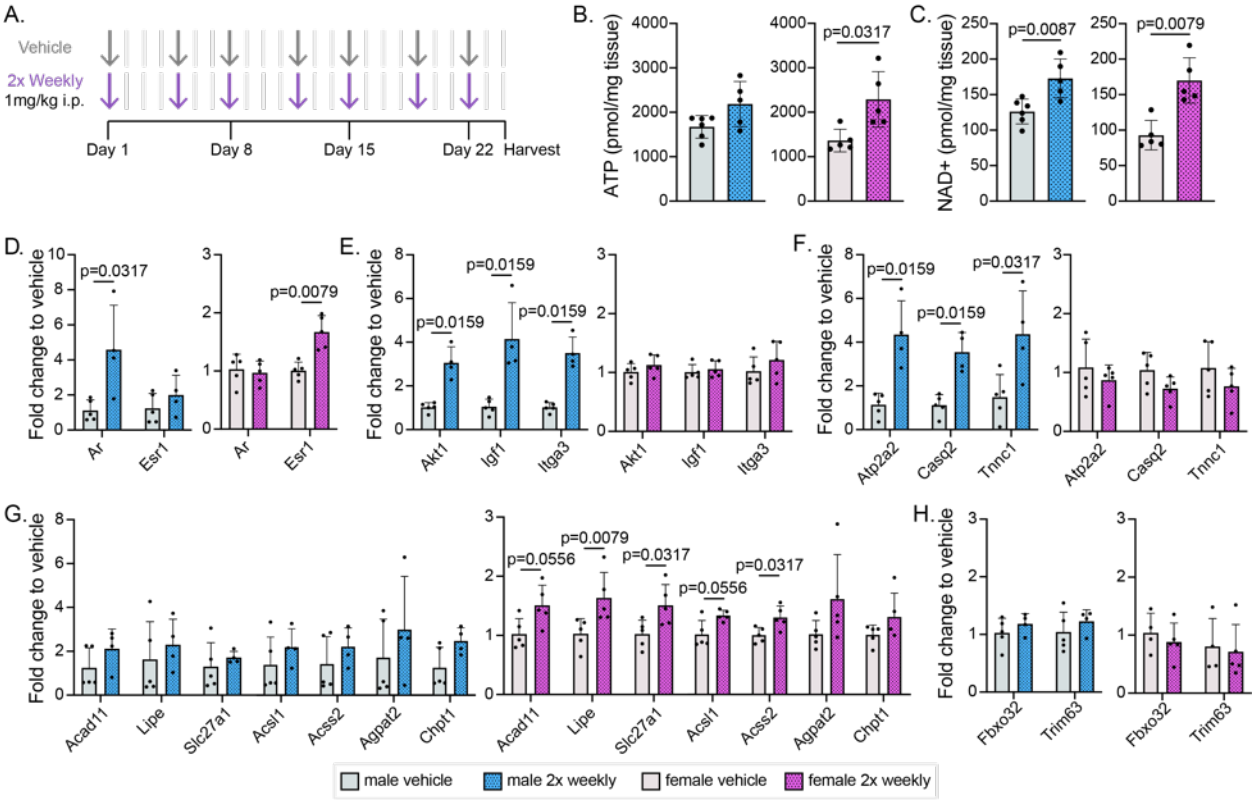

Figure 2.5 Twice-weekly prednisone exerted some of the same effects as once-weekly. **(A)** C57BL/6 mice were treated for three weeks with vehicle (DMSO) or prednisone twice a week and then analyzed. Arrows indicate i.p. injection; bars indicate no injection. **(B-C)** Female mice treated twice-weekly had increased concentrations of ATP (B) and NAD+ (C) compared to vehicle-treated animals, while males only exhibited increased NAD+. **(D-H)** Animals administered twice-weekly prednisone had sex-specific upregulation of sex steroid receptor (D), IGF1 pathway (E), calcium-handling (F), and some lipid metabolism (G) genes. **(H)** Atrogene expression was not upregulated by twice-weekly prednisone. (A-H) Mann-Whitney.

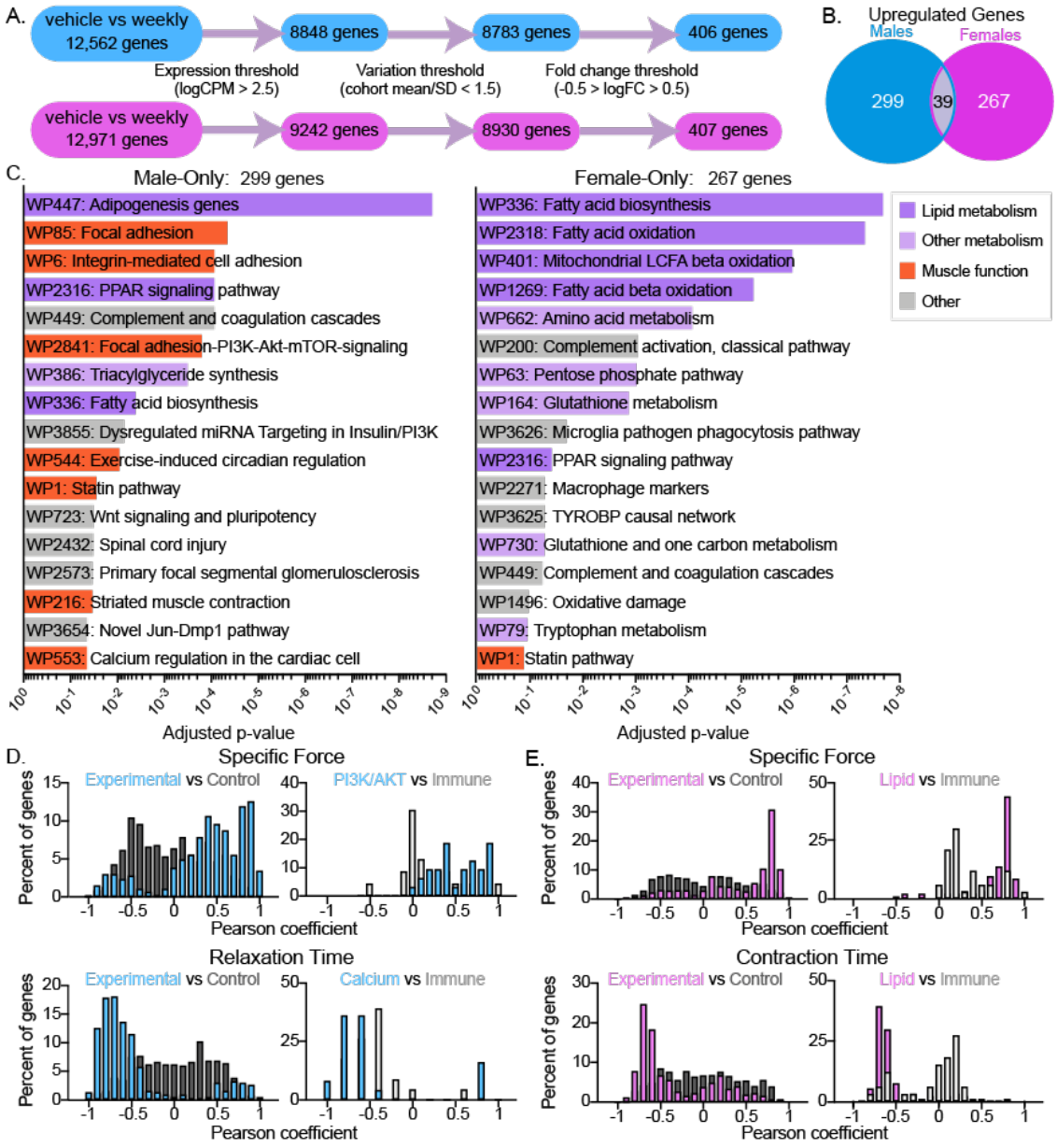

Figure 2.6 Weekly prednisone elicited distinct transcriptional responses in male versus female muscle. **(A)** Transcriptome comparison of once weekly prednisone-treated muscle to vehicle-treated for both sexes; prednisone-responsive genes were identified as being above expression and fold change thresholds and below a variation threshold. N=3 animals per group. **(B)** The majority of prednisone-responsive upregulated genes were unique to one sex. **(C)** Gene ontology of male- and female-only upregulated genes shows differential pathway enrichment. **(D-E)** Pheno-RNA analysis of genes shows high correlation between gene expression and physiological response in males (D) and females (E). Genes responsive to weekly prednisone are more correlated with physiological response than a shuffled control group and genes in pathways of interest are more correlated with physiological response than prednisone-responsive genes involved in other cellular processes.

weekly prednisone were more highly correlated with specific force and relaxation time in males (Figure 2.6D) and specific force and contraction time in females (Figure 2.6E). When we asked how well certain pathways correlated with physiological outcome, we found that PI3K/AKT signaling genes in males were more correlated with specific force than immune signaling genes, despite both pathways being significantly upregulated in response to weekly prednisone. Calcium-handling genes were more correlated with relaxation time than immune signaling genes in males. In females, lipid metabolism genes were more correlated with specific force and contraction time than immune signaling genes. Overall, we found that sex-specific transcriptional responses to weekly prednisone highly correlated with improvements in skeletal muscle performance.

The IGF1/PI3K pathway is upstream of mTOR and is known to regulate myofiber hypertrophy (Coleman et al. 1995, Musarò et al. 2001, Lai et al. 2004). Quantitative RT-PCR confirmed upregulation of IGF1/PI3K pathway member genes in male but not female muscle (Figure 2.7A). mTOR is downstream of IGF1/PI3K, where it phosphorylates target proteins S6K and 4EBP1 (Ma and Blenis 2009). Total protein levels of S6K and 4EBP1 were significantly increased in weekly-treated male muscle but were decreased in weekly prednisone-treated female muscle (Figure 2.7B). Levels of phosphorylated 4EBP1 were slightly increased in weekly prednisone-treated male muscles, resulting in a maintained ratio of p4EBP1/4EBP1. Female muscle had an increase in the ratio of p4EBP1/4EBP1, perhaps to compensate for a reduction in total 4EBP1. We measured myofiber cross-sectional area in the tibialis anterior muscle, as an indicator of hypertrophy; weekly prednisone-treated males trended towards increased cross-sectional area compared to vehicle-treated males, while there was no difference between female groups (Figure 2.7C).

Gene ontology of genes upregulated in male muscle also identified enrichment of genes involved in calcium handling (Figure 2.6C). Quantitative RT-PCR confirmed that genes

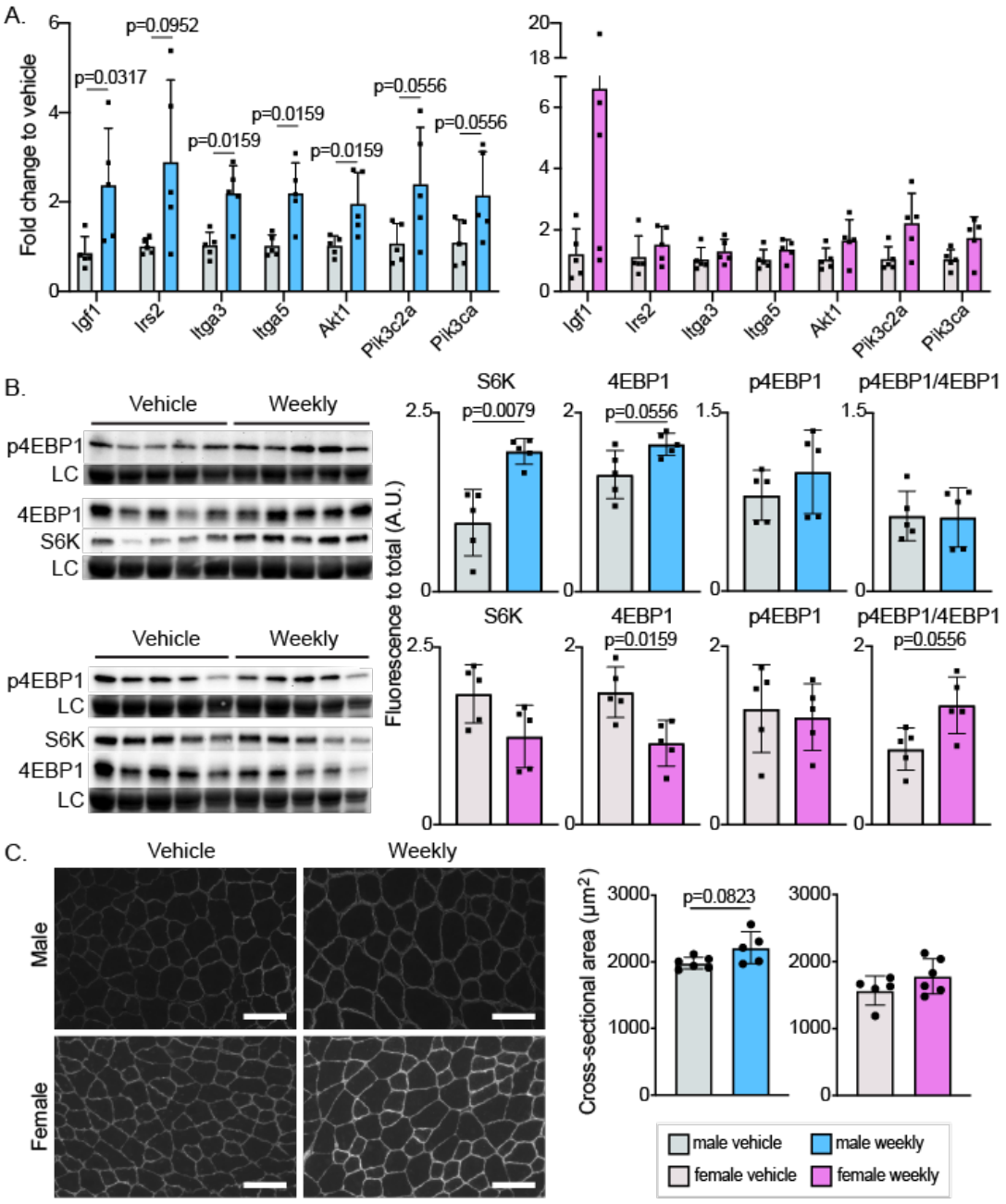

Figure 2.7 Weekly prednisone treatment activated the IGF1/PI3K pathway in males but not females. (A) Genes encoding IGF1/PI3K pathway members had increased expression in the muscle (quadriceps) of weekly-treated male muscle but not female muscle by qPCR. **(B)** Total protein levels of mTOR targets S6K and 4EBP1 were increased in the gastrocnemius of weekly-treated males but decreased in weekly-treated females. LC = loading control. **(C)** Myofiber cross-sectional area in the tibialis anterior trended larger in weekly-treated compared to vehicle-treated males but was unchanged in females. (A-C) Mann-Whitney; white bar = 100μm.

encoding SERCA2 (*Atp2a2*), calsequestrin (*Casq2*), troponin (*Tnnc1, Tnni1, Tnnt1*), and the $Ca_v3.2$ channel (*Cacna1h*) were upregulated by weekly glucocorticoid treatment in males but were unchanged or downregulated in females (Figure 2.8A). Protein levels of calsequestrin isoforms 1 and 2 were not changed in response to weekly treatment in males, although SERCA2 did appear to be elevated (Figure 2.8B). In contrast, levels of both calsequestrin isoforms were reduced in weekly-treated females. To evaluate how these gene expression and protein changes might impact calcium handling, we investigated calcium transients at 40Hz in isolated flexor digitorum brevis myofibers. Weekly-treated males had an increased calcium release rate compared to vehicle-treated males, while females had no change (Figure 2.8C). Weekly-treated males also had more fibers reach 50% rise with the first electrical stimulus compared to vehicle-treated males, while weekly-treated females had fewer (Figure 2.8D). Overall, these data suggest that males had improved calcium handling and increased IGF1/PI3K pathway activity in response to weekly glucocorticoid treatment, while females did not.

2.4.3 Weekly treatment changes lipid metabolism in females resulting in increased endurance

Gene ontology of genes upregulated in female muscle after weekly prednisone highlighted enrichment of lipid metabolism-related pathways (Figure 2.6C). Although expression of some lipid metabolism genes was slightly increased in weekly-treated males, weekly-treated females exhibited a unique and strong upregulation of genes involved in beta oxidation, lipid droplet formation, lipolysis, and fatty acid binding and transportation (Figure 2.9A). These expression shifts were confirmed by quantitative RT-PCR (Figure 2.10). After four weeks of glucocorticoid treatment, weekly-treated females had decreased whole body percent fat mass while weekly-treated males were unchanged compared to vehicle-treated

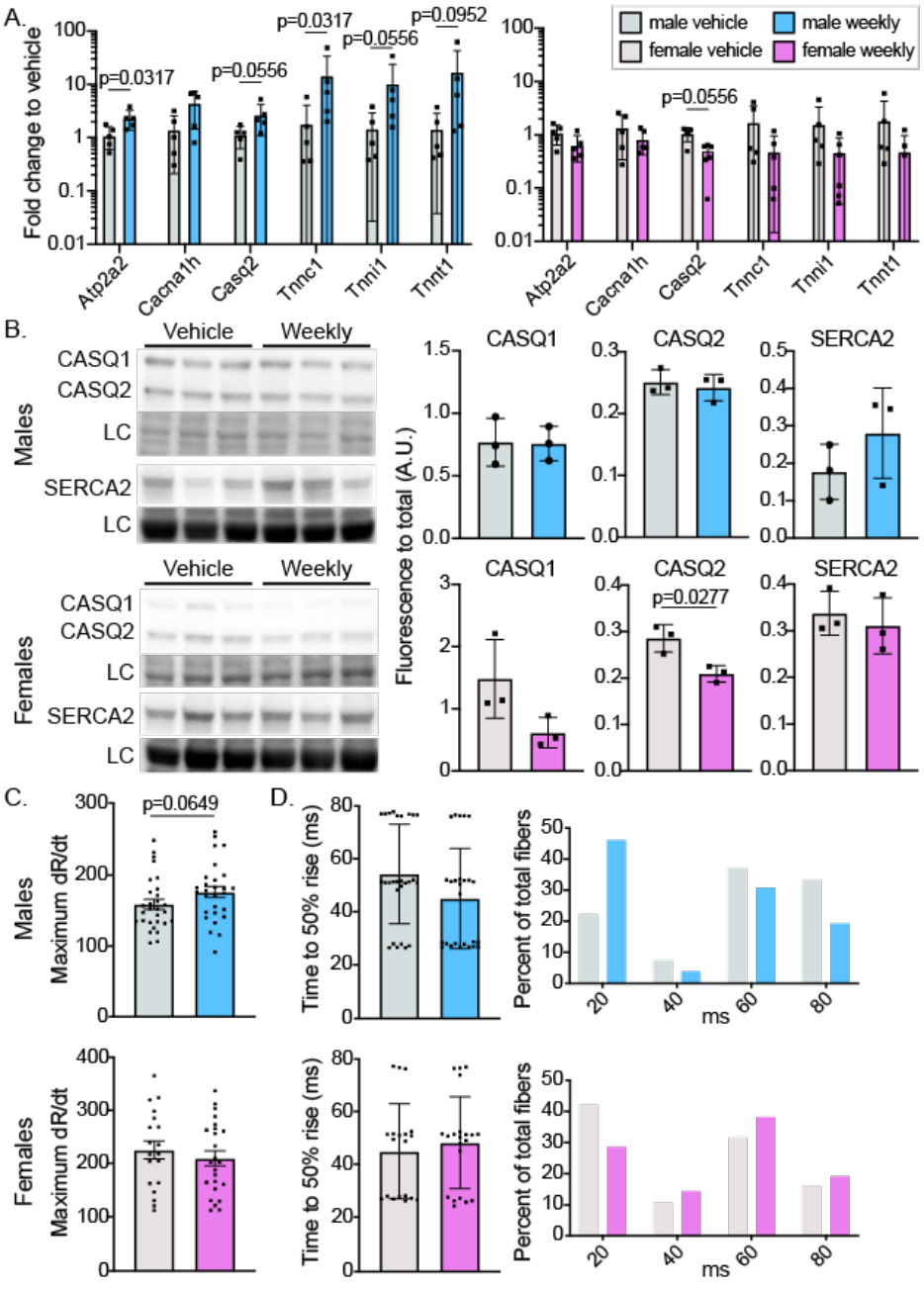

Figure 2.8 Weekly prednisone treatment improved calcium handling in males but not females. (A) Genes encoding calcium handling proteins were upregulated in the quadriceps of weekly-treated males but downregulated in weekly-treated females by qPCR. **(B)** Protein levels of CASQ1, CASQ2, and SERCA2 were unchanged or increased in the gastrocnemius of weekly-treated males, but decreased in weekly-treated females compared to vehicle-treated animals. LC = loading control. **(C-D)** Calcium transient measurements in isolated flexor digitorum brevis fibers trended toward a faster maximum rate of calcium release (C) and time to 50% rise (D) in weekly-treated males and no change in weekly-treated females compared to vehicle-treated animals. (A,C) Mann Whitney, (B) Welch's t-test.

