ABSTRACT

Effects of Resistance Exercise Load on Androgen Receptor–DNA Binding, Androgen Regulated Gene Expression, and β-Catenin Mediation in Human Skeletal Muscle

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The purpose of the study was to determine the effect low (LL) and high (HL) load resistance exercise on serum total and free testosterone, and muscle testosterone, dihydrotestosterone (DHT), androgen receptor (AR), and AR-DNA binding, β-catenin, and AR-regulated/responsive gene expression. Ten participants completed LL and HL resistance exercise with samples collected pre-, 3h post-, and 24h post-exercise. Separate 2x3 factorial repeated measures ANOVAs were completed. Serum free and total testosterone significantly decreased 3h post-exercise with free testosterone remaining significantly decreased 24h post-exercise in both conditions ($p<.05$). No significant differences were observed in muscle AR, testosterone, or DHT in either condition ($p>.05$). In response to HL, AR-DNA binding significantly increased at 3h post-exercise ($p<.05$), whereas no significant differences were observed in response to LL ($p>.05$). Muscle β-catenin content was significantly greater in HL condition ($p<.05$). No significant/practical differences were observed in *AR*, *MyoD*, *MYOG*, *IGF-1Ea*, or *p21-cip1* expression ($p>.05$).

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by

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TABLE OF CONTENTS

LIST OF FIGURES vi
LIST OF TABLES vii
ACKNOWLEDGMENTS ix
CHAPTER ONE 1
Introduction 1
Purpose 6
Hypotheses 6
Delimitations 7
Limitations 8
Assumptions 8
Definition of Terms 9
CHAPTER TWO 11
Review of Literature 11
Androgen Receptor Structure, Function, and Signaling 11
β-Catenin and AR interactions 16
Resistance Exercise Load and Acute AR Signaling Response 21
CHAPTER THREE 26
Methods 26
Experimental Approach 26
Participants 27
Study Site 28
Independent and Dependent Variables 28
Participant Entry Protocol 28
Dietary Analysis 29
Hydration Analysis 30
Muscle Biopsies 30
Blood Sampling 31
Resistance Exercise Max Testing 31
Resistance Exercise Protocol 32
Serum Hormone Assessment 33
Intramuscular Protein and Hormone Assessment 33
mRNA Gene Expression Assessment 34

Statistical Analysis 36
CHAPTER FOUR 37
Results 37
Dietary Analysis 37
Resistance Exercise Volume and Rating of Perceived Exertion 37
Total Body Water Analysis 38
Packed Cell Volume Analysis 38
Delayed Onset Muscle Soreness Analysis 39
Intramuscular Androgen Receptor-DNA Binding 40
Intramuscular β-Catenin Content 41
Intramuscular Androgen Receptor Protein Content 42
Intramuscular Testosterone Concentration 44
Intramuscular Dihydrotestosterone Concentration 45
Serum Total Testosterone Concentration 46
Serum Free Testosterone Concentration 47
Intramuscular Androgen Receptor (AR) mRNA Expression 48
Intramuscular Myoblast Determination Protein 1 (MyoD) mRNA Expression 49
Intramuscular Myogenin (MYOG) mRNA Expression 50
Intramuscular Insulin-Like Growth Factor-1Ea (IGF-1Ea) mRNA Expression 51
Intramuscular Cyclin-Dependent Kinase Inhibitor 1 (p21-cip1) mRNA Expression52
CHAPTER FIVE 54
Discussion 54
Baseline Testing Measures 54
Resistance Exercise Load-Mediated AR Signaling 54
β-Catenin and AR Signaling 62
Conclusion 70
BIBLIOGRAPHY 73

LIST OF FIGURES

Figure 4.1 Changes in Delayed Onset Muscle Soreness 40

Figure 4.2 Changes in Intramuscular AR-DNA Binding Activity 41

Figure 4.3 Changes in Intramuscular β-Catenin Content...42

Figure 4.4 Changes in Intramuscular Androgen Receptor Protein Content 43

Figure 4.5 Changes in Intramuscular Testosterone Concentration...................................44

Figure 4.6 Changes in Intramuscular Dihydrotestosterone Concentration........................45

Figure 4.7 Changes in Serum Total Testosterone Concentration 47

Figure 4.8 Changes in Serum Free Testosterone Concentration.......................................48

Figure 4.9 Changes in Intramuscular Androgen Receptor (*AR*) mRNA Expression.........49

Figure 4.10 Changes in Intramuscular Myoblast Determination Protein 1 (*MyoD*) mRNA Expression 50

Figure 4.11 Changes in Intramuscular Myogenin (*MYOG*) mRNA Expression 51

Figure 4.12 Changes in Intramuscular Insulin-Like Growth Factor-1Ea (*IGF-1Ea*) mRNA Expression 52

Figure 4.13 Changes in Intramuscular Cyclin-Dependent Kinase Inhibitor 1 (*p21-cip1*) mRNA Expression 53

Figure 5.1 Proposed Cytoplasmic Androgen-Dependent AR Activation by β-Catenin 66

Figure 5.2 Proposed Nucleoplasmic Androgen-Dependent AR Activation by β-Catenin 67

Figure 5.3 Proposed Cytoplasmic Androgen-Independent AR Activation by β-Catenin 68

LIST OF TABLES

Table 3.1 Participant Descriptives 28

Table 3.2 Primer Sequences For Genes of Interest 35

Table 4.1 Total Calories and Macronutrient Intake 37

Table 4.2 Training Volume and Changes in Rating of Perceived Exertion 38

Table 4.3 Total Body Water Responses 38

Table 4.4 Packed Cell Volume Responses 39

Table 4.5 Changes in Delayed Onset Muscle Soreness 39

Table 4.6 Changes in Intramuscular AR-DNA Binding Activity 41

Table 4.7 Changes in Intramuscular β-Catenin Content 42

Table 4.8 Changes in Intramuscular Androgen Receptor Protein Content 43

Table 4.9 Changes in Intramuscular Testosterone Concentration 44

Table 4.10 Changes in Intramuscular Dihydrotestosterone Concentration 45

Table 4.11 Changes in Serum Total Testosterone Concentration 46

Table 4.12 Changes in Serum Free Testosterone Concentration 48

Table 4.13 Changes in Intramuscular Androgen Receptor (*AR*) mRNA Expression 49

Table 4.14 Changes in Intramuscular Myoblast Determination Protein 1 (*MyoD*) mRNA Expression 50

Table 4.15 Changes in Intramuscular Myogenin (*MYOG*) mRNA Expression 51

Table 4.16 Changes in Intramuscular Insulin-Like Growth Factor-1Ea (*IGF-1Ea*) mRNA Expression 52

Table 4.17 Changes in Intramuscular Cyclin-Dependent Kinase Inhibitor 1 (*p21-cip1*) mRNA Expression 53