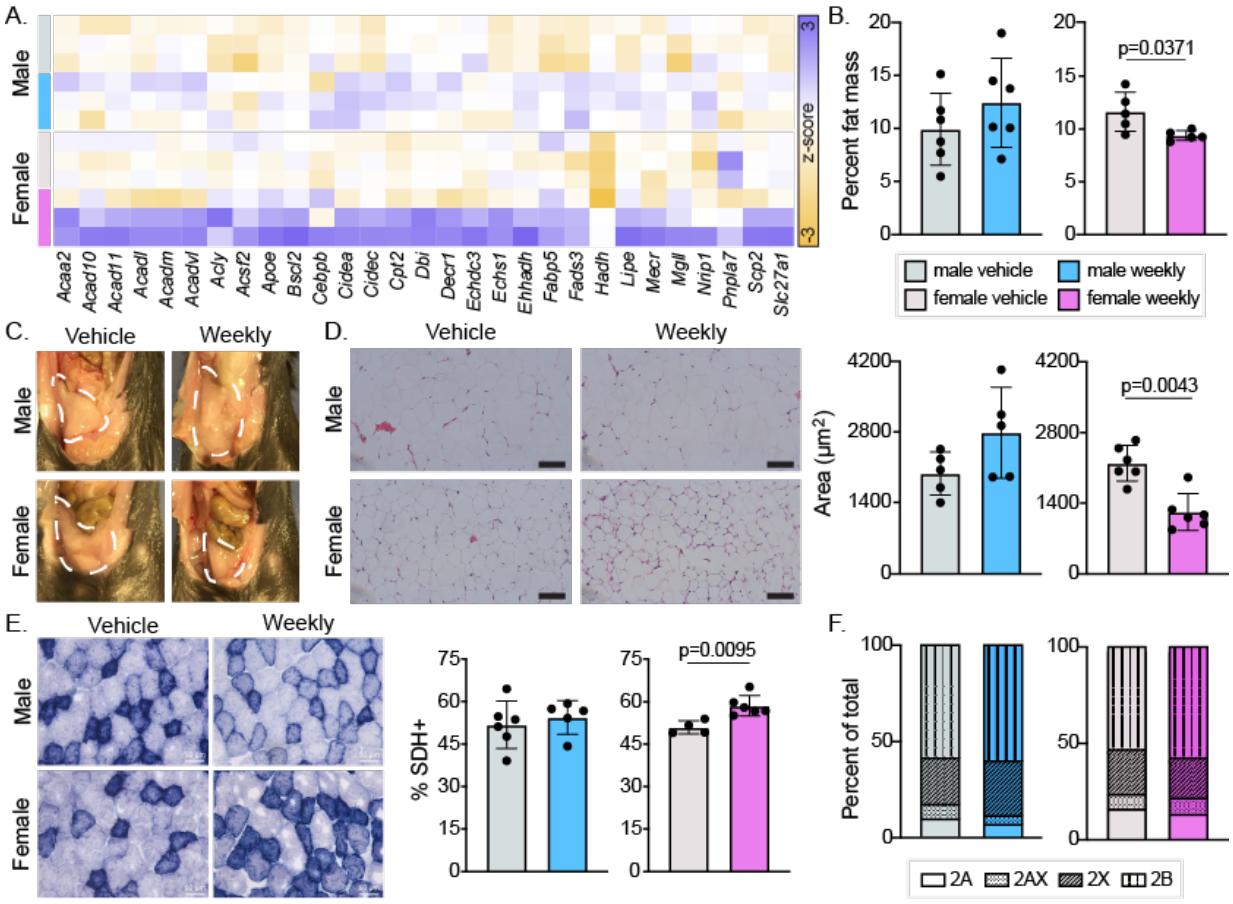

Figure 2.9 Weekly prednisone treatment improved lipid metabolism in females but not males. (A) Results from RNA sequence analysis revealed increased expression of lipid metabolism-related genes in weekly-prednisone treated females compared to vehicle-treated females and weekly-prednisone treated males. **(B)** Body composition analysis after four weeks of treatment demonstrated reduced whole body percent fat mass in once weekly prednisone treated females, while comparably treated males had no change. **(C-D)** The visceral fat pad of weekly-treated females was smaller than vehicle-treated females and weekly-treated males (C) and the adipocytes in this fat pad had significantly reduced cross-sectional area (D). **(E)** Weekly-treated females had an increased proportion of SDH+ fibers in the tibialis anterior compared to vehicle-treated females while the males exhibited no change. **(F)** Neither sex had changes in fiber type proportion following weekly treatment. (A-B, D-E) Mann-Whitney; black bar = 100μm; white bar = 50μm.

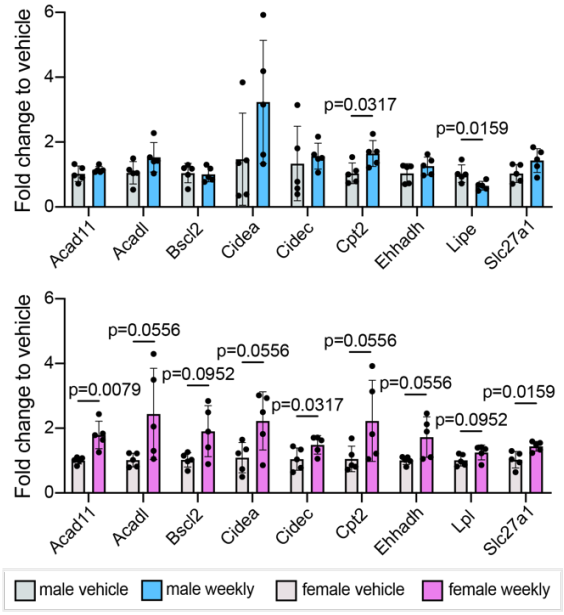

Figure 2.10 qPCR validation of lipid metabolism genes. Lipid metabolism genes were significantly upregulated in weekly-treated females but were mostly unchanged in weekly-treated males. P-value determined by Mann-Whitney.

animals (Figure 2.9B). These differences could be seen in the size of the visceral fat pad, which was much smaller in weekly-treated females compared to vehicle-treated females and all males (Figure 2.9C). Interestingly, this difference appears to have been driven by adipocyte size; the cross-sectional area of adipocytes in the visceral fat pad was significantly reduced in weekly-treated females (Figure 2.9D). Reflecting a change in metabolic state, an increased percentage of myofibers in the tibialis anterior muscles of weekly-treated females were positive for succinate dehydrogenase enzymatic activity (Figure 2.9E), without any associated change in fiber type (Figure 2.9F).

To investigate the impact of weekly prednisone and associated changes in lipid metabolism on the skeletal muscle lipid profile, we performed untargeted lipidomics on whole quadriceps from vehicle- and weekly-treated females. Overall, only 26 (4.8%) of 536 lipid compounds profiled were significantly different between vehicle- and weekly-treated females,

most of which were glycerides and phospholipids. Triglycerides are the predominant form of stored lipid in skeletal muscle and are funneled into beta oxidation through their catabolism into free fatty acids. Quantitative RT-PCR demonstrated upregulation of genes encoding enzymes involved in glyceride catabolism and acyl-CoA synthesis after weekly prednisone (Figure 2.11A). Di- and triglyceride species were significantly decreased in weekly-treated females (Figure 2.11B, Table 2.4), further suggesting that this pathway was activated by weekly prednisone. The phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the main components of skeletal muscle mitochondrial membranes (Tsalouhidou et al. 2006, Stefanyk et al. 2010). Genes encoding key enzymes involved in the synthesis of PC were upregulated by weekly prednisone (Figure 2.11C), and PC and PE species were significantly increased in weekly-treated females (Figure 2.11D, Table 2.4). These data indicate a subtle but significant shift in skeletal muscle lipid metabolism following weekly prednisone.

We next texted whether endurance exercise, which is known to be influenced by lipid metabolism (Hurley et al. 1986, Mendenhall et al. 1994, Phillips et al. 1996), was altered by this drug regimen. Based on the observed profile in weekly prednisone-treated female mice, we tested whether exercise endurance was altered by this drug regimen. We treated a cohort of female mice with prednisone for three months and then assessed the running distance to exhaustion at three time points. The previously noted shift in body composition towards increased lean mass was maintained through three months of treatment (Figure 2.12A). Weekly-treated females had a significantly increased distance to exhaustion after six months of weekly prednisone (Figure 2.12B) with fewer stimuli required per kilometer run (Figure 2.12C). Although their visceral fat pad was no longer significantly smaller than vehicle-treated females after nine months of treatment (Figure 2.12D), weekly-treated females still had significantly smaller adipocytes (Figure 2.12E). Some but not all the of lipid metabolism genes transcriptionally upregulated by four weeks of weekly prednisone were still upregulated in

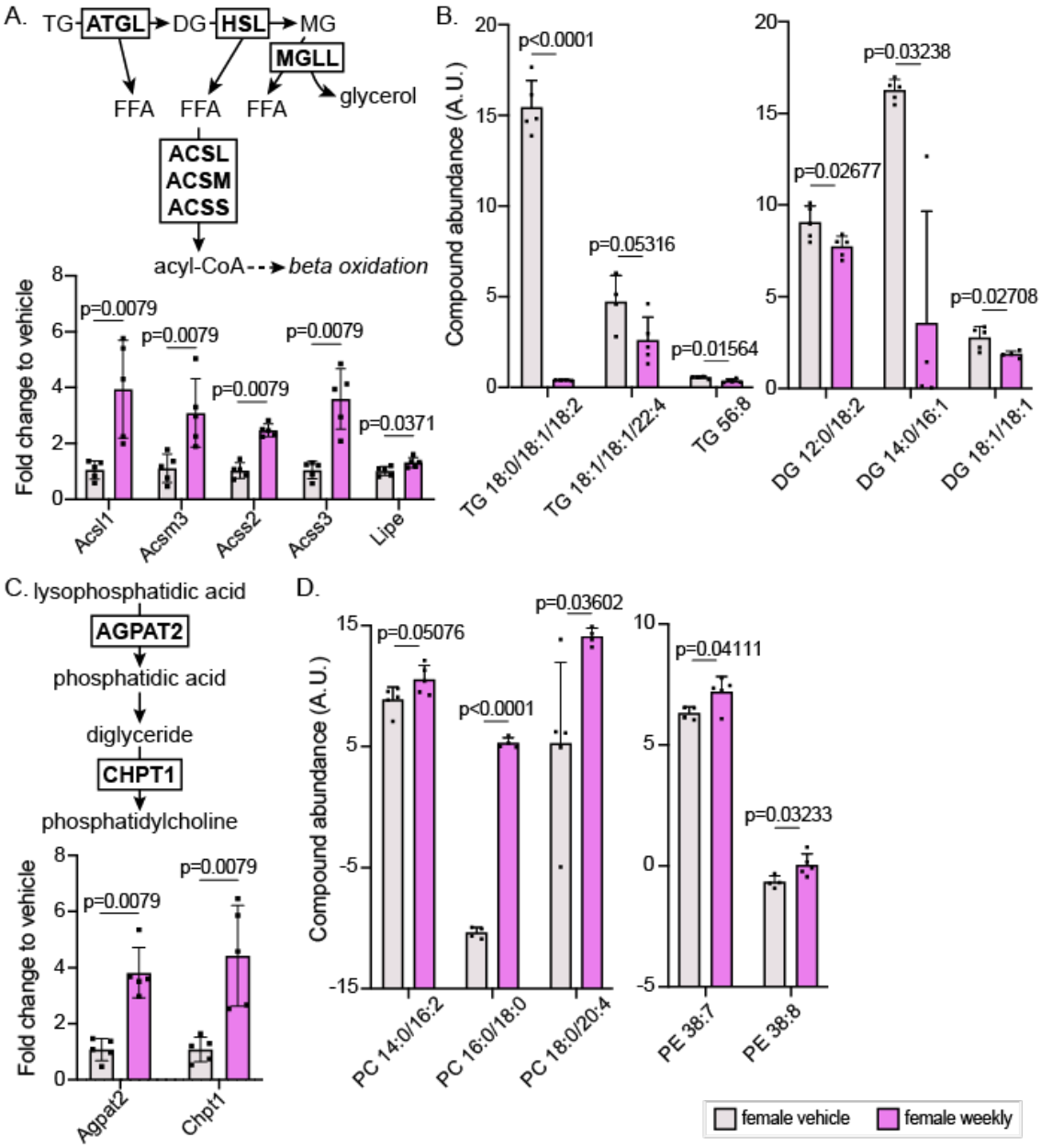

Figure 2.11 Weekly prednisone-treated females had increased glyceride catabolism and mitochondrial phospholipid abundance. **(A)** Muscles (quadriceps) from weekly-prednisone treated females had significantly increased expression of enzymes involved in triglyceride catabolism and acyl-CoA synthesis. **(B)** Females also had significantly reduced abundance of triglyceride and diglyceride species in the quadriceps. **(C)** Enzymes involved in phosphatidylcholine synthesis were significantly upregulated by weekly prednisone treatment. **(D)** Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) abundance was increased in weekly-prednisone treated females compared to vehicle-treated. Top three most abundant lipid compounds are presented as [number of carbon atoms]:[number of double bonds]; less abundant compounds listed in **Table 2.4**. (A,C) Mann-Whitney; (B,D) Welch's t-test.

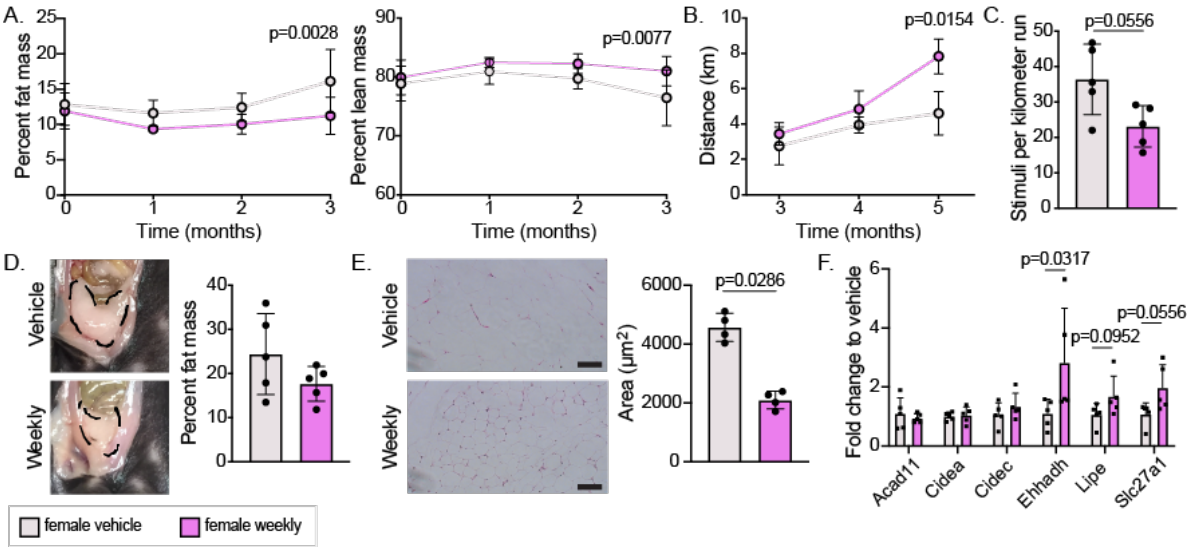

Figure 2.12 Long-term weekly prednisone treatment increased endurance in female mice. **(A)** Weekly-prednisone treated females had reduced whole body percent fat mass and increased percent lean mass after three months of treatment. **(B)** Weekly-prednisone treated females showed increased distance to exhaustion in a treadmill run-to-exhaustion test compared to vehicle-treated females. **(C)** Weekly-prednisone treated females required fewer stimuli per kilometer run than vehicle-treated. **(D)** After nine months of treatment, weekly-treated females had a slightly smaller visceral fat pad and moderately reduced percent fat mass than vehicle-treated females. **(E)** Adipocytes in the visceral fat pad of weekly-treated females had significantly reduced cross-sectional area compared to vehicle-treated females. **(F)** Some lipid metabolism genes continued to be upregulated in response to weekly prednisone. (A-B) Two-way ANOVA; (C, E-F) Mann-Whitney; black bar = 100μm.

female muscle after nine months of treatment (Figure 2.12F). Overall, we observed significant changes in the lipid metabolism of weekly-treated females, including increases in expression of beta oxidation genes, triglyceride catabolism, and abundance of mitochondrial membrane phospholipids as well as decreased whole body percent fat mass, resulting in improved endurance.

2.4.4 The sex-specific response to weekly prednisone is abrogated by sex steroid antagonism

Glucocorticoids regulate target gene expression through binding to GREs, a process moderated by many co-factors including the androgen receptor (AR) and estrogen receptor

(ER). Both testosterone and estrogen were slightly increased in the serum in response to weekly treatment (Figure 2.13A) and expression of the genes encoding their receptors was sex-specifically responsive to weekly prednisone (Figure 2.13B). To investigate the role of these receptors in the response to weekly prednisone, we co-treated male mice with the AR competitive antagonist flutamide and female mice with the ER competitive antagonist fulvestrant in addition to vehicle or weekly prednisone starting at 10 weeks of age (Figure 2.13C). Four weeks of daily flutamide treatment significantly reduced body weight and prostate wet weight, while daily fulvestrant significantly reduced uterus wet weight but did not affect body weight (Figure 2.14A-B). When administered in conjunction with sex steroid antagonists, weekly prednisone failed to increased ATP and NAD+ in muscle (Figure 2.13D, E) and did not reduce whole body percent fat mass or adipocyte cross-sectional area in female mice (Figure 2.13F, Fig 14C). Weekly prednisone did not stimulate upregulation of *Ar* (Figure 2.13G), IGF1/PI3K pathway (Figure 2.13H), or calcium-handling genes (Figure 2.13I) in males co-treated with flutamide, suggesting that AR is required for the male-specific transcriptional response to weekly prednisone. Intriguingly, both *Ar* and *Esr1* were upregulated in females co-treated with fulvestrant (Figure 2.13G), as were the IGF/PI3K pathway members *Igf1* and *Itga3* (Figure 2.13H) and the lipid transporter *Slc27a1* (Figure 2.13J). Other lipid metabolism genes previously demonstrated to be responsive to weekly prednisone were not upregulated when females were co-treated with fulvestrant, suggesting that only part of the female-specific transcriptional response to weekly prednisone is modulated by ER.

2.4.5 Weekly prednisone induces sex-specific chromatin changes

To further investigate the sex-specific response to weekly prednisone, we performed chromatin immunoprecipitation (ChIP) for GR using vehicle- and weekly-treated muscles. We investigated established or likely GREs in the following target genes: *Cidea, Cidec, Esr1,*

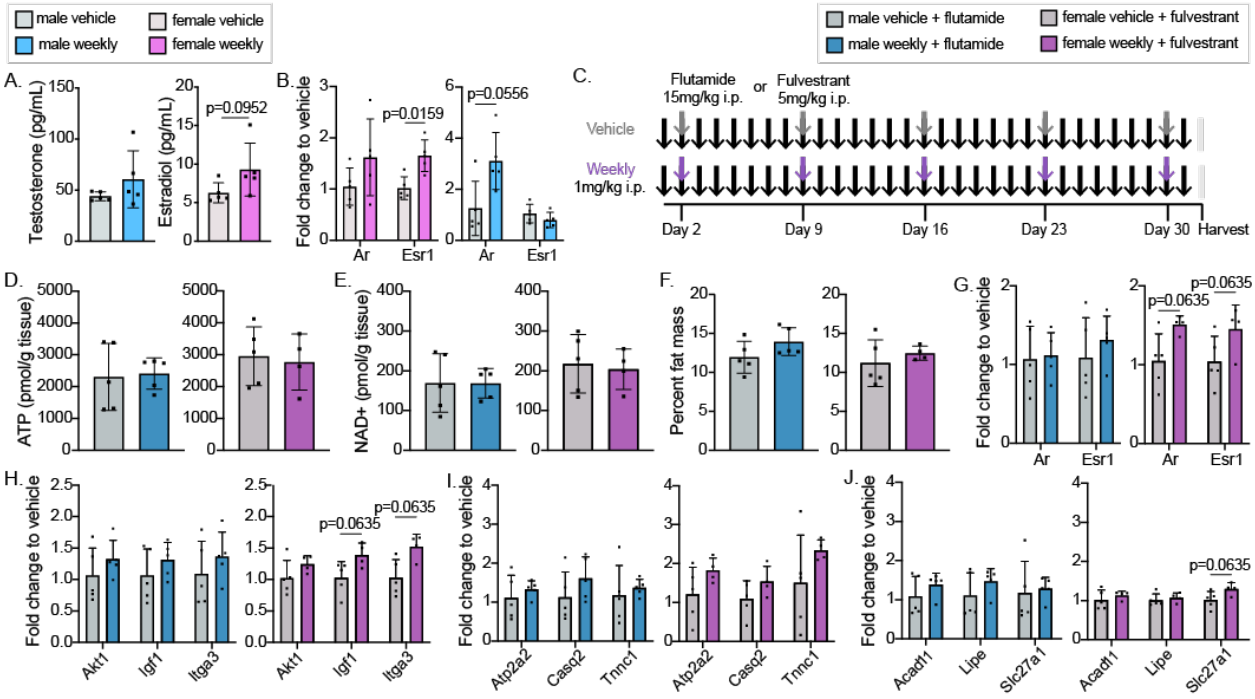

Figure 2.13 Sex steroid receptor antagonism attenuated the effects of weekly prednisone. **(A)** Circulating levels of testosterone and estrogen were increased in weekly-prednisone treated animals. **(B)** Expression of the gene encoding the androgen receptor (*Ar*) was increased in weekly prednisone-treated males, while expression of the gene encoding estrogen receptor α (*Esr1*) was increased in weekly-treated females. **(C)** C57BL/6 mice were co-treated for four weeks with sex steroid inhibitors (males: flutamide, females: fulvestrant). Concomitantly, half of the cohort received weekly prednisone or vehicle. Arrows indicate i.p. injections. **(D-E)** Weekly-prednisone treated mice had no change in concentrations of ATP (D) and NAD+ (E) compared to vehicle-treated animals. **(F)** Weekly-treated animals had no change in whole body percent fat mass when co-treated with sex steroid inhibitors. **(G-J)** After four weeks of weekly prednisone, males co-treated with flutamide had no change in gene expression profiles for sex steroid receptors (G), IGF1 pathway (H), calcium-handling (I), or lipid metabolism (J). Females co-treated with fulvestrant had some increased sex steroid receptor (G), IGF1 pathway (H), and lipid metabolism (J) gene expression. (A-B, D-J) Mann-Whitney.

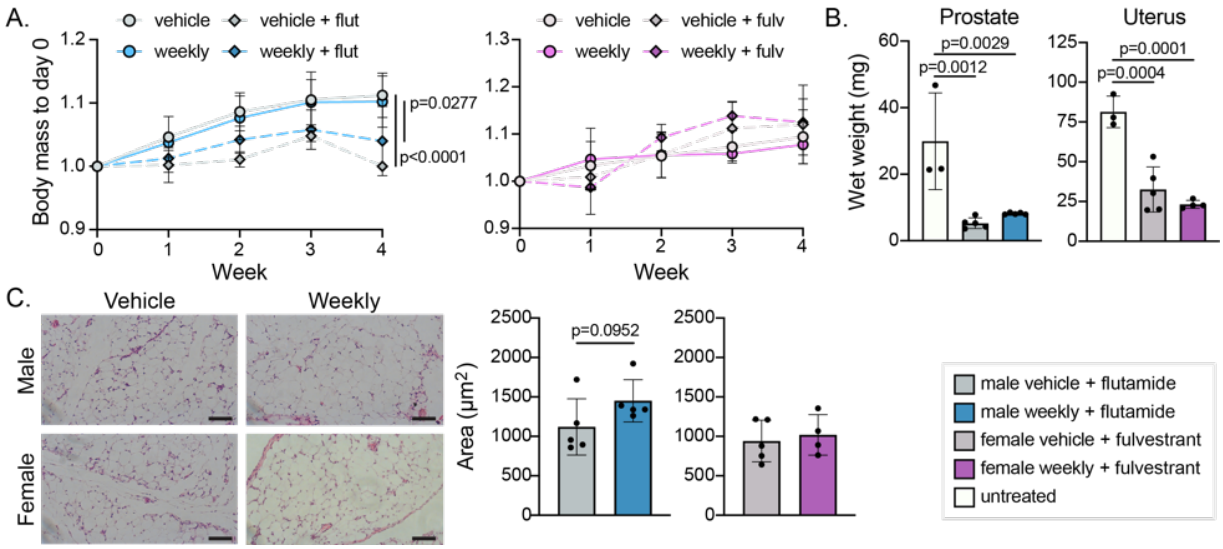

Figure 2.14 Sex steroid receptor inhibition affected body mass and reproductive organ size. (A) Male mice treated with flutamide for four weeks had significantly reduced body weight, while females treated with fulvestrant had no change in body mass. **(B)** Both male and female mice had significantly reduced reproductive organ wet weight after four weeks of sex steroid inhibitor treatment. **(C)** Visceral fat pad adipocytes had significantly increased cross-sectional area in males after co-treatment with flutamide and weekly prednisone, while female adipocytes did not change in size. (A) Two-way ANOVA; (B) One-way ANOVA; (C) Mann-Whitney; black bar = 100µm

Pik3c2a, Pik3ca, and *Tnnc1* (Yu et al. 2010, Sasse et al. 2017, Falcón et al. 2018, Hemmer et al. 2019). For the genes without previously identified GREs (*Akt1, Ar, Slc27a1, Tnnt1),* we investigated binding at sequences matching the GR consensus motif, ACAnnnTGT, located within 10kb of the transcriptional start site of the target gene. GREs near lipid metabolism genes had increased GR binding in weekly prednisone-treated females compared to vehicle, while weekly prednisone-treated males had no changes in GR occupancy (Figure 2.15A). For male muscle, GREs near IGF1/PI3K pathway and calcium-handling genes were enriched (Figure 2.15B-C), including a well-characterized GRE known to modulate *Cacna1h* expression. These results demonstrate sex-specific GR binding profiles consistent with the sexually dimorphic transcriptional patterns.

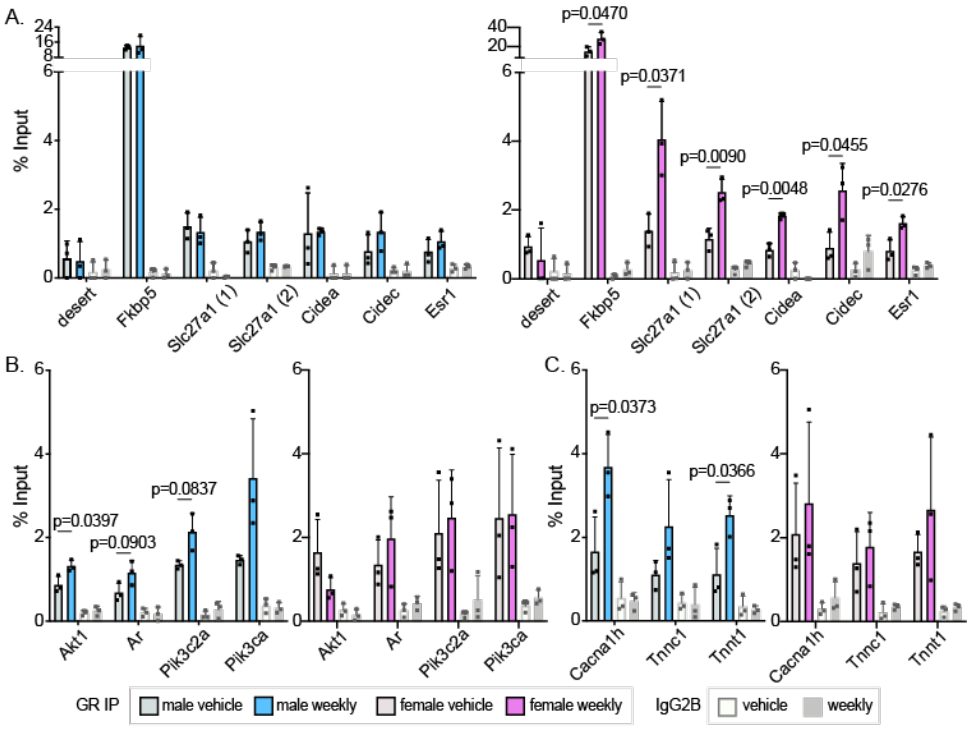

Figure 2.15 Sex-specific GR binding patterns in skeletal muscle treated with weekly prednisone. (A) ChIP-qPCR showed low occupancy of GR at a negative control locus on chromosome 5 (desert) and high occupancy at the canonical GRE near *Fkbp5* in both males and females. Putative GREs near lipid metabolism genes had increased occupancy of GR in weekly-treated females compared to vehicle-treated females; males had no change in GR occupancy. **(B-C)** ChIP-qPCR showed increased occupancy at putative GREs near IGF1/PI3K pathway (B) and calcium-handling (C) genes in weekly-treated males, while females had no change in binding with treatment. (A-C) Welch's t-test.

We investigated GR binding two days after the final weekly injection; our findings accordingly represent a relatively short-term response to prednisone, which has total body clearance in 24-36hrs in adult humans (Magee et al. 2001). To better understand how weekly prednisone affects chromatin architecture and how long those changes are maintained, we treated a cohort of mice for four weeks and then harvested quadriceps four, six, and eight days after the final injection (Figure 2.16A). We performed ChIPseq for the histone modification H3K27ac on isolated myofibers to identify active enhancers (Figure 2.17A-C). We were particularly interested in enhancers present four days after the final weekly injection but absent

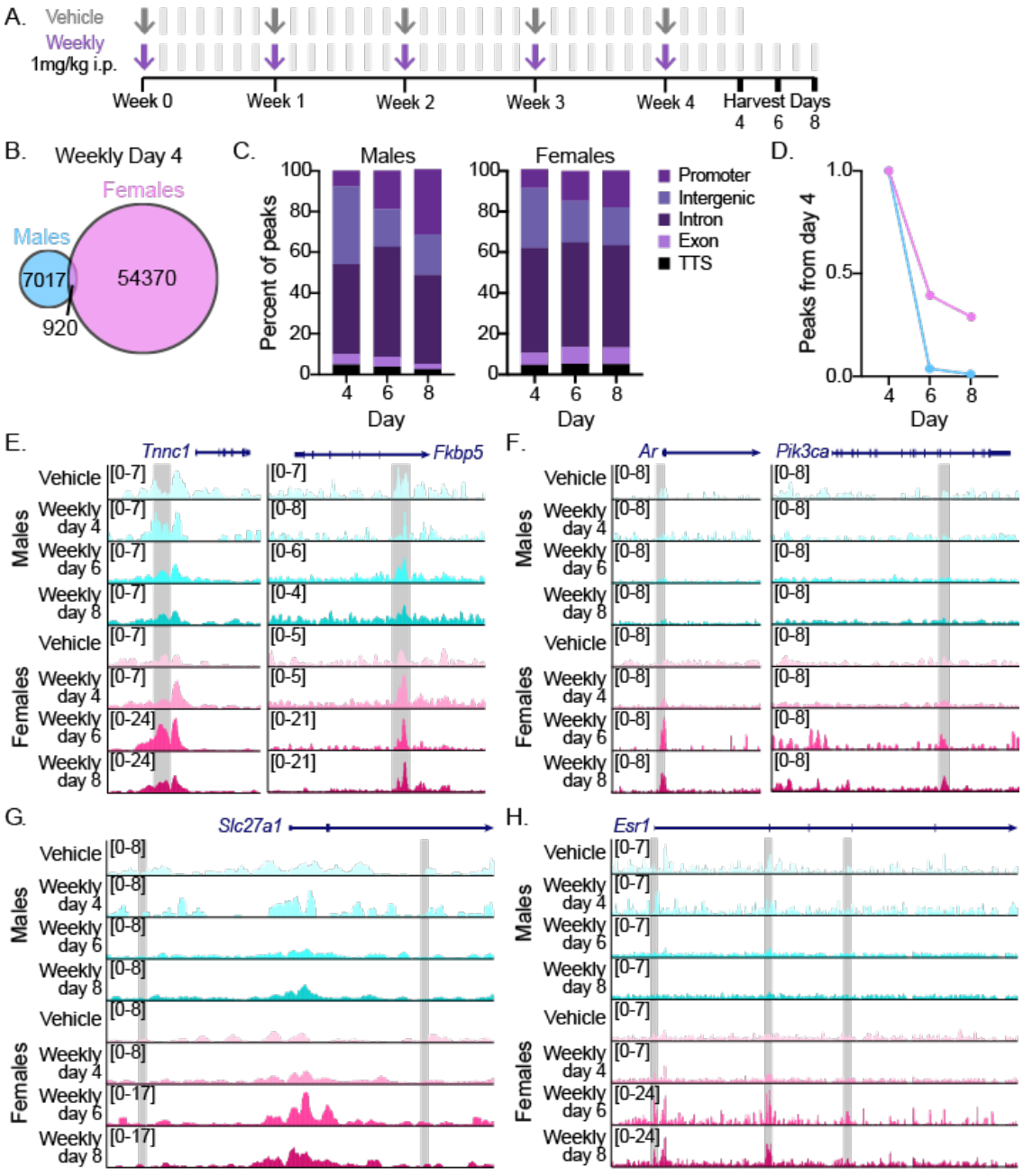

Figure 2.16 Skeletal muscle sex-specific enhancer landscape after weekly prednisone. **(A)** C57BL/6 mice were treated for four weeks with vehicle or weekly prednisone and then analyzed at four, six, or eight days after the final injection. Arrows indicate i.p. injections; bars indicate no injection. **(B-C)** Enhancers present four days after final prednisone injection but not present in vehicle-treated myofibers were mostly unique to one sex (C) and predominantly intergenic or intronic (C). **(D)** Percent of enhancers present four days after final prednisone injection that were still present after six and eight days. **(E-G)** ChIPseq tracks showing presence (E) or absence (F-G) of H3K27ac enrichment at putative GREs (grey boxes). **(H)** ChIPseq tracks showing female-specific enhancer peaks (grey boxes) near *Esr1*.

in vehicle-treated samples, which we identified as prednisone-responsive enhancers (PREs). PREs mostly intergenic or intronic and few were shared between males and females (Figure 2.16B-C). We queried how many PREs remained at six or eight days after the final injection. Although the vast majority of male PREs were lost by day 6, females maintained some PREs out to day 8 (Figure 2.16D). When we assigned the 500 top-ranked PREs to their nearest promoter, we found that males had PREs near genes implicated in muscle function and nuclear receptor signaling, while females had PREs near genes involved in IGF1/PI3K signaling (Figure 2.17D). Those PREs maintained in females at day 8 were near genes involved in both lipid metabolism and IGF1/PI3K signaling (Figure 2.17E). Similar to a previous investigation of dystrophic male muscle (Quattrocelli et al. 2019), the most significant transcription factor motifs enriched in long-lived PREs in both sexes were MEF2 family members (Figure 2.17F-G).