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CHAPTER ONE

Introduction

Androgens, such as testosterone and dihydrotestosterone (DHT), play a pivotal role in muscle specific gene and protein expression, which can ultimately lead to skeletal muscle hypertrophy (Fink et al., 2018). Primarily, androgens exert their anabolic effects through the bloodstream where they interact with androgen receptors (AR) in skeletal muscle. Specifically, free/unbound testosterone diffuses across the sarcolemma in skeletal muscle, where a portion is converted to the more biologically active dihydrotestosterone (DHT) by the enzyme, 5α-reductase. Due to DHT being more biologically active, it is able to bind to the nuclear hormone AR with a 2-fold higher affinity and a decreased dissociation rate of 5-fold compared to testosterone (Grino et al., 1990). Once bound by the androgen, the activated AR undergoes a conformational change causing a dissociation from the heterocomplex of heat shock proteins and other co-chaperones, ultimately resulting in dimerization. After dissociation, it is then considered an active AR complex and is translocated into the nucleus where it binds to the androgen response element (ARE). The ARE is a highly conserved palindromic, dihexameric motif organized as inverted repeats of 5′-AGAACA-3′-like motifs with a three-nucleotide spacer present in promoters or enhancers of genes targeted by the AR (Denayer et al., 2010). The androgen receptor (*AR*) gene, the p21 cyclin-dependent kinase inhibitor protein 1 (*p21-cip1*) gene, and the insulin-like growth factor-1 (*IGF-IEa*) gene have all been found to contain AREs (Roberts et al., 2009; Grad et al., 1999; Lu et al., 2000; Wu et al., 2007). Interestingly, the myogenin

(*MYOG*) and myoblast determination protein 1 (*MyoD*) genes do not contain AREs but do appear to be induced by androgen treatment (Montano et al. 2007; Lee, 2002). This AR-DNA binding results in up-regulation of these genes (and others) which play significant roles in skeletal muscle anabolism and contribute to hypertrophy of the muscle (Kicman, 2010).

AR activation and subsequent DNA-binding has been shown to increase in response to resistance exercise (Spillane et al., 2015). It is thought the acute increase in serum/muscle androgen and/or AR protein concentrations are responsible for the up-regulation in AR signaling observed. However, data showing increases in AR signaling with concomitant increases in serum/muscle androgen concentrations and AR content are inconsistent. A number of studies have found acute increases in serum androgen concentrations in response to a single bout of resistance exercise (Ahtiainen et al., 2011; Ahtiainen et al., 2004; Cadore et al., 2008; Hakkinen & Pakarinen, 1993; Kraemer et al., 1991; Kraemer et al., 1990; Morton et al., 2016; Roberts et al., 2009; Spiering et al., 2009; Vingren et al., 2009; Walker et al., 2011; West et al., 2009; Willoughby & Taylor, 2004). Other studies have found significant decreases or no change in serum androgen concentrations in response to a single bout of resistance exercise (Bamman et al., 2001; Fry & Lohnes, 2010; Gonzalez et al., 2015; Goto et al., 2011; Harber et al., 2004; Spillane et al., 2015; Wu & Lin, 2010). Additionally, increases in AR mRNA or protein expression in response to acute resistance exercise have been observed (Bamman et al., 2001; Lee et al., 2003; Willoughby & Taylor, 2004). However, AR mRNA and protein expression have been reported to decrease or not significantly change in a number of studies as well (Ahtiainen et al., 2011; Hulmi et al., 2008; Lee et al., 2003; Ratamess et al., 2005; Spiering

et al., 2009; Spillane et al., 2015; Vingren et al., 2009). This data suggests that increases in AR signaling may not be primarily dependent on rises in androgens or AR protein concentrations. However, this inconsistent response may be due to the variability in resistance exercise protocols and lack of controlling program design variables.

Moreover, recent research suggests increases in AR signaling in response to mechanical loading may be due to up-regulation in specific AR-interacting proteins (Kim & Lee, 2009; Ueda et al., 2002; Yang et al., 2014; Spillane et al., 2015). These AR-interacting proteins may be responsible for the increase in AR signaling due to modulating AR binding affinity and/or activating the AR in a ligand-dependent or -independent manner. Specifically, β-catenin, an important multifunctional protein involved in wingless-type MMTV integration site (Wnt) signaling, has been shown to be a transcriptional co-activator of the AR (Wang et al., 2008). Data suggests β-catenin is able to interact with the AF-2 region located within the ligand binding domain (LBD) of the AR (Song et al. 2003). This interaction has been shown to play a crucial role in regulating receptor binding affinity and AR activation through facilitating NH2-terminal domain (NTD) and COOH-terminal ligand-binding domain (LBD) interactions and heat shock protein dissociation (Wang et al., 2008; Mulholland et al., 2005). Furthermore, β-catenin's ability to activate the AR increases muscle specific gene expression, which in turn can play an important regulatory role in skeletal muscle growth. Currently, there is extremely limited data investigating β-catenin-AR interactions in resistance exercise models. Thus far, the existing data suggest that β-catenin-mediated AR signaling does appear to be responsive to resistance exercise (Leal et al. 2011; Spillane et al., 2015). However, more research is needed to elucidate the mechanisms and whether manipulating program design variables influences this response.

Generally it is thought that resistance exercise involving higher intensity and volume are needed to elicit an appropriate stimulus for increasing AR signaling activity. Although, resistance exercise load may be the key variable responsible for increasing AR activity, provided it is accompanied with appropriate training volume and intensity. This is due to the overwhelming role load has on recruitment of motor units and the subsequent fiber type specific metabolic and/or contractile stress placed on skeletal muscle. However, research investigating load's impact on muscular adaptations are commonly misinterpreted due to suboptimal methodological approaches and inconsistencies in terminology. For example, much of the literature does not clearly define and differentiate between intensity and load. In resistance exercise, intensity is commonly defined as a percent of 1 repetition maximum (1RM) (Haff & Triplett, 2016). However, it is more appropriate to define this as "load". Intensity may be more appropriately defined as the number of repetitions performed at a given repetition maximum (Cintineo et al., 2018). For example, performing 10 repetitions at a 10RM (which is approximately 75% of 1RM) until volitional failure would be considered an intensity of 100%. These semantic differences and lack of clearly defining these variables have contributed to some of the confusion in this area.

Rarely is the effect of load on skeletal muscle adaptations examined without being affected by confounding variables such as volume or intensity. This is problematic since volume and intensity have both been shown to be important factors influencing androgenic hormone responses following resistance training (Kraemer & Ratamess, 2005). For example, a study by Morton et al. (2016) investigated the impact of varying loads on resistance training-mediated hypertrophy and strength gains over a 12-week training period as well as the acute hormonal response (Morton et al., 2016). They concluded neither low

(30-50% 1RM) nor high (75%-90% 1RM) load, or the accompanying acute hormonal response to resistance exercise, preferentially increased skeletal muscle hypertrophy or strength. In this study, intensity was controlled between groups by all sets being performed to volitional failure. However, the researchers failed to equate volume. They stated the high load group only performed ~62% of the total volume performed by the low load group. Therefore, these conclusions concerning load must be made with caution being the additional volume performed by the low load group may have confounded these findings. Future research investigating the effects of resistance exercise load on skeletal muscle adaptations need to better control these program design variables in order to more accurately discern the impact of load.

Although multiple models have shown increases in AR signaling in response to resistance exercise, it is still unclear the mechanisms in which resistance exercise load may alter AR signaling activity and subsequent gene and protein expression. Moreover, much of the research that has been conducted in this area is misinterpreted due to suboptimal methodological approaches and inconsistencies in terminology. Therefore, the purpose of this study was to investigate the impact of load, in a volume- and intensity- equated manner, on AR-DNA binding and AR-regulated gene expression. Additionally, the influence of resistance exercise load on serum/muscle androgenic hormone concentrations, AR protein content, and muscle β-catenin content was measured to determine their potential load-dependence and influence on AR signaling activity.