We then investigated H3K27ac enrichment at GREs with sex-specific binding two days after final injection (Figure 2.15). Three of these GREs overlapped an enhancer that was present in all animals, including vehicle, suggesting these enhancers had baseline activity that was further modulated by weekly prednisone (grey box, Fig 16E, Fig 17B). Some GREs with sex-specific GR binding in males at day 2 did not have H3K27ac enrichment at day 4 (Figure 2.16F), suggesting that these enhancers were transiently responsive to weekly prednisone. Transient enhancer occupancy was also observed at female-specific GREs (Figure 2.16G). Reflecting the overall longevity of the female response to weekly prednisone, we identified PREs near female-specific genes that were maintained out to day 8 (Figure 2.16H). Overall, these results suggest that male and female skeletal muscle undergo differential chromatin remodeling to weekly prednisone. While males have a relatively transient response to weekly prednisone that mostly disappeared by six days after the end of treatment, female skeletal muscle had more persistent chromatin marks that were evident for longer than a week after cessation of treatment. Intriguingly, these longer-lived enhancers annotate not only to genes

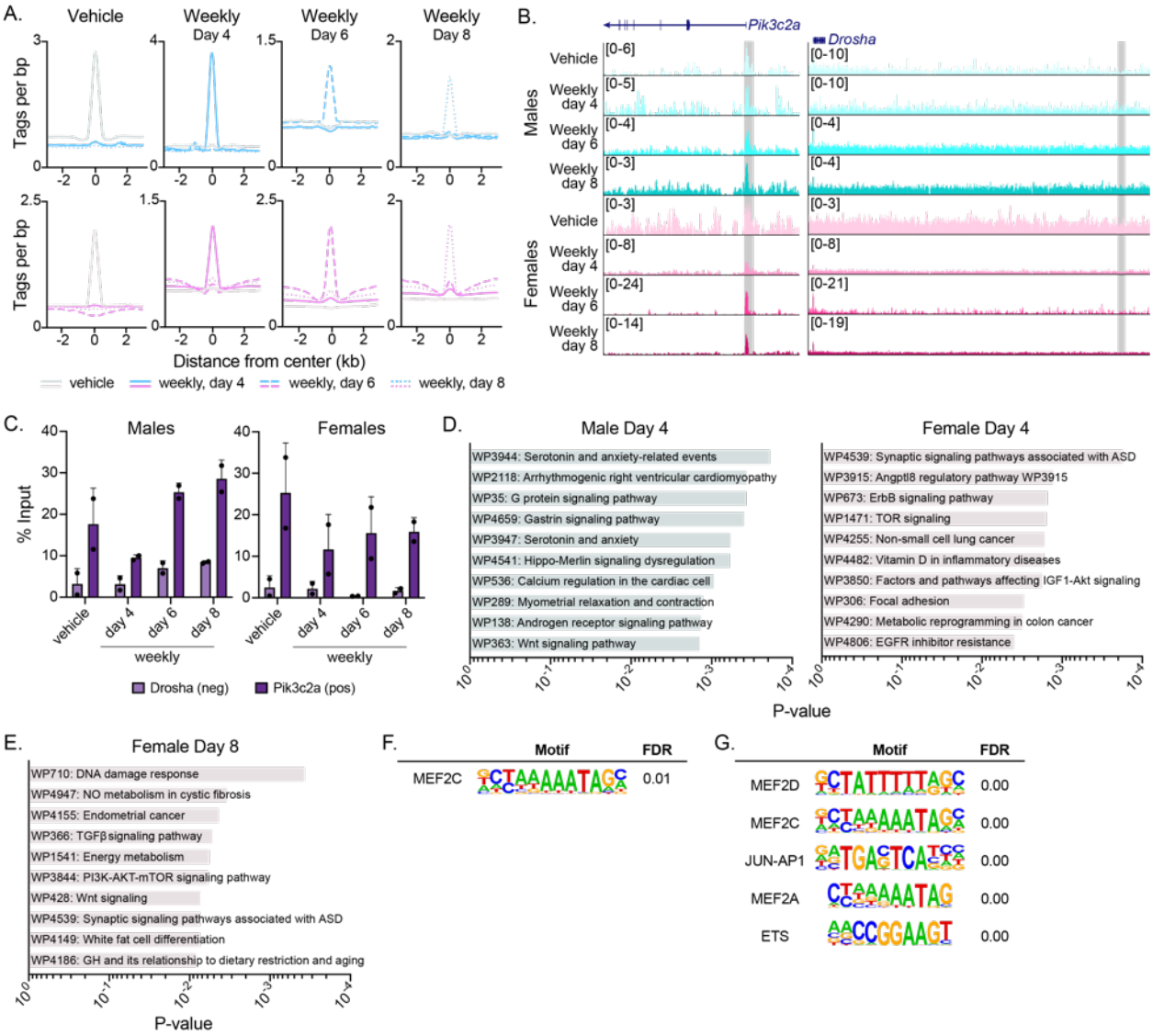

Figure 2.17 H3K27ac ChIPseq in isolated myofibers. **(A)** Tag density distribution plots for peaks unique to each time point; top row = males, bottom row = females. **(B-C)** ChIPseq tracks (B) and qPCR validation (C) of loci called as either a peak at all time points (*Pik3c2a*) or not called as a peak at any time point (*Drosha*). **(D)** Gene ontology of top 500 enhancers by peak score in male and female myofibers four days after last prednisone injection. **(E)** Gene ontology of top 500 enhancers by peak score that were maintained out to day eight in females. **(F-G)** Most significantly enriched motifs in male (F) and female (G) enhancers that were maintained out to day eight after last injection.

involved in female-specific pathways such as lipid metabolism but also male-specific pathways such as IGF1/PI3K signaling.

2.5 Discussion

2.5.1 Weekly prednisone avoids triggering the atrophy pathways

Weekly prednisone enhanced muscle performance while daily prednisone triggered significant muscle atrophy. We observed significant improvements in specific force compared to vehicle-treated animals, similar to studies of anabolic steroids such as testosterone (Prezant et al. 1993) and nandrolone (Salmons 1992, Lewis et al. 1999). Although there was considerable overlap of the transcriptional profiles from weekly and daily prednisone-treated muscle, only daily prednisone exposure produced atrogene upregulation and canonical glucocorticoid-induced atrophy (Bodine et al. 2001). Atrogene upregulation was accompanied by profound repression of mitochondrial respiratory chain genes in daily prednisone-treated mice, and this is consistent with the known role of chronic glucocorticoid treatment in the suppression of mitochondrial oxidation (Lee et al. 2013). Whether reduction of mitochondrial oxidation genes results from the direct action of GR or an indirect side effect of atrophy is not known. Once-weekly prednisone did not elicit the same atrogene upregulation as daily steroid treatment, thus providing many of the positive adaptive changes without the adverse consequences.

2.5.2 Sexual dimorphism in response to weekly prednisone

The vast majority of genes responsive to weekly prednisone were upregulated uniquely in one sex. Skeletal muscle is known to be one of the most sexually dimorphic tissues in the body. Males and females exhibit major differences ranging from fiber type composition to metabolism and gene expression (Friedlander et al. 1998, Horton et al. 1998, Carter et al. 2001, Moran et al. 2007, Haizlip et al. 2015). Studies investigating the molecular effects of

glucocorticoids on skeletal muscle have often been evaluated in a single sex (Bodine et al. 2001, Morrison-Nozik et al. 2015, Quattrocelli et al. 2017) or with results from both sexes analyzed in aggregate, missing the biological effects of sex-specific responses (Braun et al. 2013, Quattrocelli et al. 2017, Quattrocelli et al. 2019). Studies in liver (Duma et al. 2010) and more recently in skin (Baida et al. 2020) suggest males and females can have distinct responses to synthetic glucocorticoid treatment. Male muscle was characterized by increased IGF1/PI3K signaling activation, a pathway strongly implicated in muscle growth (Musarò et al. 2001). We also identified a novel role for calcium handling in the male response to weekly prednisone. Glucocorticoids have been shown to increase store-operated calcium entry (Itagaki et al. 2010) as well as contractile force in isolated cardiomyocytes (Wester et al. 2019) and the mouse model of DMD (Baltgalvis et al. 2009), providing additional support for this mechanism of enhanced performance. Although we observed male-specific improvements in gene expression and calcium release rate, both sexes responded to weekly prednisone with significantly decreased muscle relaxation time. We suspect that these improvements in female skeletal muscle performance were the result of increased ATP, the cytoplasmic concentration of which is known to mediate the time course and amplitude of tetanic force (Dutka and Lamb 2004). The benefits offered by prednisone-induced increased ATP content in female muscle might be only observable by whole-muscle mechanics and too subtle to be evaluated in single myofibers, and further study is required to investigate this important relationship. Although we did not directly identify the source of increased ATP, ATP production in muscle has been shown to be stimulated by IGF1 signaling (Das et al. 2015), mitochondrial calcium taken up from the sarcoplasmic reticulum (Territo et al. 2000, Glancy et al. 2013, Zhou et al. 2016), and mitochondrial membrane phospholipid composition (Heden et al. 2019), all of which were improved by weekly prednisone. However, further investigation is needed to clarify exactly how these pathways specifically modulate specific force independent of fiber size.

2.5.3 Female-specific improvements in lipid metabolism and endurance

Glucocorticoids are well-described mediators of white adipose tissue, modulating both adipocyte differentiation and fasting- and hormone-induced lipolysis (Macfarlane et al. 2008, Lee et al. 2018). Acute or short-term exposure to glucocorticoids in particular has been identified as a mediator of lipolysis from adipocytes (Slavin et al. 1994, Xu et al. 2009, Ramshanker et al. 2019). We observed reductions in adipocyte size, visceral fat pad size, and whole body percent fat mass in weekly-treated females but not males. Skeletal muscle of weekly-treated females exhibited strong upregulation of lipid metabolism-related genes previously reported as glucocorticoid-responsive in adipose tissue (Yu et al. 2010) and as having sexually dimorphic expression in skeletal muscle (Berthon et al. 1998, Kiens et al. 2004, Roepstorff et al. 2006, Maher et al. 2009, Maher et al. 2010). This baseline sexual dimorphism has been proposed as an explanation for why, although endurance exercise instigates a shift towards increased lipid utilization in both sexes, the effect is stronger in females, who have a lower respiratory exchange ratio and utilize 25-50% less glycogen than males depending on exercise type and muscle group (Tarnopolsky et al. 1990, Tarnopolsky et al. 1995, Esbjörnsson-Liljedahl et al. 1999, Carter et al. 2001). The lipid profile of weekly-treated females exhibited improvements that mirror those observed in trained muscle. Both PC and PE are responsive to exercise intervention (Lee et al. 2018) and addition of PE to the skeletal muscle mitochondrial membrane has been shown to promote mitochondrial respiratory capacity (Heden et al. 2019). We also observed increased expression of PC synthesis enzymes, including *Chpt1*, which has been shown by skeletal muscle-specific knockout to be crucial for exercise tolerance (Funai et al. 2016). Sexual dimorphism in skeletal muscle lipid metabolism gene expression has been partly attributed to differences in fiber type, and female muscle tends to have a higher percentage of oxidative fibers than male muscle (Eason et al. 2000, Staron et al. 2000, Daniels

et al. 2008, Welle et al. 2008, Haizlip et al. 2015). We did observe that female muscle had a slightly higher percentage of type 2A fibers than male muscle (15.2% and 9.8% of the tibialis anterior muscle, respectively), but we observed no significant changes in fiber composition in response to weekly prednisone. The relatively small contribution of type 2A fibers is likely insufficient to account for the functional improvements after weekly prednisone.

Sex hormones influence muscle size and contractility, metabolism, and response to exercise (Moran et al. 2006, Moran et al. 2007, Esbjornsson et al. 2012, West et al. 2012, Deane et al. 2013). Glucocorticoid and sex steroid receptors are members of the same superfamily, capable of cooperatively binding (Chen et al. 1997) or sharing co-factors (Bolt et al. 2013), and we hypothesized that AR and ER were responsible for directing sex-specific GR binding. When AR was antagonized, male muscle failed to respond to weekly prednisone, suggesting that AR is the co-factor mediating the male response to weekly prednisone. Intriguingly, weekly prednisone administered to female mice treated with an ER antagonist resulted in upregulation of previously male-specific genes such as *Ar* and *Igf1*. We also found that female myofibers treated with weekly prednisone alone had H3K27ac enrichment at male-specific GREs (Figure 2.16F). It is possible that the female-specific transcriptional profile is the result of direct activation of female-specific genes and repression of male-specific genes; further investigation is needed to clarify if ER is the direct mediator of both of these gene regulatory programs.

2.5.4 Implications for clinical use of glucocorticoids

This work focused on the muscle response to weekly prednisone exposure between male and females, but glucocorticoids are primarily prescribed as anti-inflammatory drugs. Whether there are sexually dimorphic differences in inflammation response is possible, and may be more relevant in the disease or injury context. Other adverse effects of glucocorticoids may

also exhibit sexually dimorphic response. For example, central obesity is a common side effect of chronic glucocorticoid treatment (Desmet and De Bosscher 2017) but our data show a reduction in fat mass and adipocyte size only in females. Osteoporosis is a highly significant adverse consequence of steroid use, and bone is also known to have strong hormonal influence and sexual dimorphism. Glucocorticoid-induced osteoporosis is in part driven by decreased osteoblast function through suppression of IGF1 (Delany and Canalis 1995), expression of which was significantly upregulated in weekly-treated males but not females.

Overall, our data indicate that weekly glucocorticoid treatment is beneficial to skeletal muscle health and performance. Although male and female mice exhibit similar improvements in strength, the mechanisms by which they achieve this are very different, driven by distinct gene expression profiles associated with specific differences in chromatin occupancy of the GR. This work has considerable implications given the widespread nature of chronic glucocorticoid use.

Chapter 3. Peripheral artery disease skeletal muscle transcriptomes differ based on sex and responsiveness to exercise intervention

3.1 Overview

Peripheral artery disease (PAD) is the progressive narrowing or blockage of arteries in the lower extremities, leading to reduced blood flow and discomfort when walking. Supervised exercise therapy is one treatment for PAD that improves walking distance and quality of life; however, some PAD patients do not respond to exercise intervention and there is currently no way to distinguish exercise responders from non-responders prior to therapy. To better understand how skeletal muscle of PAD patients responds to exercise intervention, we generated and analyzed transcriptome profiling to compare matched pairs of exercise responders to non-responders before and after exercise intervention. We found that male and female PAD patients had distinct transcriptomic profiles with little overlap between the sexes. Prior to exercise, male non-responders had increased expression of genes involved in fibrosis, extracellular matrix, and vascular function compared to responders. In contrast, female non-responders were characterized by decreased expression of mitochondrial genes. Although both male and female responders had improvements in walking distance, myofiber cross-sectional area, and capillary density after exercise, neither sex exhibited substantial transcriptional changes, suggesting that exercise responsiveness is mediated by pre-existing differences in skeletal muscle.

This chapter was a collaboration with the laboratories of Drs. Mary McDermott and Charlotte Peterson. I contributed to study design, analyzed RNA sequencing generated by the NUSeq Core Facility, and drafted the manuscript. R. Grace Walton isolated RNA from patient biopsies. Dr. Kate Kosmac performed histological analyses. Dr. Rob Sufit performed muscle biopsies. Mary McDermott conceived experiments and consented patients. Charlotte Peterson

conceived experiments and assisted with analyses. Dr. Elizabeth McNally contributed to study design, was responsible for data integrity and analyses, and edited the manuscript.

3.2 Introduction

Lower extremity peripheral artery disease (PAD) is the third leading cause of atherosclerotic cardiovascular morbidity and affects over 200 million people worldwide (Hirsch et al. 2012, Fowkes et al. 2013). Atherosclerotic blockages in the lower limbs reduce blood flow and lead to discomfort when walking that can be alleviated by rest. Individuals with PAD walk shorter distances in a six-minute walk test and have a slower walking velocity in a four-meter walk test than people without PAD (McDermott et al. 2002) and these metrics continue to decline without intervention (McDermott et al. 2004, McDermott et al. 2015). Although exercise therapy has been found to prevent or reverse mobility loss and reduce ischemia-induced discomfort, not all patients respond to exercise therapy (Treat-Jacobson et al. 2019).

Patients with PAD have pathology of the affected lower limbs that is characterized by fibrosis, myofiber degeneration, and mitochondrial dysfunction (Makitie and Teravainen 1977, Marbini et al. 1986, Hedberg et al. 1988, Regensteiner et al. 1993, Brass and Hiatt 2000, Pipinos et al. 2003, McDermott et al. 2004, Pipinos et al. 2007, Koutakis et al. 2010, McDermott et al. 2012, Cluff et al. 2013, Weiss et al. 2013). Myofibers of patients with PAD have features suggestive of denervative atrophy (England et al. 1992, Regensteiner et al. 1993, Hiatt et al. 1996) and individuals with PAD who do not exercise have greater denervation in calf muscle after twelve weeks compared to those who do (Hiatt et al. 1996). Ankle-brachial index (ABI), a measure of systolic pressure difference between the upper and lower limbs that is classically used to diagnose PAD, is associated with decreased calf muscle area and increased fatty muscle infiltration (McDermott et al. 2007). PAD is also associated with reduced mitochondrial activity and increased mitochondrial DNA damage in calf muscle (Pipinos et al. 2008, Thaveau

et al. 2009, Weiss et al. 2013) as well as abnormalities in sarcomeric proteins such as actin, myosin, and desmin (Sjostrom et al. 1980, Koutakis et al. 2015). Muscle performance can be partly rescued by revascularization (West et al. 2012). However, it is unclear if all vasculature is equally influential, as some studies have reported a negative association between capillary density and muscle function (McGuigan et al. 2001, Askew et al. 2005, Ho et al. 2006, Jones et al. 2012, Baum et al. 2016, White et al. 2016, Kosmac et al. 2020), while others have reported the opposite or no effect at all (White et al. 2016).

Mitigating and managing risk factors such as smoking and diabetes is central to improving outcomes with PAD. Exercise therapy is also recommended to improve quality of life for individuals with PAD. Although the extent of the benefit is dependent on claudication, supervised exercise therapy generally improves peak walking distance and distance walked to claudication onset (Lyu et al. 2016, Treat-Jacobson et al. 2019). The benefits are dependent to an extent on intensity of workload, duration of activity, and claudication intensity (Treat-Jacobson et al. 2019). However, there are PAD patients who receive no benefit from exercise. It is unclear whether exercise is simply non-beneficial or some patients are unable to respond to exercise (Zakari et al. 2018). There is currently no reliable method to distinguish an exercise responder from a non-responder. Although some studies have reported minor associations with sex or mitochondrial function, others have found no association between exercise responsiveness and age, sex, ABI, race, or presence of diabetes mellitus or claudication symptoms (McDermott et al. 2009, McDermott et al. 2013, Gardner et al. 2014, Gardner et al. 2016, van Schaardenburgh et al. 2017, McDermott et al. 2020).

To identify potential factors that could distinguish between responders and non-responders prior to exercise intervention, gastrocnemius biopsies were performed on individuals with PAD before and after exercise intervention. RNA-sequencing was carried out to evaluate transcriptional differences. We found minimal changes to the skeletal muscle transcriptome of

responders after exercise intervention. However, we did observe that responders and non-responders had divergent transcriptomes prior to exercise intervention, with differences that were unique to each sex. These data suggest that responsiveness to exercise may not be the result of substantial skeletal muscle remodeling but instead might reflect innate differences in skeletal muscle health prior to intervention. We were also able to identify sex-specific biomarkers that could potentially differentiate between responders and non-responders prior to exercise.

3.3 Materials and methods

3.3.1 Participant inclusion and exclusion criteria

The institutional review board at Northwestern University, Tulane University, University of Minnesota, and University of Pittsburgh approved the trial protocols (McDermott et al. 2017, McDermott et al. 2021). Participants provided written consent. Participants were recruited to one of three clinical trials: LITE (McDermott et al. 2021), PROPEL, and TELEX (McDermott et al. 2017). Participants were included based on ABI of 0.90 or less at their baseline visit. Potential participants with ABI > 0.90 were eligible if evidence of PAD was demonstrated by a hospital-affiliated vascular laboratory report or lower extremity angiogram. Potential participants with baseline ABI between 0.90 and 1.00 and those with normal ABI and prior lower extremity revascularization were eligible if their ABI decreased by 20% after a heel-rise test, consisting of 50 heel rises at one rise per second (Amirhamzeh et al. 1997). Potential participants in the LITE trial were eligible if they exhibited ischemic leg symptoms during walking that were not consistent with classic claudication symptoms, such as pain in the buttocks or thighs but not calves. Potential participants in all three trials were excluded if they had below- or above-knee amputation; wheelchair confinement; use of a walking aid other than a cane; walking impairment for a reason other than PAD; foot ulcer or critical limb ischemia; significant visual or hearing

impediment; major surgery or revascularization during the previous three months; or major surgery planned to occur in the next six months. Potential participants were also excluded if they had a Mini-Mental State Examination score less than 23 (Heun et al. 1998). Potential participants in the PROPEL or TELEX trials were excluded if they had Parkinson disease; required oxygen during activity; had abnormal baseline stress findings; or did not complete the run-in. Potential participants in the LITE trial were excluded if they had inability or unwillingness to attend exercise sessions; major surgery planned in the next twelve months; or major medical illness. Participants were also excluded from the LITE trial if they were unable to walk sufficiently slowly to avoid ischemic leg symptoms or if they did not experience ischemic leg symptoms during walking.

3.3.2 ABI measurement

A handheld Doppler probe (Pocket Drop II, Nicolet Biomedical Inc) was used to measure systolic pressure twice in the right and left brachial, dorsalis pedis, and posterior tibial arteries. ABI was calculated by dividing the mean of the dorsalis pedis and posterior tibial pressures in each leg by the mean of the four brachial pressures.

3.3.3 Medical history, race, and demographics

Medical history, race, and demographics were obtained by questionnaire. Information on race was based on patient self-report using an open-ended question and was classified with fixed categories. Height and weight were recorded and body mass index was calculated as weight in kilograms divided by height in meters squared.

3.3.4 Randomization

For the PROPEL and TELEX trials, participants were randomized using R to one of four groups: supervised exercise with granulocyte-macrophage colony-stimulating factor, control with granulocyte-macrophage colony-stimulated factor, exercise alone, or control alone. Randomization was stratified by diabetes mellitus. Block randomization was used, with block sizes randomly selected from 8 and 12. Only participants in the exercise alone and attention control alone groups were used for this study. For the LITE trial, participants were randomized to low-intensity exercise, high-intensity exercise, or nonexercised control groups in a ratio of 120:120:65 using SAS (SAS Institute Inc) and a randomly permuted block method using block sizes of 61, stratified by study site and consent for muscle biopsy.

3.3.5 Exercise intervention

For the PROPEL and TELEX trials, treadmill exercise was provided three times weekly with an exercise physiologist. Walking exercise duration was increased gradually until 50 minutes of exercise per session was achieved. Participants were asked to exercise to maximal ischemic leg symptoms. Participants randomized to control attended weekly one-hour educational sessions by Northwestern faculty on health topics including cancer screening, immunizations, and hypertension. For the LITE trials, participants randomized to the exercise groups met with a coach for four weeks and were taught to use an accelerometer to monitor their walking exercise intensity. For each participant, accelerometer counts corresponding to high- and low-intensity walking exercise were determined by five minutes of walking with and without ischemic discomfort, respectively. Participants randomized to high- or low-intensity groups then walked for exercise at their predetermined accelerometer counts in an unsupervised setting five times per week for up to 50 minutes per session while wearing the accelerometer to document exercise time and intensity. Accelerometer data were viewable to a

coach who telephoned participants weekly for twelve months and helped them adhere to their prescribed exercise. Participants randomized to the control group attended four weeks of weekly one-hour educational settings and then received twelve months of weekly educational phone calls. Topics included cancer screening and Medicare Part D.

3.3.6 Six-minute walk test

Participants walked in a 100ft hallway after receiving instructions to cover as much distance as possible in six minutes. All participants received identical instructions from a script read by a research coordinator who was unaware of the participant's group assignment. The distance completed after six minutes was recorded. The minimum clinically important difference in people with PAD ranges from 8m to 20m (Gardner et al. 2018, McDermott et al. 2021).

3.3.7 Calf muscle biopsy

An open muscle biopsy was performed in the medial head of the gastrocnemius muscle at baseline and at follow-up after exercise intervention in participants that provided written informed consent. Anesthesia was achieved with subcutaneous lidocaine. Muscle was frozen at -80°C.

3.3.8 RNA sequencing and analysis

RNA was isolated from biopsies using the RNeasy Lipid Tissue Mini Kit (74804, Qiagen). Prior to sequencing, RNA was quantitated with a Qubit fluorometer (Q33216, Thermo Fisher) and the quality of each sample was assessed by Bioanalyzer (G2943, Agilent Technologies). Libraries were prepared from approximately 1µg RNA per sample using the TruSeq Stranded mRNA Library Prep Kit (20020595, Illumina). Libraries were then sequenced as 100bp single-end reads using the Illumina HiSeq 4000 System (Illumina). Reads were aligned to the hg38

genome assembly (GRCh38 from Ensembl v78) with TopHat v2.1.0 using Bowtie2 v2.2.6 (Langmead et al. 2009, Trapnell et al. 2009). Raw read counts per gene were quantified with HTSeq (Anders et al. 2015). Differential gene expression was assessed as log2 fold change between groups using the Bioconductor package EdgeR (Robinson et al. 2010, McCarthy et al. 2012). To account for matched responder-non-responder pairs pre-exercise and matched samples from the same individuals pre- and post-exercise, the paired design function of EdgeR was used. Genes of interest were identified as being above an expression threshold of log2 counts per million (logCPM) > 2.5 and having an absolute log2 fold change > 0.5. Multidimensional scaling (MDS) was plotted from gene counts per million using EdgeR. All of the following R analyses were performed in R 4.0.3 (Team 2020) and RStudio 1.3 (Team 2020). Principal component analysis was performed on gene counts per million using the prcomp function from the R package stats (Team 2020). The percent contribution of each gene to each principal component was identified using the get_pca_var function from the R package factoextra (Kassambara and Mundt 2020). Genes with the highest contribution to principal component 1 were identified as having a sum contribution of 50% when ranked by percent contribution (males, 355 genes; females, 256 genes). Genes were then categorized as belonging to signaling pathways or having cellular functions based on tissue-specific expression in GTEx (Consortium 2013) and protein function as compiled by GeneCards (Stelzer et al. 2016) and the NCBI Gene database (Coordinators 2018). Venn diagrams were produced using the Venn function from the R package Vennerable (Swinton 2020). Heatmaps were generated using the heatmap.2 function from the R package gplots (Warnes et al. 2020) as a z-score from the normal log-transformed median and standard deviation of gene counts per million. Gene ontology (GO) was assessed from lists of co-regulated genes using EnrichR (Chen et al. 2013, Kuleshov et al. 2016). GO terms of interest were identified as having an adjusted p-value < 0.1.

3.3.9 Histology and immunofluorescence microscopy

Capillary density and total extracellular matrix (ECM) were quantified on the same cryosection. Briefly, cryosections were dried, rehydrated, and washed twice with PBS. Sections were blocked for 1 hour in 2.5% NHS and then incubated for 90min with a biotinylated lectin mix (Ulex europaeus, B-1065-2; Griffonia simplicifolia, B-1105-2, Vector Laboratories) (Kirkeby et al. 1993). Sections were then washed and incubated for 1 hour with streptavidin-AlexaFluor488 (S-11223, ThermoFisher) to visualize capillaries. Following capillary labeling, sections were fixed with 4% PFA, incubated with Texas Red conjugated α-wheat germ agglutinin (W21405; ThermoFisher) for 2 hours, and then mounted with Vectashield (Emde et al. 2014). Stitched images of whole muscle cross-sections were acquired with an Olympus BX61VS (Olympus Microscopy) at 20X. Capillaries were quantified manually as number per muscle fiber. WGA was quantified in Fiji (Schindelin et al. 2012) from three regions of interest per section. Briefly, a threshold was set for positive staining and the percent area in a region of interest above this threshold was measured. Fiber size was determined as minimum ferret diameter using MyoVision (Wen et al. 2018). Fibrosis was evaluated from Masson-Trichrome staining of cryosections imaged with a Zeiss Axio Observer.Z1 (Zeiss) at 20X (Plan-Apochromat 20x/0.8, Zeiss).

3.3.10 Statistical analyses

Statistical analyses were performed with Prism (Graphpad). When comparing two groups, Welch's *t* test was used. A p-value less than or equal to 0.05 was considered significant, although all p-values less than 0.1 are reported in figures. Dot plots and marked line plots depict mean ± SD.

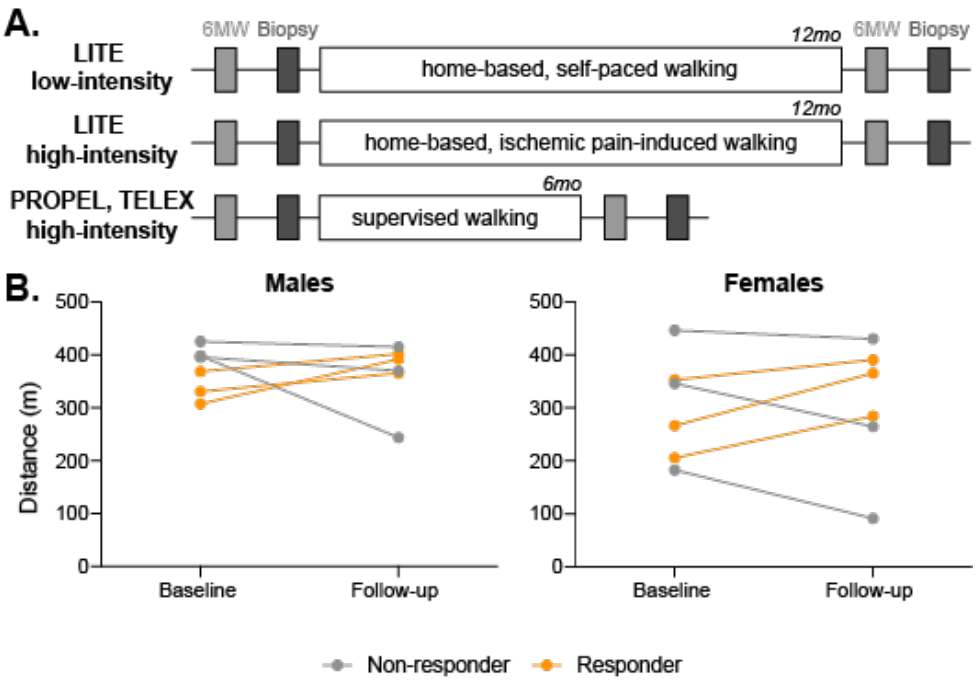

Figure 3.1 Responders had improved six-minute walk distance after exercise intervention. (A) Diagram of exercise intervention. LITE participants underwent twelve months of self-directed low-intensity, self-paced walking or high-intensity, ischemic pain-inducing walking. PROPEL and TELEX participants underwent in six months of supervised treadmill walking three times per week. Six-minute walk distance and gastrocnemius muscle biopsy were performed prior to and after exercise intervention at two separate appointments. **(B)** Six-minute walk distance in meters of each participant at baseline and after exercise intervention.

3.4 Results

3.4.1 Individuals with PAD can be responsive or non-responsive to exercise intervention

A PAD cohort was assembled from patients who received exercise interventions through one of three clinical trials: Low Intensity Exercise Intervention (LITE) (McDermott et al. 2021), Progenitor Cell Release Plus Exercise (PROPEL) (McDermott et al. 2017), or Telmisartan Plus Exercise (TELEX). LITE participants underwent twelve months of home-based walking exercise, while PROPEL and TELEX participants received six months of supervised walking exercise therapy (Figure 3.1A). Six pairs of participants (3 male, 3 female) were matched as responder and non-responder pairs based on age, sex, race, ankle brachial index, body mass index, and

presence of diabetes mellitus (Table 3.1). Non-responders were characterized by a decrease in six-minute walk distance after exercise intervention, while responders increased (Figure 3.1B).

3.4.2 Responders exhibit minimal transcriptional changes after exercise intervention

We initially asked how the skeletal muscle transcriptome was altered by exercise. To investigate this, we performed RNA-sequencing on biopsies from gastrocnemius muscle before and after exercise intervention. To determine the transcriptional signature of exercised muscle, we generated a list of genes of interest that were up- or down-regulated after exercise in responders but were unchanged in non-responders (Figure 3.2A). Few genes were responsive to exercise in both males and females, with only a handful changing in both sexes (Figure 3.2A-B). Principal component analysis of the genes of interest did not separate pre- from post-exercise samples (Figure 3.2C). Gene ontology found that female responders had increased expression of genes involved in lipid metabolism after exercise but no other strong trends (Figure 3.3). To determine if exercise intervention had a profound impact on the sarcomere, the basic contractile unit of skeletal muscle, we compared the log2 fold change after exercise of genes encoding sarcomeric proteins in non-responders versus responders (Figure 3.2D). We found that the majority of sarcomeric proteins were unchanged in both responders and non-responders after exercise (41/48 in males, 40/48 in females, grey dots). Expression of the embryonic and neonatal myosin heavy chain isoforms MYH3 and MYH8 is a marker of regenerating muscle (Schiaffino et al. 2015). Although *MYH8* expression was upregulated in responders of both sexes, it was also upregulated in female non-responders. Responders of both sexes had downregulation of *MYH3* after exercise. The strongest transcriptional signature after exercise was increased expression of lipid metabolism-related genes in females, including those encoding the adipokines adiponectin and leptin (Figure 3.2E). To investigate overall muscle morphology, we stained muscle biopsy sections for wheat germ agglutinin (WGA) and

Table 3.1 Matched responder and non-responder pairs. Study participants were matched as pairs of responders and non-responders based on baseline age, sex, race, baseline diabetes, and ABI. 6MW = six-minute walk.