Purpose

The purpose of the study is two fold. The primary purpose was to determine the effect of single bouts of volume- and intensity-equated low load (LL) and high load (HL) full body resistance exercise on AR-DNA binding, serum/muscle testosterone and dihydrotestosterone, AR muscle protein content, AR-DNA binding, and androgen regulated mRNA gene expression (*AR, MyoD, MYOG, IGF-1Ea, p21-cip*). A secondary purpose was to determine the effect of resistance exercise on skeletal muscle β-catenin concentrations in order to determine its impact on mediating AR-DNA binding in the absence/presence of increases in serum/muscle androgen and AR concentrations. The specific aims of the study were to determine if: 1) acute androgenic hormone activity, androgen receptor response, AR-DNA binding, and AR-regulated and –responsive gene and protein expression differed with varying loading schemes, 2) muscle β-catenin concentrations influenced AR-DNA binding in response to resistance exercise.

Hypotheses

H1: There would be no significant differences in serum and muscle androgenic hormone concentrations, androgen receptor protein content, AR-DNA binding, and androgen mediated gene expression across all time points.

H2: There would be no significant differences in serum and muscle androgenic hormone concentrations, androgen receptor protein content, AR-DNA binding, and androgen mediated gene expression between different resistance exercise load conditions across all time points.

H3: There would be no significant differences in skeletal muscle β-catenin concentrations across all time points.

H4: There would be no significant difference in skeletal muscle β-catenin concentrations between different resistance exercise load conditions across all time points.

H5: There would be no significant relationship between skeletal muscle β-catenin concentrations and AR-DNA binding across all time points.

Delimitations

1. Apparently healthy males between the ages of 18 to 35 who were familiar with intense resistance training. (Participants who have been resistance training for more than a year.)

2. Participants were recruited from Baylor University and within the surrounding Waco, TX area by flyers and online advertisements.

3. Participants were excluded from the study if they have consumed any dietary supplement (except a multivitamin) or any pharmaceutical that is used as a potential ergogenic aid for three months prior to the study.

4. All participants were considered low risk for cardiovascular disease, with no contraindication to exercise as outlined by the American College of Sports Medicine (ACSM).

5. All participants were tested at the Baylor Laboratory for Exercise Science and Technology (BLEST) and Exercise & Biochemical Nutrition Laboratory (EBNL) location under the supervision of the investigator in accordance with the Helsinki Code after signed university approved informed consent documents.

Limitations

1. Inferences were limited to the time points at which samples are collected. Hormone and metabolite concentrations may have been affected with the intervals of collection.

2. Each participant's diet, sleep, and activity level prior to each resistance training bout may have influenced the results of the study.

3. The psychological factors surrounding each participant such as the motivation to finish the resistance training test to the best of their ability may have potentially affected the results of the training study.

Assumptions

1. All laboratory equipment was functioning properly to produce valid and reliable measurements. Proper calibration and the use of trained research staff would minimize any potential for error.

2. Participants would put forth maximal effort during the maximal strength testing sessions.

3. All participants would comply to carry out the respective resistance training protocol, at the desired intensity throughout the study.

4. All participants would maintain their normal dietary habits throughout the study.

5. All participants would not undergo any resistance training and also abstain from aerobic activity during the study.

Definition of Terms

Androgen: Any natural or synthetic steroid hormone that promotes the development and maintenance of the male sex characteristics; primarily exerts its effects through interactions with the androgen receptor

Androgen Receptor (AR): Nuclear receptor activated by binding any of the androgenic hormones in the cytoplasm and then translocating into the nucleus where it binds to the target gene and up-regulates transcriptional activity

Androgen Response Element (ARE): Short sequence of DNA within the promoter of specific androgen-regulated genes; binds to activated androgen receptor complex and regulates transcriptional activity

AR-DNA Binding: The action of the activated androgen receptor complex binding to the androgen response element (ARE) in the target gene to exert its genomic effects

Armadillo Repeats (ARM): Repetitive amino acid binding sequence of about 40 residues in length that is found in β-catenin

β-Catenin: Multifunctional signaling protein involved in regulation and coordination of cell–cell adhesion, gene transcription, and tissue development; androgen receptor co-activating protein

Cytoplasm: Semifluid substance of a cell that is external to the nuclear membrane and internal to the cellular membrane which contains filaments, proteins, ions, and macromolecular structures

Dihydrotestosterone (DHT): Steroid hormone synthesized from testosterone by the enzyme, 5α-reductase, in bodily tissue; primarily exerts its effects through interactions with the androgen receptor

Intensity: Number of repetitions performed at a given repetition maximum

Load: Percent (%) of 1-repetition maximum (1RM)

Nucleoplasm: Semifluid substance of a cell enveloped by the nuclear membrane

Skeletal Muscle Hypertrophy: Increase in size of skeletal muscle cells through a growth in size of its component cells

Testosterone: Steroid hormone that stimulates development of male sex characteristics; primarily exerts its effects through interactions with the androgen receptor

Volume: Total amount of work performed (i.e. sets x repetitions x load)

Wingless-Type MMTV Integration Site (Wnt) Ligands: Family of secreted cysteine-rich glycoproteins that activate intracellular signaling pathways by binding to one of several Frizzled family receptors, ultimately resulting in accumulation of cytoplasmic β-catenin

CHAPTER TWO

Review of Literature

Resistance exercise has been shown to potently increase skeletal muscle strength and hypertrophy (Kraemer & Ratamass, 2004). One mechanism proposed to mediate resistance exercise-induced skeletal muscle hypertrophy is up-regulating AR signaling through increasing androgen-AR interactions. Therefore, the acute hormonal and receptor response to resistance exercise has been theorized to play a key role in AR signaling increases (Kraemer et al., 2017; Mangine et al., 2017). Additionally, others suggest increases in a number of AR-interacting proteins may be responsible for the up-regulation in AR signaling (Kim & Lee, 2009; Ueda et al., 2002; Yang et al., 2014; Weigel & Zhang, 1998). Therefore, this review will summarize basic AR structure, function, and signaling in addition to examining the evidence relevant to resistance exercise's influence on AR signaling.

Androgen Receptor Structure, Function, and Signaling

The AR is categorized as a Class I member of the steroid nuclear hormone receptor superfamily along with receptors for hormones such as progestins, corticosteroids, and estrogens (Detera-Wadleigh & Fanning, 1994). Eight exons code for a 90 KB AR gene located on the X chromosome (Lubahn et al., 1988). Structurally, the AR is a ligand-activated transcription factor made up of a DNA binding domain (DBD), a COOH-terminal ligand-binding domain (LBD), an NH2-terminal domain (NTD), and a hinge region. The NTD is a unique ligand-independent domain which plays a large functional role in the AR

due to the presence of the transcriptional activation function-1 (AF-1) domain. The AF-1 contains three AR phosphorylation sites and is required for maximal activation of the AR (Callewaert et al., 2006). Various growth factors and other co-regulators are thought to be recruited and able to phosphorylate these sites and initiate activation of the AR. For example, during transactivation, the AR makes specific protein-protein interactions with several basal transcription factors such as TATA-box-binding protein and transcription factor IIF at the AF-1 region. This protein-protein interaction plays an important role in transcriptional activation by the AR. Therefore, deletion of the AF-1 domain has been shown to significantly decrease the transcriptional capacity of the AR (Bevan et al., 1999). A second ligand-dependent domain called activation function-2 (AF-2) is located in the LBD. The AF-2 domain is important for forming the co-regulator binding site and mediates direct interactions between the NTD and LBD by binding specific short amino acid sequence motifs within the NTD (Schaufele et al., 2005). The AF-2 domain allows the LBD of the AR to form the ligand-binding pocket and mediate the interaction between the AR and chaperone proteins. In turn, this facilitates domain interactions between the LBD and NTD to stabilize the bound androgen (Fang et al., 1996). Deletion or mutation of the AF-2 domain has also been shown to dramatically decrease transcriptional activation of the AR in response to ligand (He et al., 2004).

The DBD is the most highly conserved region between the different members of the steroid hormone nuclear receptor family that is comprised of two zinc finger motifs that are responsible for DNA recognition and dimerization. These two zinc finger motifs facilitate DNA binding of the AR to the ARE in AR-regulated genes, thereby allowing the AF-1 and AF-2 of the NTD and LBD to stimulate or repress the transcription of these

genes. Interestingly, due to the highly conserved nature of the DBD, it has been shown that binding of selective androgen response elements (AREs) allow the specific activation of the AR. For example, the ARE in the promoter region of the probasin gene is specifically recognized by the AR but not by other steroid hormone nuclear receptors, such as the glucocorticoid receptor (Schoenmakers et al., 2000). The DBD is linked to the LBD by a hinge region. The hinge region is a lysine rich region that has been shown to be crucial for nuclear translocation of the AR. Deletion of this region has been shown to eliminate transcriptional activity and nuclear translocation of the AR in the presence of ligand (Zhou et al., 1996). Also, located within the LBD and between the hinge region and DBD, important signal sequences have been identified that play roles in AR trafficking into and out of the nucleus. These signals include a nuclear localization signal NLS that is responsible for import of the receptor into the nucleus, and a nuclear export signal NES that is responsible for exporting the AR to the cytoplasm upon ligand withdrawal (Davey & Grossmann, 2016).

Testosterone enters into circulation following synthesis by the testicular leydig cells and (to a minor extent) the adrenal cortex in males and by the ovaries in females (Ruiz-Cortes, 2012). Total testosterone in circulation is comprised of bound and unbound/free testosterone. Testosterone that is bound to sex-hormone binding globulin (SHBG) and albumin is termed bound testosterone. Testosterone not bound to any protein is termed free testosterone. In men, approximately 2% of total testosterone is free testosterone and is the most biologically active (Rommerts, 2004). Due to its lipophilic properties, circulating free testosterone diffuses across the sarcolemma in skeletal muscle, where a portion is converted to the more biologically active dihydrotestosterone (DHT) by the enzyme, 5α-

reductase. Due to DHT being more biologically active it is able to bind to the nuclear hormone AR with a 2-fold higher affinity and a decreased dissociation rate of 5-fold compared to testosterone (Grino et al., 1990). As previously discussed, the AF-2 domain mediates interactions between the NTD and LBD resulting in the formation of a ligand binding pocket. This allows for AR chaperone and co-regulating proteins to interact and modify AR binding affinity. Once bound, the activated AR undergoes a conformational change which causes dissociation from the heterocomplex of heat shock proteins and other co-chaperones resulting in dimerization. After dissociation, it is then considered an active androgen receptor complex and is translocated into the nucleus. Research has shown many AR interacting proteins play a pivotal role in the trafficking of the AR into the nucleus in addition to regulating ligand binding affinity (Pratt & Toft, 1997). Specifically, AR trafficking following translocation into the nucleus is due to binding of heat shock protein 90 and 27 and their respective co-chaperones, Cdc37, Bag-1L and FKBP52 (Pratt et al., 2004; Zoubeidi et al., 2007). Once translocated into the nucleus, the activated androgen receptor complex binds to the androgen response element (ARE). The ARE is made of a palindromic six base-pair element called a core recognition sequence located in the promoter region of the androgen-responsive gene. The AR binds as a homodimer to inverted repeats of the 5'-TGTTCT-3' core sequence motifs, separated by a 3 base pair spacer (5'-GAACAnnnTGTTCT-3') (Khorasanizadeh & Rastinejad, 2001; Spillane et al., 2015). This AR-DNA binding results in increasing muscle specific gene expression and plays a significant role in anabolic and anti-catabolic processes in skeletal muscle.

A number of genes have been identified which contain an ARE and are up-regulated due to the genomic actions of the activated AR (Grad et al., 1999; Lu et al., 2000; Wu et

al., 2007). These genes include the AR (*AR*) gene, the p21 cyclin-dependent kinase inhibitor protein 1 (*p21-Cip1*) gene, the insulin-like growth factor-1 (*IGF-1Ea*) gene and the insulin-like growth factor-1 receptor (IGF-1R) gene. Interestingly, the myogenin (*MYOG*) and myoblast determination protein 1 (*MyoD*) genes do not contain AREs but do appear to be induced by androgen treatment (Montano et al. 2007; Lee, 2002). Collectively, these genes play crucial roles in regulating skeletal muscle metabolism along with other important cellular functions (Rana et al., 2014; Roberts et al., 2009). However, of the few studies conducted thus far investigating the impact of resistance exercise on AR-regulated gene expression, there appears to be inconsistent findings. Roberts et al. (2009) showed no significant changes in *AR*, *IGF-1Ea*, *p21-Cip1*, or *MYOG* gene expression 24 hours following resistance exercise in older or younger men. On the contrary, Bamman et al. (2001) found significant increases in *IGF-1* and *AR* gene expression at 48 hours following a bout of resistance exercise (Bamman et al., 2001). These inconsistencies may be due to a variety of methodological differences between studies including sampling windows, resistance exercise protocols, and properly controlling confounding variables. Therefore, there is a need to investigate this phenomenon at a transcriptional level. Additionally, more research is needed to better understand the mechanistic role AR-regulatory proteins play in influencing this response. The activated androgen receptor complex has also been shown to impact transcriptional capacity through non-DNA binding-dependent pathways. Specifically, activation of extracellular signal-regulated kinase (ERK), protein kinase B (Akt), and mitogen-activated protein kinase (MAPK) have all been shown to be up-regulated in the absence of DNA binding (Davey & Grossmann, 2016; Estrada et al., 2003). This suggests the active AR complex may increase transcriptional capacity without

DNA binding by its ability to influence other important signaling pathways. Interestingly, while majority of research has been conducted in vitro, in vivo studies have shown increased signaling occurs too rapidly following androgen administration to have arisen via AR-DNA binding to regulate the transcription and translation of target genes.

Ligand-independent activation of the AR has been shown by a number of different AR-interacting proteins and growth factors (Kim & Lee, 2009; Ueda et al., 2002; Yang et al., 2014; Weigel & Zhang, 1998). Over 400 proteins have been shown to interact with the AR and act as co-activators, -repressors, or –regulators (i.e. β-catenin, heat shock protein 90, etc.). Due to their clinical relevance in many clinical conditions (i.e. prostate cancer), these AR interacting proteins have been extensively studied. However, there is a paucity of literature pertaining to AR interacting proteins' impact on AR signaling in response to exercise, specifically resistance exercise.