ID	Study	Sex	Race	Exercise response	Six-minute walk (m)			Age	DM	ABI	BMI	Smoker	Rate
					Pre	Post	Change						
25	LITE*	M	Black	Non-responder	398.7	243.8	-154.8	77	N	0.58	27.4	Current	95.5
31				Responder	368.8	402.3	33.5	67	N	0.84	24.5	Current	97.8
91	PROPEL	M	Black	Non-responder	396.2	370.0	-26.2	68	N	0.75	21.7	Current	81.4
78				Responder	331.0	365.8	34.7	63	N	0.59	21.5	Current	91.3
75	LITE[t]	M	White	Non-responder	425.5	415.1	-10.3	67	N	0.46	22.5	Former	95.7
62				Responder	307.8	392.0	84.1	62	Y	0.57	44.0	Former	93.3
5	LITE*	F	Black	Non-responder	446.5	430.1	-16.4	54	Y	0.74	19.6	Current	95.6
74				Responder	205.7	284.4	78.6	59	Y	0.74	37.2	Current	75.6
15	PROPEL	F	Black	Non-responder	182.9	91.4	-91.4	59	Y	0.41	41.8	Current	56.8
42				Responder	266.4	365.8	99.3	66	Y	0.52	27.1	Current	62.0
86	TELEX	F	Black	Non-responder	345.9	264.6	-81.3	61	N	0.45	35.5	Current	93.0
63				Responder	353.3	390.8	37.4	64	N	0.60	24.2	Current	76.1

DM = diabetes mellitus
BMI = body mass index
ABI = ankle brachial index
Rate = adherence rate
*high-intensity exercise
[t]low-intensity exercise

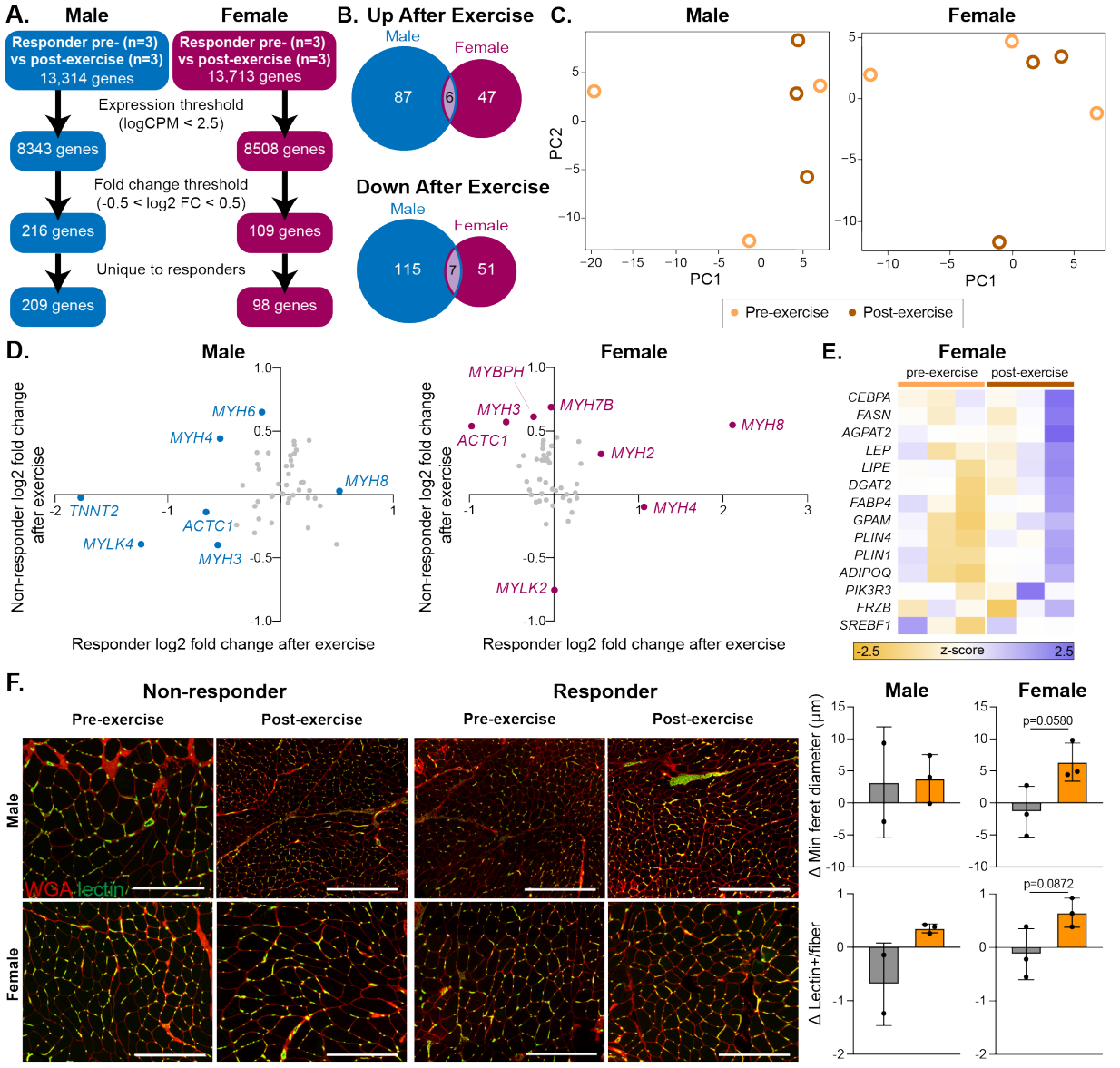

Figure 3.2 Responders exhibited minimal transcriptional changes after exercise intervention. (A) RNASeq results were filtered with expression and fold change thresholds to identify genes of interest after exercise unique to responders. **(B)** Venn diagram analysis of genes up- or downregulated in responders after exercise showed little overlap between males and females. **(C)** Principal component analysis did not separate responders pre- and post-exercise. **(D)** A comparison of sarcomeric gene expression after exercise in responders versus non-responders revealed that the majority had no transcriptional response to exercise in responders and non-responders (-0.5 < log2 FC < 0.5; grey dots). **(E)** Heatmap of RNASeq gene counts per million showing expression of lipid metabolism genes in female responders pre- and post-exercise. **(F)** Staining with WGA and lectin showed that female responders had significantly increased change in minimum ferret diameter and lectin-positive fibers after exercise by Welch's t-test. Male responders trended towards increased change in lectin-positive fibers after exercise. Scale bar = 100μm.

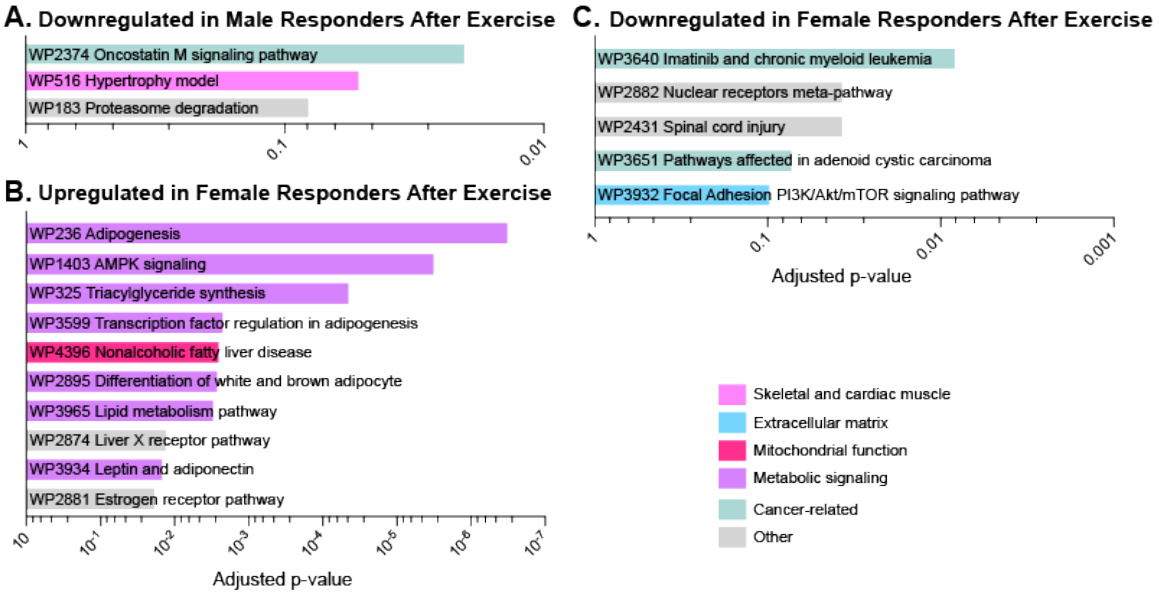

Figure 3.3 Gene ontology analysis of genes changed in responders after exercise. (A-C) Enriched pathways downregulated in male (A), upregulated in female (B), and downregulated in female (C) responders after exercise.

lectin (Figure 3.2F). Female responders had an increase in myofiber cross-sectional area after exercise intervention, while female non-responders had no change. Both male responders and non-responders had a modest increase in cross-sectional area after exercise. Responders of both sexes had increased capillary density after exercise, as measured by lectin+ foci per myofiber; non-responders had either no change or a reduction in capillary density. Overall, these results suggest that exercise intervention led to alterations in muscle morphology in responders that did not occur in non-responders; however, this was not accompanied by any profound transcriptional changes.

3.4.3 Responders and non-responders have differences in skeletal muscle transcription prior to exercise intervention

Having observed minimal changes after exercise intervention, we hypothesized that there might be initial differences in the muscle of responders and non-responders that could

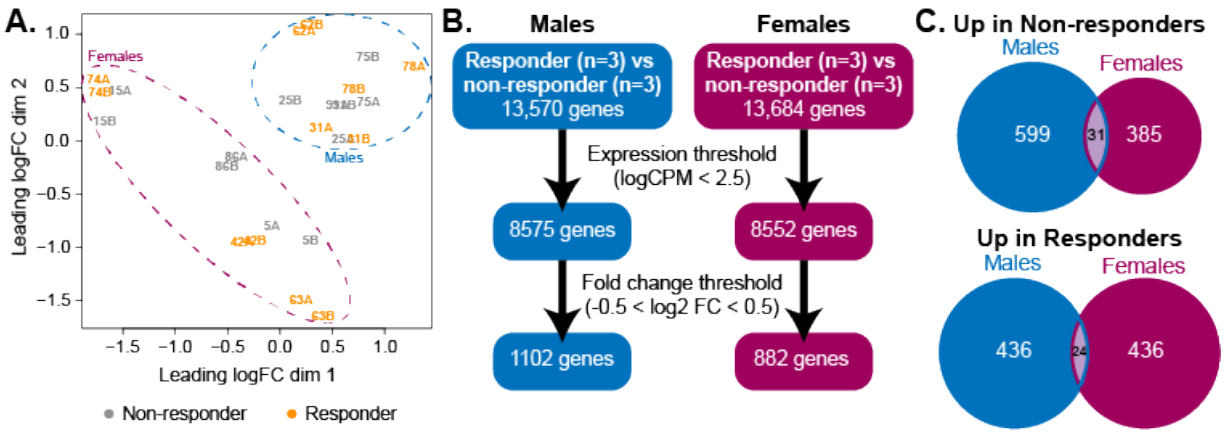

Figure 3.4 Responders and non-responders had divergent skeletal muscle transcriptomes prior to exercise intervention. (A) Multidimensional scaling plot of RNASeq normalized gene counts per million showed clustering based on individual and sex. A = pre-exercise biopsy, B = post-exercise biopsy. **(B)** RNASeq results were filtered with expression and fold change thresholds to identify genes of interest distinguishing responders from non-responders prior to exercise. **(C)** Venn diagram analysis of genes upregulated in either responders or non-responders prior to exercise showed little overlap between males and females.

influence their responsiveness to exercise intervention. Multidimensional scaling showed a high degree of concordance between the pre (A) and post (B) exercise samples from each individual (Figure 3.4A). Participants clustered based on sex but not exercise responsiveness. We generated a list of genes of interest by filtering for minimum expression and fold change thresholds (Figure 3.4B) and found very little overlap between males and females (Figure 3.4C). Gene ontology analysis found that male non-responders had higher expression of genes involved in extracellular matrix (blue) and vasculature (indigo) while male responders had enrichment of pathways involved in skeletal and cardiac muscle (pink, Fig 3.5). Female non-responders had higher expression of genes involved in immune signaling (green) and lipid metabolism pathways (purple) while female responders were highly enriched in mitochondrial (red) and skeletal and cardiac muscle terms (pink). When we performed a principal component analysis of the genes of interest that were differentially expressed between responders and non-responders, we found that principal component 1 (PC1) separated non-responders from

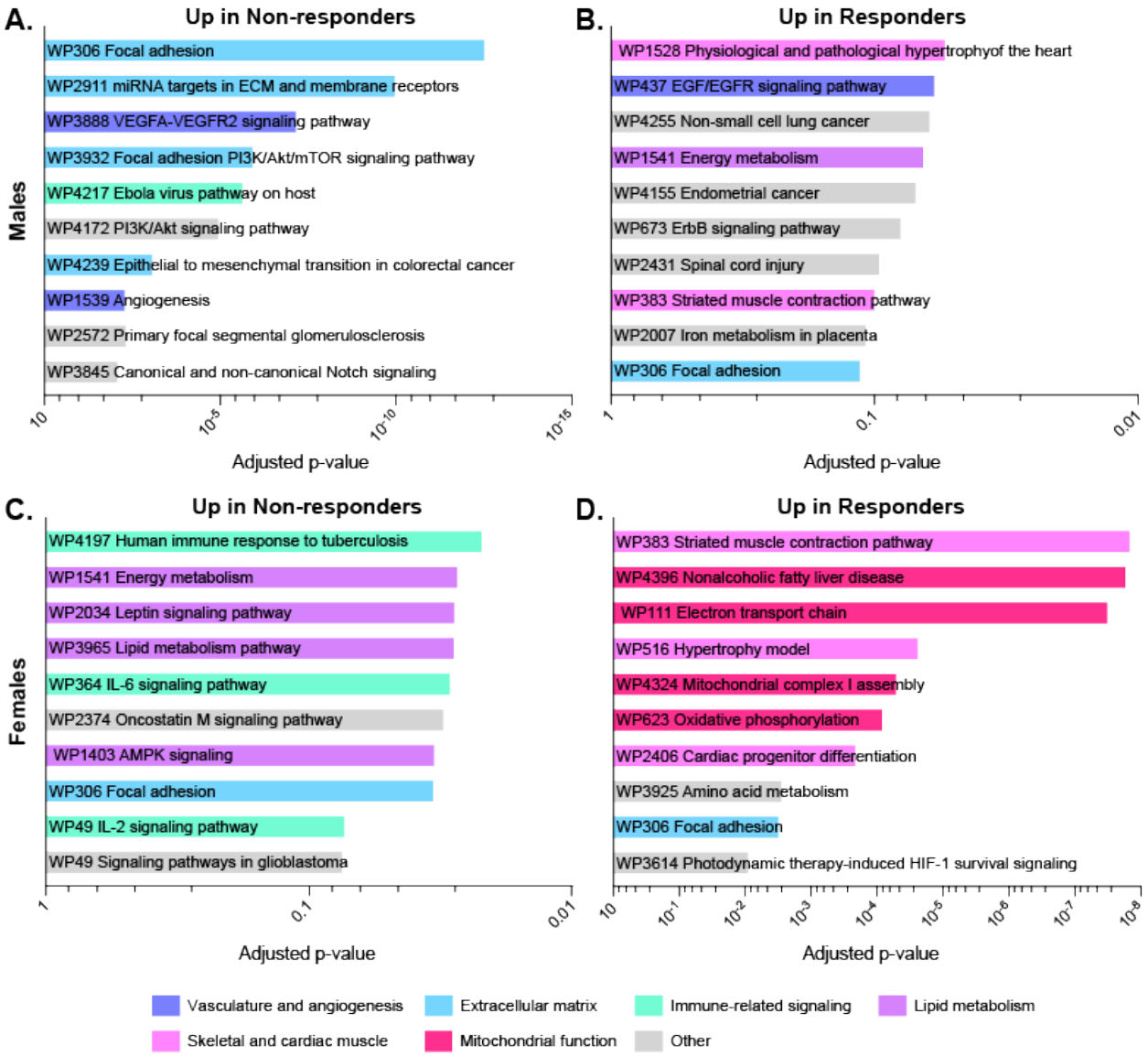

Figure 3.5 Gene ontology prior to exercise. (A-B) Gene pathways that were divergent between male non-responders (A) and responders (B) prior to exercise were related to extracellular matrix and vasculature. **(C-D)** Gene pathways that were divergent between female non-responders (C) and responders (D) included lipid metabolism and mitochondrial function.

responders in both males and females (Figure 3.6A, D). We identified the genes with the highest percent contribution to PC1 and annotated them for their relevance to major cellular functions on the basis of the protein they encoded and the tissue in which they were expressed. In males, there were a large number of genes involved in angiogenesis and endothelial and smooth muscle cell function, contributing to a distinctive vasculature signature in non-responders (Figure 3.6B-C). We also found that male non-responders had increased expression of extracellular matrix, fibrosis, and cytoskeleton genes as well as genes characteristic of non-myofiber cells, such as neurons and immune cells. In females, we found that non-responders had increased expression of smooth muscle genes and genes involved in regulating transcription and translation, while responders had increased expression of genes involved in mitochondrial function (Figure 3.6E-F). A few pathways related to cell cycle progression and protein trafficking and degradation were enriched in both male and female non-responders, although the specific genes differed between the sexes (Figure 3.7). Together, these data suggest that skeletal muscle of PAD patients that are not responsive to exercise has a transcriptome distinct from responders prior to exercise intervention. Male responsiveness might be the result of reduced fibrosis, while female responsiveness might rely on mitochondrial function.

3.4.4 Candidate biomarkers to distinguish between responders and non-responders prior to exercise

Extensive fibrosis in liver or muscle can be identified without invasive biopsies through the use of protein serum biomarkers (Valva et al. 2011, Yilmaz and Eren 2019, Al-Khalili Szigyarto 2020, Tsuji et al. 2020). As we had observed that male non-responders could be distinguished from responders prior to exercise by higher expression of genes involved in ECM formation and fibrosis, we asked whether genes encoding these biomarkers were differentially

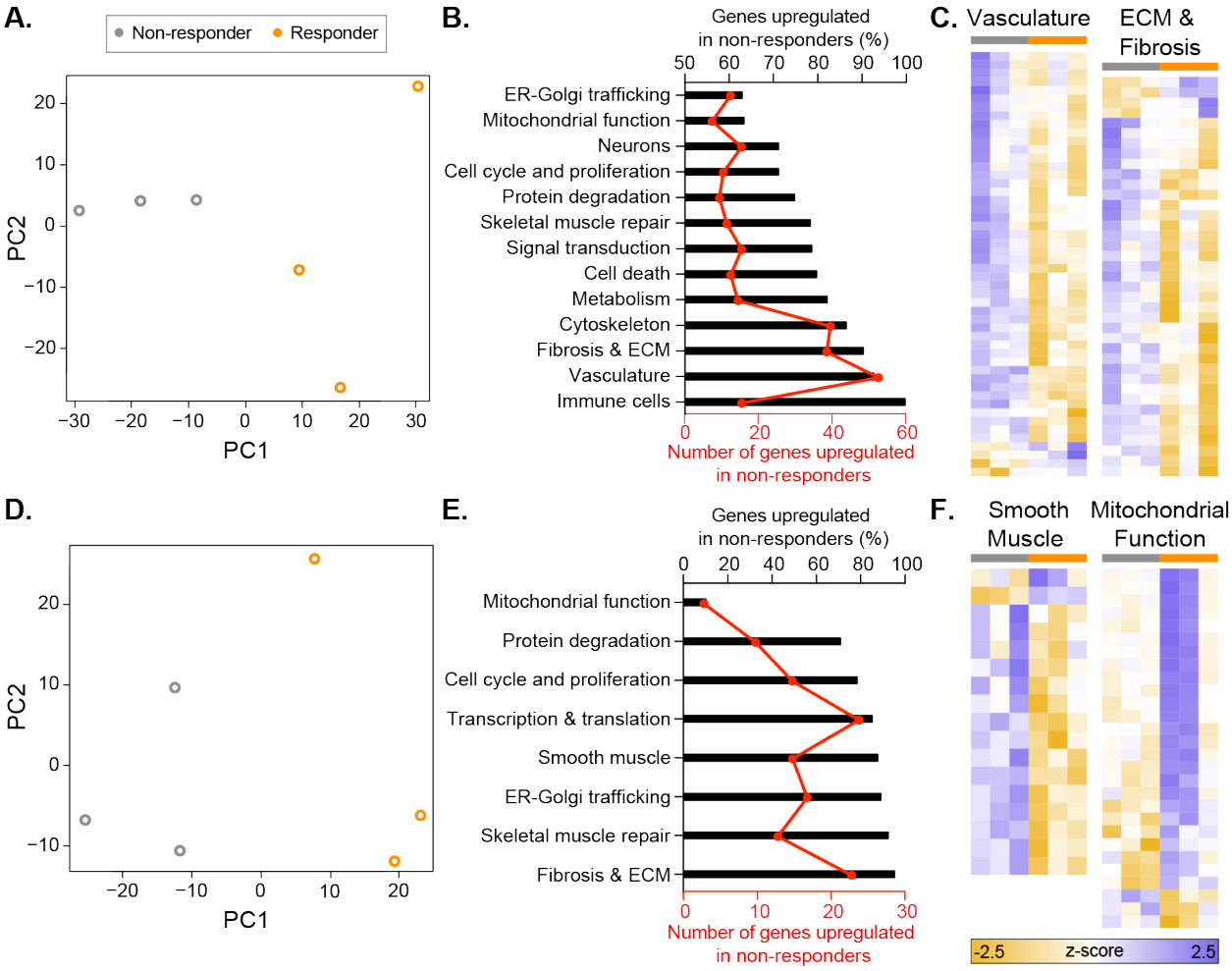

Figure 3.6 Responders and non-responders were separated prior to exercise by divergent gene expression patterns. (A) PC1 separated male responders from non-responders. **(B-C)** Genes that made the highest contribution to PC1 in males were sorted by their relevance to cellular functions (B), including vasculature and cytoskeleton (C). **(D)** PC1 separated female responders from non-responders. **(E-F)** Genes that made the highest contribution to PC1 in females were sorted by their relevance to cellular functions (E), including smooth muscle and mitochondrial function (F).

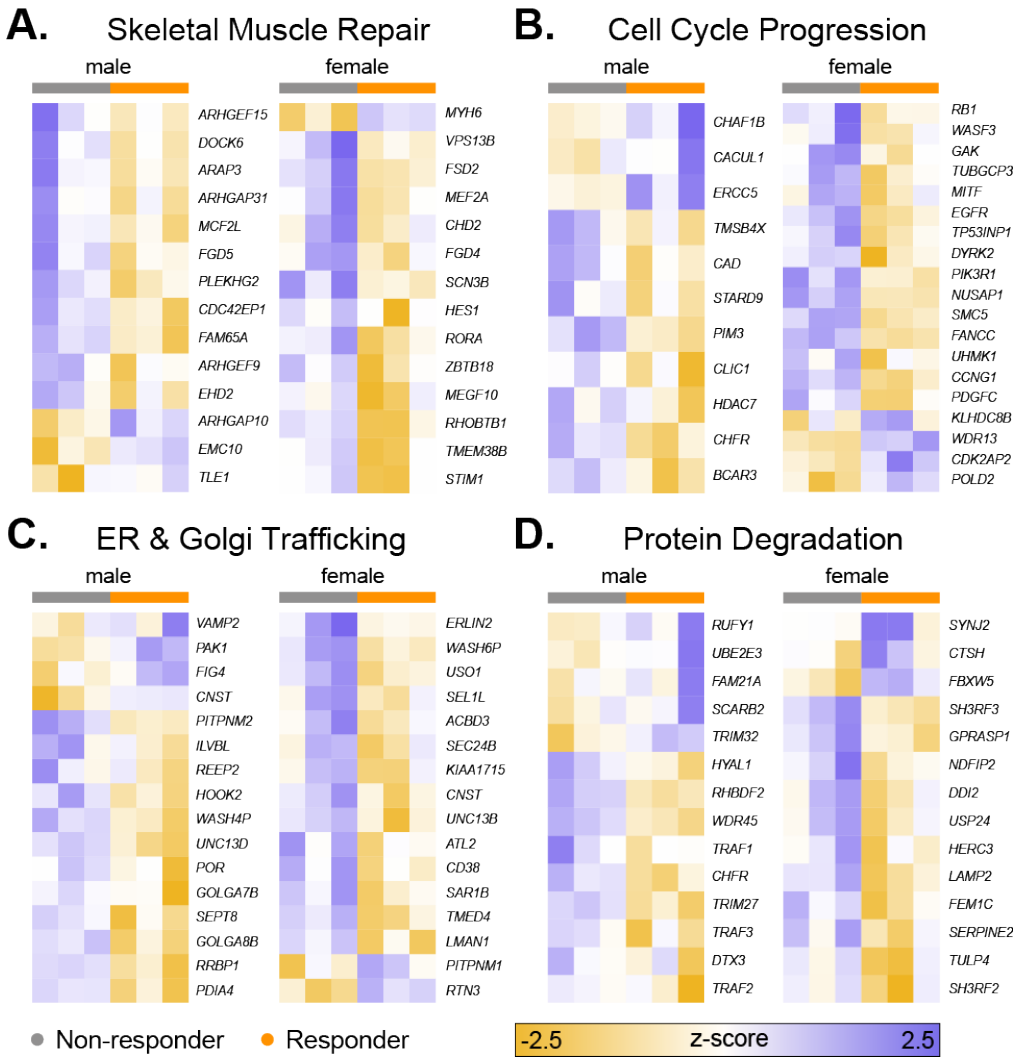

Figure 3.7 Pathways enriched in both sexes prior to exercise. (A-D) Pathways where non-responders exhibited higher expression of relevant genes prior to exercise include skeletal muscle repair (A), cell cycle progression (B), ER and Golgi trafficking (C) and protein degradation (D).

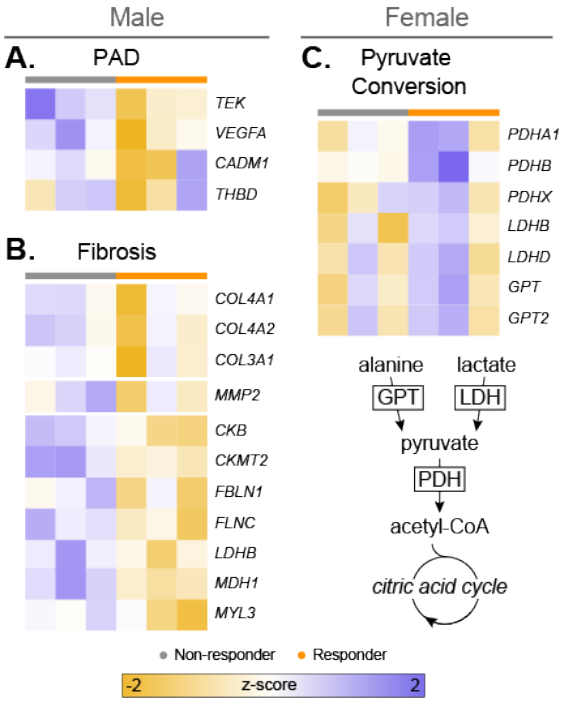

Figure 3.8 Candidate biomarkers to distinguish responders from non-responders prior to exercise. (A-B) Heatmaps of RNASeq gene counts per million showed divergent expression of genes encoding biomarkers of peripheral artery disease (A) and fibrosis (B) in male non-responders and responders prior to exercise intervention. **(C)** Genes encoding enzymes catalyzing the synthesis of pyruvate from alanine and lactate were upregulated in female responders versus non-responders prior to exercise.

expressed prior to exercise. We found that male non-responders expressed genes encoding fibrosis biomarkers associated with PAD (Figure 3.8A) and liver and muscle disease (Figure 3.8B) more highly than male responders. We also observed that female responders could be distinguished from non-responders by higher expression of mitochondrial respiratory chain members. Circulating levels of metabolites such as pyruvate, lactate, and alanine can be used as biomarkers of mitochondrial dysfunction (Boenzi and Diodato 2018). We found that female responders expressed higher levels of the enzymes that catalyze the creation of these metabolites (Figure 3.8C). Overall, we have identified several candidate biomarkers that might be used to distinguish responders from non-responders prior to exercise without the need for a skeletal muscle biopsy.

3.5 Discussion

3.5.1 Responders have few transcriptional changes in response to exercise intervention

Despite improvements in six-minute walk distance, myofiber cross-sectional area, and capillary density, the transcriptional profile of PAD patients responsive to exercise intervention was not greatly altered by exercise. One of the few sarcomeric genes responsive to exercise in both male and female patients was *MYH8*. *MYH8* encodes a neonatal myosin heavy chain that, along with MYH3 and MYL4, comprises a trio of developmental sarcomeric proteins that are re-expressed during muscle regeneration (Schiaffino et al. 2015), a process that occurs under the control of the myogenic regulatory factors MYOD, MYF5, MRF4 (*MYF6*) and myogenin (*MYOG*) (Hernandez-Hernandez et al. 2017). Expression of *Myh8* is increased after acute hindlimb ischemia in mice and expression of both *Myh8* and *Myh3* has been shown to be upregulated after chronic exercise in a mouse model of diabetic PAD (Lee et al. 2004, Nagase et al. 2017). However, we did not find that exercise increased expression of other myogenic genes in responders; there was no change in expression of *MYOG*, *MYOD1*, or *MYF6*, neither *MYL4* nor *MYF5* were expressed, and expression of *MYH3* was actually downregulated after exercise (Figure 3.2D). Thus, it appears unlikely that exercise responsiveness was the result of increased expression of myogenic and regenerative factors. We did observe a heterogenous but substantial increase in expression of lipid metabolism genes in female responders after exercise (Figure 3.2E). Exercise has been previously shown to improve expression of lipogenic enzymes and increase triglyceride synthesis in the skeletal muscle of women with diabetes mellitus (Schenk and Horowitz 2007). Female responders thus may respond to walking exercise therapy, an endurance-type exercise, with improvements in oxidative metabolism. Thorough interrogation of the metabolic state of female responders and non-responders before and after exercise intervention will be required to address this hypothesis. With that being said,

our data overall suggest that responsiveness to exercise is likely mediated by initial factors that exist in skeletal muscle prior to exercise intervention.

3.5.2 Sexual dimorphism in peripheral artery disease

Prior to exercise intervention, responders and non-responders had distinct transcriptional profiles that we found to be divergent between sexes. Skeletal muscle is a highly sexually dimorphic tissue, with males and females exhibiting differences in gene expression, metabolism, and exercise performance that are at least partially mediated by sex hormones (Tarnopolsky et al. 1990, Miller et al. 1993, Lindle et al. 1997, Friedlander et al. 1998, Horton et al. 1998, Carter et al. 2001, Welle et al. 2008, Wust et al. 2008). Sex is also known to be a contributing variable to atherosclerosis, where men generally have a worse risk factor profile, greater plaque burden, and more markers of inflammation (Man et al. 2020). However, the mechanism(s) responsible for these differences are poorly understood due to the limited number of studies analyzing sex differences in vascular disease (Lu et al. 2018, Ramirez and Hibbert 2018, Lu et al. 2020, Man et al. 2020). Similarly, the extent to which sex contributes to PAD is unclear. While some groups have reported that women are more likely to have asymptomatic PAD, atypical leg symptoms, and higher rates of functional impairment and mobility loss, others have found no difference between men and women (Gardner 2002, McDermott et al. 2003, Collins et al. 2006, Sigvant et al. 2007, Gardner et al. 2009, McDermott et al. 2011). Overall, it is difficult to establish any role that sex might play in PAD because the majority of clinical trial participants are men (Hirsch et al. 2012). Our findings suggest that responsiveness to exercise may be the result of different mechanisms in men versus women. Increased expression of immune cell markers in male non-responders might reflect increased presence of inflammation, which is associated with plaque rupture and acute ischemia (Libby 2002). Higher expression of vascular, immune, and neuronal cell markers in male non-

responders may reflect an increased proportion of non-myofiber cell types, which could reduce the impact of exercise-induced changes in skeletal muscle. Female responders had increased expression of mitochondrial respiratory chain members as well as mitochondrial genes, which could be suggestive of either increased numbers of mitochondria or better mitochondrial function. As the skeletal muscle adaptation to endurance exercise is predominantly the result of improvements in oxidative metabolism (Holloszy 1967, Gollnick et al. 1973, Holloszy and Booth 1976), female responders may be better prepared to react to exercise intervention than non-responders.

3.5.3 Use of biomarkers to distinguish exercise responders from non-responders

Male non-responders had distinct upregulation of genes involved in extracellular matrix formation and fibrosis compared to responders prior to exercise. A few of the genes contributing to this transcriptional signature encode proteins that serve as serum protein biomarkers, enabling their identification without an invasive biopsy. There are few biomarkers associated with peripheral artery disease. Those that have been proposed include angiogenic markers such as soluble TIE2 and VEGF, markers of endothelial dysfunction such as soluble cell adhesion molecule 1, and coagulation factors such as thrombomodulin (Cooke and Wilson 2010). Chronic hepatitis B and hepatitis C are accompanied by profound liver fibrosis. Increased type IV collage 7s and increased procollagen type III peptide are associated with fibrosis staging in chronic hepatitis B and correlate with worse fibrosis in adults with chronic hepatitis C (Valva et al. 2011, Tsuji et al. 2020). Patients with nonalcoholic fatty liver disease have liver fibrosis that can be distinguished by increased circulating MMP2 (Yilmaz and Eren 2019). Duchenne muscular dystrophy (DMD) is characterized by skeletal muscle fibrosis that is associated with a number of elevated serum factors, including creatine kinase, fibulin 1, filamin C, lactate dehydrogenase, malate dehydrogenase, and myosin light chain 3 (Al-Khalili Szigyarto

2020). The genes encoding these markers are all upregulated in male non-responders prior to exercise, suggesting that they may be able to identify male patients responsive to exercise prior to intervention.

Female responders at baseline exhibited upregulation of genes encoding mitochondrial respiratory chain members, suggesting that responders might be distinguishable from non-responders by improved mitochondrial function. In addition to these mitochondrial genes, female responders had strong upregulation of genes encoding subunits of pyruvate dehydrogenase, lactate dehydrogenase, and alanine aminotransferase. These enzymes regulate the conversion of lactate and alanine into pyruvate as well as the conversion of pyruvate into acetyl-CoA, which directs pyruvate into the citric acid cycle. Mitochondrial dysfunction can result in increased serum pyruvate, lactate, and alanine (Boenzi and Diodato 2018). A recent investigation of a large group of prospective general-population cohorts found that higher concentrations of lactate and pyruvate were strongly associated with incident of PAD (Tikkanen et al. 2021). Female responders might be thus distinguishable by lower levels of pyruvate, lactate, and alanine than non-responders. For all of the candidate biomarkers we have proposed, further investigation is needed to determine whether these transcriptional signatures reflect detectable changes in circulating protein. Should these biomarkers prove suitable for use in the clinic, they may be able to identify non-responders to exercise intervention, allowing for a more tailored approach to care.

Chapter 4. Discussion

4.1 Overview

It has been observed for many years that skeletal muscle is highly adaptable, but how sex influences skeletal muscle adaptation in response to various stimuli, including glucocorticoid steroids and exercise, remains poorly described. Overall, this thesis presents two models in which male and female muscle respond differently to ergogenic stimuli. We have reported a novel paradigm in which weekly glucocorticoids exert sex-specific effects in mice through interactions between the glucocorticoid receptor and sex steroid receptors, leading to epigenetic and transcriptomic changes. We have also reported sex-specific transcriptional responses to exercise in the context of peripheral artery disease. In this chapter, I discuss how these findings contribute to our understanding of the ways in which sex influences skeletal muscle health and performance.

4.2 Discussion

4.2.1 Using mice to model skeletal muscle performance

Although glucocorticoid steroids have been banned by the World Anti-Doping Agency as potentially performance-enhancing drugs for over three decades, investigations into the influence of glucocorticoids on human athletic performance have produced inconsistent results (Vernec et al. 2020). While acute, low doses of glucocorticoids have not been shown to improve athletic performance, 50-60mg doses administered daily for 1-3 weeks have led to improvements in resistance to fatigue and force production (Arlettaz et al. 2007, Collomp et al. 2008, Le Panse et al. 2009, Zorgati et al. 2014). We treated a group of male and female mice once a week for four weeks with a comparable dose (1mg/kg) and observed that weekly prednisone was able to significantly improve specific force and contraction and relaxation time in the tibialis anterior muscle of both sexes. In males, this beneficial outcome was the result of

improvements in protein synthesis and calcium handling (Figure 4.1). However, improvements in female muscle performance were due to modulation of white adipose lipolysis and skeletal muscle lipid metabolism (Figure 4.2). These findings could have striking implications for the nearly one million Americans who are chronic users of glucocorticoid steroids, but we must first ask how our results might translate from mouse to human skeletal muscle.

Humans and mice have substantial differences in body size, developmental timespan, and cell biology, making it difficult to render a perfect comparison of skeletal muscle structure and function between the two species. Several features of skeletal muscle such as myofiber shortening velocity, myosin heavy chain kinetics, actin translocation speed, optimal fiber length, and myonuclear domain size scale proportionately with body size (Hook et al. 2001, Pellegrino et al. 2003, Andruchov et al. 2004, Marx et al. 2006, Bicer and Reiser 2007, Eng et al. 2008, Liu et al. 2009, Hu et al. 2017). Others, such as length of fiber excursion (Hu et al. 2017) and tendon and fascia thickness (Partridge 2013), do not. Still other aspects of human skeletal muscle biology are recapitulated by mouse muscle without adjusting for scale; the distance between the center of a myofiber and the nearest capillary is similar between mice and humans, as are satellite cell proliferation rate and migration speed (Partridge 2013). In studying the hindlimbs specifically we often face the difficult task of reconciling differences in locomotion. Mice, non-cursorial quadrupeds, have increased flexibility of the knee and hip during locomotion when compared to cursorial bipedal humans (Jenkins 1971, Kadaba et al. 1990, Akay et al. 2014). These fundamental differences in gait have implications for the biomechanical load experienced by rodents versus humans during hindlimb use, including exercise (Hu et al. 2017).