β-Catenin and AR interactions

The multifunctional wingless-type MMTV integration site (Wnt)-signaling protein, β-catenin, has been suggested to largely impact stability, activation, and transcriptional activity of the AR. The canonical Wnt signaling pathway plays a crucial role in gene expression, cell adhesion, and tissue development (Mulholland et al., 2005). Through a series of regulatory steps, Wnt signaling increases cytoplasm levels of the multifunctional protein β-catenin. β-catenin is able to translocate into the nucleus and act as transcription factor where it interacts with T-cell specific transcription factor/lymphoid enchancer-binding factor 1 (TCF/LEF-1) family proteins and increases Wnt-specific gene expression. However, β-catenin is also able to act as a transcriptional co-activator of the AR (Wang et al., 2008). This nuclear interaction between β-catenin and the AR can modulate gene

expression in androgen target tissues, which in turn can play a regulatory role in skeletal muscle growth. Due to its co-regulatory role in AR signaling, β-catenin has been investigated in AR-mediated diseases (i.e. prostate cancer), however, its implications in improving skeletal muscle anabolism has only recently been studied.

β-catenin levels are regulated by Wnt extracellular ligands and their ability to bind to the transmembrane cysteine-rich family of receptors, Frizzled (Bejsovec, 2005). Activation of the Frizzled receptor via Wnt proteins results in subsequent phosphorylation of low-density lipoprotein receptor-related proteins 5 and 6 (LRP-5 and LRP-6). These binding events lead to the receptor kinases phosphorylating the cytoplasmic mediator, Dishevelled. Additionally, this recruits the serine/threonine kinase glycogen synthase kinase-3 (GSK3), casein kinase I isoforms (CKI), and the scaffold protein axin to the plasma membrane near the Frizzled and LRP receptor proteins (Kypta & Waxman, 2012). This leads to disruption of the destruction complex, which is comprised of GSK-3, CKI, axin, adenomatous polyposis coli protein (APC), and F-box/WD repeat containing protein 1A (also known as β-transducin repeat containing E3 ubiquitin protein ligase). The destruction complex regulates proteasomal degradation through phosphorylation and ubiquitination of β-catenin. Mutations in APC and other components of the complex have been shown to lead to development of various diseases including colorectal and prostate cancer (Barker & Clevers, 2006). Down-regulating destruction complex activity encourages accumulation of β-catenin in the cytosol and facilitates nuclear translocation to interact with other proteins such as the AR. In the nucleus, β-catenin canonically associates with TCF/LEF-1 along with co-activating proteins B-cell lymphoma 9 protein (Bcl-9), pygopus homologs 1 & 2 and cAMP response element binding protein (CREB). This up-

regulates transcription of specific genes that contain TCF/LEF-1 binding sites such as c-Myc, cyclin D1, and paired-like homeodomain transcription factor 2.

AR and β-catenin protein interactions are not entirely well understood. However, data suggests β-catenin is able to interact with the LBD of the AR due to specific structural aspects of this domain (Mulholland et al., 2005). β-catenin's unique ability to interact with the LBD suggests that it may play a crucial role in AR conformational changes along with androgen binding and dissociation of heat shock proteins. Specifically, within the AF-2 region of the LBD, LXXLL (L=leucine, X=any other amino acid) binding motifs have been shown to interact with Armadillo (ARM) repeats 1-6 of β-catenin, in addition to transcriptional regulator transcriptional initiation factor-2 (TIF-2) binding, and NTD and LBD interactions (Song et al., 2003). This was shown by mutations in ARM repeats 5 or 6 eliminating binding, co-activation, and nuclear translocation interactions with the AR (Fajun Yang et al., 2002). Interestingly, ARM repeats 5 and 6 also bind to TCF4 and the cell adhesive protein E-cadherin. Therefore, it has been suggested that competitive binding for these ARM repeats may be a method of regulating AR/β-catenin transcriptional capacity. On the contrary, Singh and colleagues (2009) proposed that the AR is able to interact with β-catenin as well as TCF-4 (Singh et al., 2009). This suggests a co-binding of these proteins with β-catenin. Thus, further research is needed to solidify interactions between these proteins.

AR and β-catenin protein interactions are reported to be androgen dependent due to β-catenin mediated activation of the AR being enhanced in the presence of androgens (Truica et al., 2000; Yang et al., 2002). However, it has been suggested β-catenin may potentially be involved in ligand independent activation of the AR as well (Wang et al.,

2008). Thus, more research is needed to solidify these interactions, especially in human skeletal muscle. Additionally, it has been suggested that the ability of β-catenin to act as an AR co-activator is also dependent on Phosphoinositide 3-kinase (PI3K)/Akt signaling. Specifically, activated PI3K signaling promotes decreased GSK3β function and results in high levels of β-catenin (Sharma et al., 2002). As previously discussed, this impairment GSK3β function will lead to disruption of the destruction complex and subsequent decreases in β-catenin phosphorylation, ubiquitination, and degradation through proteasomal activity resulting in cytosolic β-catenin accumulation.

Resistance exercise related studies investigating β-catenin or β-catenin/AR protein responses are extremely limited. A study by Armstrong and Esser (2005) found increased nuclear β-catenin/Lef-1 expression and induction of the transcriptional targets c-Myc, cyclin D1, and paired-like homeodomain transcription factor 2 during hypertrophy of mechanically overloaded mouse plantaris skeletal muscle (Armstrong & Esser, 2005). They proposed this was mediated through PI3K/AKT signaling resulting in the phosphorylation of GSK3β and its aforementioned impact on the destruction complex. Another study by Leal and colleagues (2011) found increases in β-catenin protein content, Wnt-signaling genes, and muscle fiber cross sectional area in response to 8 weeks of power-type resistance training in humans (Leal et al., 2011). These results suggest that Wnt/β-catenin signaling is responsive to skeletal muscle mechanical overload and may play a significant role in resistance training adaptations such as increases in muscular strength and hypertrophy. Moreover, a study by Gentile et al. (2010) found that treating aged rats with testosterone or DHT resulted in repression of Axin and Axin2, negative regulators of b-catenin, indicating modulation of the b-catenin pathway (Gentile et al.,

2010). Additionally, testosterone or DHT treatment reversed the increases in fat mass and decreases in muscle mass observed with aging. These findings further expose the potential interactions between androgen signaling and β-catenin.

Last, a study by Spillane and colleagues (2015) investigated the effects of a single bout of lower-body and upper- and lower-body resistance exercise on serum testosterone and Wnt4 concentrations, and skeletal muscle testosterone, DHT, AR protein content, AR-DNA binding, and β-catenin (Spillane et al., 2015). The researchers found significant increases in AR-DNA binding, total muscle β-catenin, and AR protein content at 3-hours post upper/lower body resistance exercise and increased AR-DNA binding and total muscle β-catenin at 24-hours post exercise. Additionally, serum Wnt4 was significantly elevated 0.5-, 1-, 2-, and 3-hours post exercise indicative of an up-regulation in Wnt signaling. Interestingly, these responses were observed with no changes in serum or muscle androgen concentrations and a significant decrease in AR protein content at 24-hours post exercise. No significant changes were observed in lower-body resistance exercise. The researchers concluded that an upper- and lower-body acute bout of resistance exercise increased Wnt/β-catenin signaling and AR-DNA binding without increases in androgen content (Spillane et al., 2015). However, the variability in volume and intensity between the conditions needs to be considered before concluding a conditional impact on resistance exercise-mediated AR activation. While this study demonstrates a potential AR/β-catenin interaction and it's effects on AR-DNA binding, more research is needed to better mechanistically understand this protein interaction in skeletal muscle and their implications in the muscular strength and hypertrophy adaptations that result from resistance exercise.

Resistance Exercise Load and Acute AR Signaling Response

The many positive AR signaling-mediated outcomes in skeletal muscle from resistance exercise may be altered with manipulation of program design variables. These program design variables include exercise selection, order of exercises, training volume, rest periods, intensity, and load. While all variables have been extensively researched, exercise load has been the most frequently studied (Kraemer & Ratamess, 2004). However, much of the literature fails to properly define exercise load as well as differentiate it from exercise intensity. In resistance exercise, intensity is commonly defined as a percent of 1 repetition maximum (1RM) (Haff & Triplett, 2016). However, it has been suggested this may be more appropriately termed "load" (Cintineo et al., 2018). Intensity in regards to resistance exercise is more accurately defined as the number of repetitions performed at a given repetition maximum. For example, performing 10 repetitions at a 10RM (which is approximately 75% of 1RM) until volitional failure would be considered an intensity of 100%. Arent et al. (2005) examined specific criteria to better define intensity through the careful analysis of ratings of perceived exertion, performing sets to volitional muscular failure, and the subsequent physiological responses (Arent et al., 2005). These considerations emphasize the complexity of this variable and better discern exercise load from intensity. Moreover, resistance exercise load is typically classified in two ways: "low/light" or "high/heavy". While there is much variability in the literature in defining these classifications, low load resistance exercise is commonly defined as ≤60% 1RM, whereas high load resistance exercise is commonly defined as >60% 1RM (Schoenfeld et al., 2017). However, these classifications encompass a wide range of load percentages. Therefore, research involving a particular load percentage is typically applied to all load

percentages that would be included in its classification. For example, the findings of a study using a load of 30% 1RM are usually applied to all loads that are considered low loads (i.e. ≤60% 1RM). These statements inaccurately conclude that a load of 30% 1RM would have an identical stimulus to all other loads ≤60% 1RM. Many of these semantic differences have contributed to the poor interpretation of the literature and confusion in this area.

The acute endocrinological response to resistance exercise has been proposed to be predictive of resistance exercise mediated changes in skeletal muscle (Kraemer et al., 2017). It is thought the acute increase in serum/muscle androgen and/or AR protein concentrations are responsible for the up-regulation in AR signaling observed. A number of studies have found acute increases in serum androgen concentrations in response to resistance exercise (Ahtiainen et al., 2011; Ahtiainen et al., 2004; Cadore et al., 2008; Hakkinen & Pakarinen, 1993; Kraemer et al., 1991; Kraemer et al., 1990; Morton et al., 2016; Roberts et al., 2009; Spiering et al., 2009; Vingren et al., 2009; Walker et al., 2011; West et al., 2009; Willoughby & Taylor, 2004). Additionally, multiple studies have observed increases in AR mRNA or protein expression in response to resistance exercise (Bamman et al., 2001; Lee et al., 2003; Willoughby & Taylor, 2004). Moreover, recent publications concluded that circulating hormones are predictive of resistance exercise mediated changes in skeletal muscle (Kraemer et al., 2017; Mangine et al., 2017). In a study by Mangine et al. (2017), they examined the relationship between the endocrine response to resistance exercise and muscle hypertrophy. Interestingly, they found a significant relationship between testosterone and muscle size and concluded exercise-induced testosterone elevations seemed to be related to muscle growth. While this theory provides a logical and valid explanation of the increases in AR activity observed in

response to resistance exercise, there is a substantial amount of evidence to reject this idea (Morton et al. 2016; Spillane et al. 2015).

Many researchers have found significant decreases or no change in serum androgen concentrations in response to a single bout of resistance exercise (Bamman et al., 2001; Fry & Lohnes, 2010; Gonzalez et al., 2015; Goto et al., 2011; Harber et al., 2004; Spillane et al., 2015; Wu & Lin, 2010). Similarly, AR mRNA and protein expression also show inconsistent and wide-ranging responses following a resistance exercise bout (Ahtiainen et al., 2011; Hulmi et al., 2008; Lee et al., 2003; Ratamess et al., 2005; Spiering et al., 2009; Spillane et al., 2015; Vingren et al., 2009). These data suggest increases in AR activation and subsequent DNA binding may not be primarily dependent on rises in androgen or AR protein concentrations. Morton et al. (2016) has proposed that the acute systemic hormonal response does not determine training-mediated hypertrophy or strength gains. In this study, they ran 120 correlations, each on 49 participants, between 10 different hormones (including testosterone) and various measures of changes in muscle mass and strength (Morton et al., 2016). Cortisol was the only marker which correlated with changes in type II skeletal muscle cross sectional area. The researchers concluded testosterone had no correlation with a number of hypertrophy and strength measures. Clearly more research is needed to determine whether resistance exercise-mediated increases in androgenic hormones or AR content impact AR signaling and subsequent increases in muscle growth or muscular strength. Additionally, more data is needed to determine if increases in AR activation, through DNA binding, are observed in the absence or presence of increases in hormonal or receptor concentrations.

Furthermore, the inconsistencies in the data may be due to the variability in resistance exercise protocols and lack of properly controlling program design variables. Generally, it is thought that resistance exercise involving higher intensity and volume are needed to elicit an appropriate stimulus for increasing AR signaling activity. However, resistance exercise load may be the key variable responsible for increasing AR activity provided it is accompanied with appropriate training volume and intensity. This is due to the overwhelming role load has on recruitment of motor units and the subsequent fiber type specific metabolic and/or contractile strains elicited on skeletal muscle. However, much of the research investigating the effect of load on AR signaling is influenced by confounding variables such as intensity and volume. For example, the aforementioned Morton et al. (2016) study investigated the impact of full body resistance exercise with varying loads (30-50% 1RM vs 75-90% 1RM) on the acute hormonal response and its correlation to muscle hypertrophy and strength gains over 12 weeks of training. In this study, intensity is controlled by each set being performed to volitional failure or 100% intensity. However, the “high load” group only performed ~62% of the volume performed by the “low load” group. Therefore, while they concluded load did not preferentially impact the acute hormonal response to resistance exercise, this was confounded by their failure to control for volume between the two sessions. To the author’s knowledge, no research has investigated the impact of exercise load on AR signaling, while properly controlling for intensity and volume.