A substantial divergence between mouse and human skeletal muscle is myosin heavy chain isoform expression. Unlike mice, adult humans do not express *MYH4* and lack type IIB

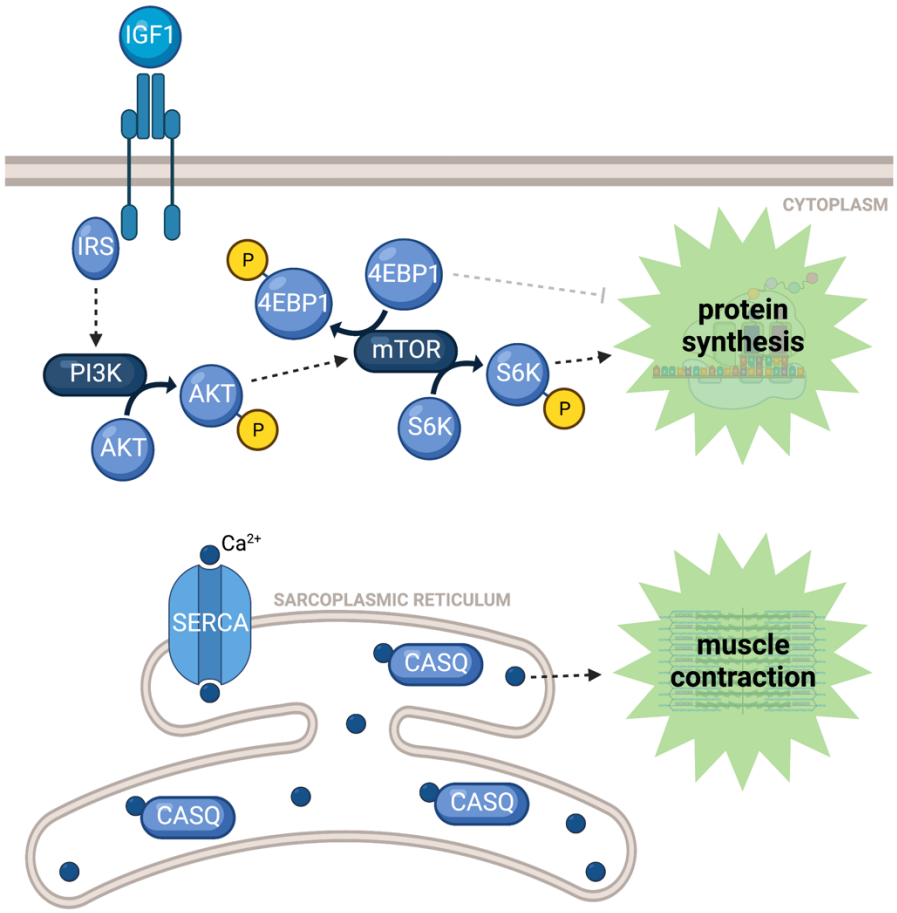

Figure 4.1 Weekly prednisone modulates multiple pathways in male skeletal muscle to improve performance. Proposed mechanistic model of weekly glucocorticoid action in male muscle. Increased IGF1 stimulates PI3K/AKT signaling to activate mTOR, which regulates protein synthesis and leads to hypertrophy. Meanwhile, increased expression of calcium-handling genes encoding SERCA and CASQ2 improves calcium-handling and muscle contraction.

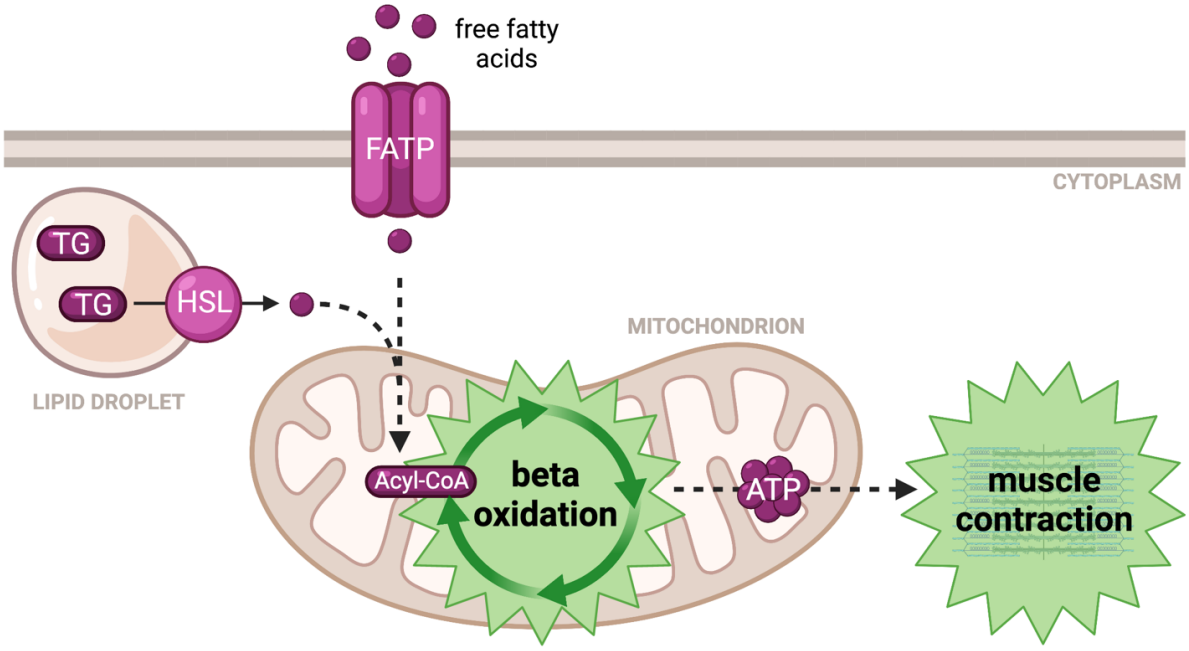

Figure 4.2 Weekly prednisone modulates lipid metabolism in female skeletal muscle. Proposed mechanistic model of weekly glucocorticoid action in female muscle. Increased lipolysis in white adipose tissue and increased glyceride catabolism from the myofiber lipid droplet led to increased fuel for beta oxidation. This ATP is then used for muscle contraction, leading to improved strength and endurance.

fibers (Smerdu et al. 1994). Accordingly, the relative proportion of fiber types differs between humans and rodents, with human muscles generally exhibiting higher percentages of type I or type IIA fibers than the direct mouse counterpart (Pellegrino et al. 2003). Human quadriceps have also been found to be more transcriptionally similar to the mouse soleus than the mouse quadriceps (Kho et al. 2006). However, human quadriceps have a mitochondrial respiratory capacity that is more similar to the mouse quadriceps than the mouse soleus (Jacobs et al. 2013), suggesting that fast fibers in humans are metabolically similar to fast fibers in mice despite differences in myosin heavy chain expression. This suggests that differences in relative fiber type percentages may not be the correct metric by which to compare human and mouse skeletal muscle metabolism.

Without treating a cohort of humans with weekly prednisone and performing RNA-sequencing on muscle biopsies, it is difficult to say whether our findings in mice will be fully recapitulated in humans. However, multiple species including mice, humans, and dogs have exhibited improvements in muscle performance after short term dosing regimens of glucocorticoids, suggesting that the beneficial effects are at least somewhat conserved across species. We have previously reported that, similar to *mdx* mice (Quattrocelli et al. 2017), DMD patients receiving weekend glucocorticoids had reduced markers of insulin resistance and higher whole body percent lean mass compared to patients receiving chronic glucocorticoids (Quattrocelli et al. 2019). Other agents that improve skeletal muscle specific force such as the anabolic steroids nandrolone and testosterone have similar effects in both rodents (Prezant et al. 1993, Lewis et al. 1999) and humans (Creutzberg et al. 2003, Casaburi et al. 2004). Crucially, human skeletal muscle expresses AR, ER, and GR, the mediators necessary for the beneficial response to weekly prednisone in mice. Overall, we predict that humans will likely receive benefit from weekly prednisone though the sex-specific mechanisms we observed in mice. However, further investigation will be needed to support this hypothesis.

4.2.2 Epigenetic response to weekly glucocorticoids

One mechanism whereby GR regulates target gene expression is through modulation of the chromatin landscape of its response elements. Both cells and mice treated with dexamethasone exhibit changes in nucleosome occupancy (Wu et al. 2015) and histone modifications, including the active enhancer mark H3K27ac (Wu et al. 2015, Guo et al. 2017, McDowell et al. 2018, Clark et al. 2019, Quagliarini et al. 2019). Although these studies have investigated exposure times to glucocorticoids ranging from a few minutes (McDowell et al. 2018) to a few days (Wang et al. 2021), there has been little investigation of the chromatin landscape after the cessation of glucocorticoid treatment. Jubb et al. (2017) examined

chromatin reorganization in macrophages exposed to 100nM dexamethasone in culture and found that cells incubated with glucocorticoids maintained significant reorganization of the *Fkbp5* locus up to 120 hours after washout. Their work, which also showcased dynamic chromatin decompaction as early as five minutes after dexamethasone treatment, suggests that glucocorticoids can quickly induce changes to genomic architecture that outlast the lifespan of the ligand.

We similarly observed that weekly prednisone induced changes in H3K27ac enrichment that persisted many days after the cessation of a course of weekly prednisone. We identified a group of enhancers that were not present in vehicle-treated animals but were enriched for H3K27ac four days after weekly prednisone treatment, which we named prednisone-response (PRE). The vast majority (98.9%) of male PREs were lost by eight days after the final weekly dose, suggesting that the male response to weekly prednisone did not stimulate long-lasting alterations to the chromatin landscape. In contrast, nearly a third (29.2%) of female PREs were still present eight days after the final weekly dose. The mechanism behind this sexual dimorphism is unclear. There is no difference in expression of the major histone deacetylases and acetyltransferases between males and females treated with either weekly prednisone or vehicle. However, it has previously been shown that alterations in skeletal muscle lipid metabolism and exercise performance are mediated via transcriptional changes that are still detectable 72 hours after physical activity (Mahoney et al. 2005, Stepto et al. 2009, Chapman et al. 2020). In contrast, the transcriptional response to resistance training—which, like the male-specific response to weekly prednisone, induces hypertrophy via mTOR (Philp et al. 2011)—is much milder. One group investigating the quadriceps transcriptomes of long-term trained athletes found that endurance-trained men had 1,097 differentially expressed genes compared to inactive male controls, while strength-trained men had only 26 (Chapman et al. 2020). Is the

longevity of female-specific chromatin remodeling the result of an endurance exercise-like adaptation? Further investigation is needed to address this possibility.

When we asked what transcription factor binding motifs were present in long-lived PREs maintained eight days after the final weekly injection, we found that both male and female myofibers had enrichment of the MEF2C consensus motif (Figure 2.17). The MEF2 family is a group of four transcriptional activators that play key roles in numerous developmental processes, including myogenesis and skeletal muscle differentiation (Potthoff and Olson 2007, Taylor and Hughes 2017). MEF2 proteins have been demonstrated to influence striated muscle growth (Lin et al. 1997, Yogev et al. 2013), fiber type, metabolism (Potthoff et al. 2007, Anderson et al. 2015), and sarcomere organization (Potthoff et al. 2007). MEF2 has also been implicated as a mediator of the skeletal muscle response to chronic exercise (Yu et al. 2001, Vissing et al. 2008, Amoasii et al. 2019). It is striking that although male and female myofibers undergo sex-specific changes to their chromatin architecture, we observed enrichment of the same motif at these sex-specific enhancers. Little is known of how biological sex influences MEF2C activity; studies investigating loss of MEF2C in rodent models have either used male animals alone (Potthoff et al. 2007, Anderson et al. 2015) or not specified sex (Lin et al. 1997, Potthoff et al. 2007). One investigation did find that endurance exercise upregulated different MEF2 proteins in men, who had a trending increase in *MEF2C*, versus women, who had significantly increased *MEF2A* expression after cycling 90min at 60% VO_2max (Vissing et al. 2008). However, it is possible that MEF2C is acting in concert with another transcription factor to direct different gene expression programs in the two sexes; as a transcriptional activator, MEF2C depends upon co-factor interactions to modulate target gene expression (Black and Olson 1998, McKinsey et al. 2002). Further study is required to determine which co-factors could be responsible for this modulation.

4.2.3 Sex steroid receptors modulate some, but not all, of the response to weekly glucocorticoids

As with most members of the nuclear receptor superfamily, GR's ability to influence tissue-specific gene regulation relies upon its interaction with co-factors, which can include other nuclear receptors. AR shares some homology with GR and is capable of forming heterodimers to regulate gene expression (Chen et al. 1997). We found that antagonism of AR completely ablated the male-specific transcriptional response to weekly prednisone, suggesting that AR might be the co-factor responsible for modulating the male response. This is concordant with the known roles of testosterone and AR in the regulation of skeletal muscle mass and contractility. It has been previously demonstrated that testosterone increases myofiber hypertrophy via IGF1/PI3K signaling (Deane et al. 2013, Hughes et al. 2016). Whole-body AR knockout significantly reduces body mass and tetanic force (MacLean et al. 2008), while gonadectomy to remove circulating testosterone has been shown to reduce muscle mass, peak twitch tension (Jiang and Klueber 1989), and IGF1 expression (Oner et al. 2008, White et al. 2013). Gonadectomy has also been shown to reduce peak Ca^{2+} transient amplitude and slow relaxation time in isolated cardiomyocytes, both of which could be rescued by treatment with testosterone (Golden et al. 2003, Curl et al. 2009). Our findings suggest that weekly prednisone stimulates GR to act in concert with AR to regulate these pathways, leading to improvements in muscle protein synthesis and contractility.

While antagonism of the androgen receptor completely abolished the male response to weekly prednisone, we observed a more nuanced response in females upon loss of their sex steroid receptor. Unlike AR, which is similar enough to GR to form heterodimers, ER cooperatively regulates gene transcription with GR through recruitment of shared co-factors such as NCoA-2, NCoA-3, and MED14 (Bolt et al. 2013). Estrogen is considered a mediator of muscle health, with roles in the maintenance of mitochondrial function and sarcomeric

contractility (Ventura-Clapier et al. 2019, McMillin et al. 2022), both of which were improved in female mice after weekly prednisone. Estrogen also plays a regulatory role in fatty acid metabolism. Postmenopausal women given estrogen supplementation have significantly reduced circulating free fatty acids (Jensen et al. 1994, Gaspard et al. 1999, Gormsen et al. 2012) and are protected from the characteristic postmenopausal abdominal adipose accumulation (Haarbo et al. 1991). Mice lacking aromatase, a key enzyme responsible for estrogen synthesis, have significantly increased adipocyte cross-sectional area and larger infra-renal and gonadal fat pads (Jones et al. 2000). We observed that ER antagonism prevented weekly prednisone from reducing adipocyte cross-sectional area and whole-body percent fat mass (Figure 2.14), suggesting that this lipolytic program is mediated by ER.

While antagonism of ER did prevent the ergogenic response to weekly prednisone, we observed only partial loss of the female-specific transcriptional program, suggesting that another co-factor may assist GR in modulating this response. One candidate is C/EBPβ, which has been shown to be a major co-factor of GR in liver (Grontved et al. 2013) and adipose tissue (Steger et al. 2010). C/EBPβ is a member of the CCAAT/enhancer binding protein family and serves as a transcriptional regulator controlling numerous cellular processes including adipogenesis, adipocyte differentiation (Cristancho and Lazar 2011), fatty acid oxidation (Du et al. 2019), and ovarian follicle development (Sterneck et al. 1997). The gene encoding C/EBPβ, *Cebpb*, was upregulated in female myofibers treated with weekly prednisone compared to vehicle-treated controls (Figure 2.9). When we used the JASPAR Scan tool (Fornes et al. 2020) to query for transcription factor binding motifs present in all five GREs with female-specific GR binding after weekly prednisone (Figure 2.15), we found the C/EBP binding motif in all GREs. In fact, C/EBPβ was the only transcription factor responsive to weekly prednisone whose motif we found in all five GREs. Although these data are highly suggestive, further work is required to fully understand the role of C/EBPβ in modulating the female response to weekly prednisone.

4.2.4 Lipid metabolism as a mechanism of ergogenic adaptation in females

Skeletal muscle relies upon the oxidative metabolism of lipids to provide fuel for contraction during submaximal exercise (Spriet and Watt 2003). As it adapts to endurance exercise training, skeletal muscle shifts its substrate utilization away from carbohydrates and towards lipids (Hurley et al. 1986). This process begins within a few days and increases in magnitude as training progresses (Mendenhall et al. 1994, Phillips et al. 1996). Intriguingly, this shift is much stronger in women (Tarnopolsky et al. 1995, Carter et al. 2001), who demonstrate greater lipid utilization than men during submaximal exercise even on the same controlled diet (Tarnopolsky et al. 1990). This sexually dimorphic response to endurance training is complex and has been attributed to both skeletal muscle intrinsic (Berthon et al. 1998, Steffensen et al. 2002, Kiens et al. 2004, Roepstorff et al. 2006, Maher et al. 2009, Maher et al. 2010) and extrinsic factors (Hellstrom et al. 1996, Horton et al. 1998, Roepstorff et al. 2002). Our observation that the female-specific response to weekly prednisone was the result of changes to lipid metabolism further emphasizes how female animals are able to leverage gains in fatty acid oxidation to improve muscle health and performance.

Intriguingly, we also observed that exercise intervention increased expression of lipid metabolism genes in female PAD patients who were responsive to exercise. Prior to exercise intervention, these female responders exhibited greater expression of mitochondrial genes compared to non-responsive females. Neither of these trends were observed in male PAD patients. It is tempting to speculate that fatty acid oxidation is a crucial feature of the beneficial response to exercise in women with PAD; perhaps female PAD patients that do not respond to exercise do not have the mitochondrial capacity for it. It is certainly true that many PAD patients present with skeletal muscle mitochondrial dysfunction; previous investigations have reported that PAD is associated with reduced mitochondrial respiration, oxidative phosphorylation activity

(Pipinos et al. 2006, White et al. 2016, Ryan et al. 2018), and mitochondrial health as measured by phosphocreatine recovery time (Anderson et al. 2009). However, all of these clinical investigations were performed in majority male cohorts, and it is difficult to know how sex influences this paradigm. It has been shown that striated muscle mitochondrial function is sexually dimorphic, as both female rabbits and healthy young women have been found to have greater fatty acid oxidative capacity than their male counterparts, with women also exhibiting increased mitochondrial volume density (McCully et al. 2007, Montero et al. 2018). Although our preliminary data is highly suggestive, further investigation is needed to understand the role that mitochondrial health and fatty acid metabolism play in mediating responsiveness to exercise female patients with PAD.

4.3 Concluding remarks

Skeletal muscle is a dynamic tissue that is able to respond to adverse or ergogenic stimuli with alterations in tissue structure, metabolism, and contractility. Although a high degree of sexual dimorphism has been reported in mammals, including rodents and humans, sex is rarely taken into account by researchers investigating the mechanisms driving the skeletal muscle adaptive response to various stimuli. In this thesis, I present two models of ergogenic skeletal muscle adaptation that are mediated by different mechanisms between males and females. Both models describe interventions to which male and female muscle have a similar response—in chapter 2, increased tetanic force; in chapter 3, improved six-minute walk distance—but further investigation revealed that the underlying mechanisms behind these improvements were the result of divergent responses between the sexes. These findings emphasize that male and female skeletal muscle are two similar but distinct organs that respond to the same stimulus with unique adaptations. This work has important implications not only for

the many millions of people who take chronic glucocorticoids or live with peripheral artery disease, but indeed for any investigation into skeletal muscle health and performance.

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