Most of the literature in this field has evaluated the serum/muscle androgen and AR response assuming they indicate increases in AR activation and subsequent DNA binding. However, there is a dearth of evidence supporting this phenomenon given it is extremely

rare for researchers to evaluate AR-DNA binding. On the contrary, resistance exercise involving full body training and higher volume has been shown to acutely increase AR activation and DNA-binding in the absence of increases in serum/muscle androgen and AR protein concentrations (Spillane et al., 2015). Therefore, an increase in AR signaling may be occurring as a result of resistance exercise but not due to hormonal or receptor mediation. As previously discussed this may be due to increases in AR-interacting proteins (i.e. β-catenin, heat shock proteins, interleukin-6, etc.) which can alter receptor binding affinity and activation. Therefore, much of the research that has been done cannot conclude the influence on AR signaling activity but only hormonal and receptor responses. This study evaluated AR activity, as well as androgenic hormones, receptor activity and β-catenin signaling, in response to different exercise loads. To the authors' knowledge, this is the first study to investigate the effects of resistance exercise load, in an intensity- and volume- equated manner, on AR-DNA binding, AR-regulated gene expression, and the influence of the androgenic hormones, AR protein content, and β-catenin concentrations.

CHAPTER THREE

Methods

Experimental Approach

Participants visited the laboratory on 3 separate occasions in the following manner: visit 1 = entry/familiarization, medical/physical activity screening, and resistance exercise max testing; visit 2 = “light” (40-50% 1RM) load (LL) resistance exercise session; visit 3 = “heavy” (70-80% 1RM) load (HL) resistance exercise session. Each visit was 7-10 days following the previous visit. In a crossover design and volume-equated manner, participants performed identical full-body resistance exercise protocols consisting of barbell bench press, horizontal leg press, seated cable row, and unilateral leg extensions. In the “low” load resistance exercise session, participants performed 40-50% of their one repetition maximum to volitional failure for each exercise. Total exercise volume (sets x repetitions x load) was calculated and equated in the “high” load resistance exercise session during visit 3. During the “high” load session, sets were performed until participants reached the volume in order to match the low load condition. This allowed for volume and intensity to be equated between the two conditions. Each session involved the gathering of data for the analysis of biochemical and hormonal markers of blood and muscle metabolite changes. This experimental approach is based on the premise that resistance exercise induced skeletal muscle hypertrophy can be achieved using “low” (30% 1RM) loads to volitional failure or “high” (>60% 1RM) loads when performed to muscular failure (Schoenfeld et al., 2017). However, while “low” or “heavy” resistance exercise load is

suggested to not preferentially induce greater hypertrophy (when performed to volitional failure), differences in AR signaling have yet to be investigated. Additionally, resistance-training experience (i.e. number of years resistance training) was also recorded to determine its impact on AR signaling activity. The proposed experimental model determined whether resistance exercise load may preferentially influence AR-DNA binding, androgen-mediated gene and protein expression, and β-catenin-mediated androgen signaling.

Participants

Ten apparently healthy, recreationally resistance-trained [regular, consistent resistance training (i.e. thrice weekly) for at least 1 year prior to the onset of the study], men between the ages of 18-35 volunteered to serve as participants in this study. Enrollment was open to men of all ethnicities. Only participants considered low risk for cardiovascular disease with no contraindications to exercise as outlined by the American College of Sports Medicine (ACSM) and have not consumed any nutritional supplements (excluding multi-vitamins) one month prior to the study were allowed to participate. All eligible participants signed university-approved informed consent documents and the Institutional Review Board granted approval for the Protection of Human Subjects in Research of Baylor University. Additionally, all experimental procedures involved in the study conformed to the ethical consideration of the Declaration of Helsinki. All mean(±SD) participant descriptives (anthropometrics, baseline health assessments, resistance training experience) are found in Table 3.1.

Table 3.1

Shows mean(±SD) of all participant descriptives.

Descriptive	Mean(±SD)
Sample Size	10
Age (y)	23.2 (±4.68)
Height (cm)	176.78 (±0.58)
Total Body Mass (kg)	87.15 (±5.77)
Lean Body Mass (kg)	70.66 (±6.62)
Bone Mineral Content (kg)	2.87 (±0.25)
Fat Mass (kg)	13.62 (±3.54)
Bodyfat (%)	15.73 (±4.30)
Resting Heart Rate (bpm)	63.6 (±9.13)
Systolic Blood Pressure (mmHg)	118.2 (±5.77)
Diastolic Blood Pressure (mmHg)	75.4 (±7.66)
Resistance Training Experience (yr)	4.68 (±1.85)

Study Site

All supervised testing and sample analyses were conducted in the Exercise and Biochemical Nutrition Laboratory (EBNL) at Baylor University.

Independent and Dependent Variables

The independent variable is resistance exercise training load in addition to resistance training experience. The dependent variables included serum/muscle androgen concentration, androgen receptor protein content, AR-DNA binding, androgen-regulated gene expression (i.e. AR gene / Androgen Receptor, p21-Cip1 / p21 cyclin-dependent kinase inhibitor protein-1, MYOG / myogenin, IGF-1 / insulin-like growth factor-1, MyoD / myoblast determination protein 1), and muscle β-catenin expression.

Participant Entry Protocol

Participants expressing interest in participating in this study were interviewed in person, on the phone, or through e-mail to determine whether they appear to qualify to participate in the study. Participants believed to meet eligibility criteria were invited to

attend an entry/familiarization/max testing session. Once reporting to the laboratory, participants were familiarized to the study protocol via a written and verbal explanation outlining the study design and then read and signed a university-approved informed consent document. Participants then completed a medical history questionnaire and underwent a general physical examination to determine whether they meet eligibility criteria. If participants met all eligibility criteria, they performed resistance exercise max testing for the exercises they performed during the resistance exercise sessions. All max testing were conducted according to the National Strength and Conditioning Association (NSCA) guidelines. At the conclusion of the familiarization/max testing session, participants were given an appointment in which to attend their first testing session. In addition, participants were instructed to refrain from exercise for 24 hours prior to each testing session and recorded their dietary intake for 48 hours prior to each resistance exercise session. Moreover, due to the diurnal nature and dietary influence of the biomarkers being investigated, participants reported to the laboratory upon waking and in a fasted state. To ensure that participants who enrolled in the study were resistance trained, the horizontal leg press one-rep max of each participant was compared to normal strength to body weight ratios. The minimum age specific strength to body weight ratio that was allowed to gain entry into the study was set at 2.82 times body weight (superior category), adapted from the Cooper Institute for Aerobics Research (1997).

Dietary Analysis

Participants were required to record their dietary intake for 48 hours prior to each of the exercise sessions. Participants' diets were not be standardized but were asked to not change their dietary habits during the course of the study. The dietary recalls were

evaluated with the Food Processor dietary assessment program (ESHA Research, Salem, OR) to determine the average daily macronutrient consumption of fat, carbohydrate, and protein in the diet for the duration of the study.

Hydration Analysis

Since previous research has demonstrated individuals who are dehydrated will have an attenuated testosterone response with resistance exercise (Judelson et al. 2008), prior to the max testing, low load, and high load resistance exercise sessions, hydration status was assessed via hematocrit and bioelectrical impedance analysis. Additionally, hematocrit was assessed during each blood draw and if needed, were used to indirectly estimate blood volume to normalize all serum markers. Blood volume was estimated based on the known blood sample volume and percent of plasma volume.

Muscle Biopsies

Percutaneous muscle biopsies (~30mg) were obtained from the middle portion of the vastus lateralis muscle of the dominant leg at the midpoint between the patella and the greater trochanter of the femur at a depth between 1 and 2 cm. After the initial biopsy, following biopsy attempts were made to extract tissue from approximately the same location as the initial biopsy by using the pre-biopsy scar, depth markings on the needle, and a successive incision that was made approximately 0.5 cm to the former from medial to lateral. After removal, adipose tissue was trimmed from the muscle specimens and was immediately frozen and stored at -80ºC for later analysis. Three muscle samples were obtained at visit 2 and 3 for a total of 6 muscle biopsies performed during the course of the

study. Biopsies were taken pre-, 3-hours post-, and 24-hours post-exercise during visits 2 and 3.

Blood Sampling

Venous blood sampling were obtained into 10ml vacutainer tubes using a 21-gauge phlebotomy needle inserted into the antecubital vein. Blood samples were allowed to stand at room temperature for 10 minutes and then centrifuged. The serum was then removed and frozen at -80°C for later analysis. Six blood samples were obtained during the course of the study. The blood samples were collected before, immediately after, 3-hour post-, and 24-hours post-exercise during visits 2 and 3.

Resistance Exercise Max Testing

To determine muscular strength and proper resistance exercise load prescription, participants performed 1-RM tests for bench press and angled leg press and 10-RM tests for bilateral leg extensions and seated cable rows in accordance with the National Strength and Conditioning Association (NSCA) recommendations. All participant's exercise testing and protocols were completed using a four-point tempo prescription that controls eccentric, amortization, concentric, and beginning of the lift time periods in an attempt to standardize repetitions. A tempo prescription of (1-0-1-0) was used to standardize the reps between exercise bouts. Additionally, leg press foot placement and bench press/seated cable row grip width was recorded and held constant over all testing conditions in order to maintain consistency. To ensure participants are moving through the full range of motion during each repetition, a goniometer was used to establish 90 degrees of knee flexion on the leg press. Moreover, during the bench press, participants were instructed to touch their chest

with the barbell and extend their elbows to full extension to constitute a completed repetition. Next, participants were required to fully extend their elbows and pull the cable attachment until it makes contact with their sternum/chest during the seated cable row. Lastly, participants were required to fully extend their knees to constitute a completed repetition during the bilateral leg extension.

Participants warmed up by completing 5 to 10 repetitions at approximately 50% of the estimated 1RM/10RM. Then participants rested for 1 minute and then completed 3 to 5 repetitions at approximately 70% of the estimated 1RM/10RM. Load was then increased conservatively and the participant attempted to lift the load for one/ten repetition(s). If the lift was successful, the participant rested for 5 minutes before attempting the next weight increment. As per NSCA guidelines, load was increased by 2.5-5% for upper body exercises and by 5-10% for lower body exercises. This procedure was continued until the participant fails to complete the lift. The 1RM/10RM was recorded as the maximum weight that the participant was able to lift for one/ten repetition(s).

Resistance Exercise Protocol

During visits 2 and 3 participants performed full-body resistance training protocols consisting of barbell bench press, horizontal leg press, seated cable row, and bilateral leg extensions. As our main experimental variable, load varied between visits 2 and 3 while keeping all other variables constant. After proper warm up, as described previously, participants performed 3 sets of each exercise at 50% 1RM and were taken to volitional failure during visit 2. During visit 3, participants performed each exercise at 80% 1RM to volitional failure. Additionally, volume was equated between the two visits. Due to the greater amount of volume that can be accumulated with a lighter load, additional sets for

each exercise were utilized (if necessary) in order to equate volume between the two visits. Moreover, when fatigue/failure occurred during a set, study personnel provided assistance to help re-rack the weight safely. In all cases, 2-4 minute rest occurred between all sets and exercises. Due to the diurnal nature of the biomarkers we are investigating, all resistance exercise protocols were performed in the morning upon waking. Additionally, in order to minimize nutritional mediation of the markers we are investigating, participants received a standardized nutrition bar 30 minutes prior to exercise (Power Bar®, Premier Nutrition Corporation, Kings Mountain, NC, USA). Lastly, in an attempt to control for variations in resistance exercise performance, skeletal muscle strength, and proper recovery, resistance exercise protocols were scheduled within 2 hours of each other and separated by 7-10 days (Wax et al., 2012).

Serum Hormone Assessment

The concentrations of androgenic serum hormones [free testosterone, and total testosterone (testosterone bound to albumin or sex hormone-binding globulin)] were assessed via commercially-available enzyme-linked immunosorbent assay (ELISA) kits (MyBiosource, San Diego, CA, USA) with a microplate reader (X-Mark, Bio-Rad, Hercules, CA, USA). The absorbances were read at a wavelength of 450 nm and unknown concentrations determined by linear regression against known standard curves using commercial software (Microplate Manager, Bio-Rad, Hercules, CA, USA).

Intramuscular Protein and Hormone Assessment

Muscle β-catenin, androgen receptor, testosterone, and dihydrotestosterone were assessed via commercially-available enzyme-linked immunosorbent assay (ELISA) kits

(MyBiosource, San Diego, CA, USA) with a microplate reader (X-Mark, Bio-Rad, Hercules, CA, USA). The absorbances were read at a wavelength of 450 nm and unknown concentrations were determined by linear regression against known standard curves using commercial software (Microplate Manager, Bio-Rad, Hercules, CA, USA).

To determine androgen receptor activation and translocation, androgen receptor-DNA binding was assessed via nuclear extracts by a commercially-available ELISA kit (Assay Bio Tech, Inc., Sunnyvale, CA, USA). This kit used a consensus DNA oligonucleotide (AGAACA) representing the ARE to first bind the AR, after which a specific polyclonal antibody interacts with the N-terminal domain of the AR. The absorbances were read at a wavelength of 450 nm using a microplate reader (iMark, Bio-Rad, Hercules, CA) and expressed relative to total protein concentration.

mRNA Gene Expression Assessment

Real-Time Quantitative Polymerase Chain Reaction (PCR) was used to quantify expression of relevant target genes (*AR, p21-Cip1, MYOG, IGF-1Ea,* and *MyoD*). Based on a previous study from our lab (Schwarz et al., 2016), total cellular RNA was extracted from biopsy samples with a monophasic solution of phenol and guanidine isothiocyanate contained within the TRI-reagent (Sigma Chemical Co., St. Louis, MO). From this, 2 μg of total skeletal muscle RNA was reverse-transcribed to synthesize cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Starting cDNA template concentration was standardized by adjusting all samples to 200 ng prior to PCR amplification.

The mRNA sequences of human skeletal muscle *AR, p21-Cip1, MYOG, IGF-1Ea,* and *MyoD* published in the NCBI Entrez Nucleotide database (www.ncbi.nlm.nih.gov)

were used to construct PCR primers using Beacon Designer software (Bio-Rad, Hercules, CA, USA), and then commercially synthesized (Integrated DNA Technologies, Coralville, IA). Due to its consideration as a constitutively expressed "housekeeping gene," and the fact that it has been shown to be an appropriate external reference standard in human skeletal muscle using real-time PCR, β-Actin was used for detecting the relative change in the quantity of mRNA. All sequences (forward and reverse) and accession numbers of the genes of interest and β-Actin are listed in Table 3.2.

Table 3.2

Shows primer sequences used for genes of interest (GOI).

GOI	Sequence (Forward and Reverse)	Accession Number
AR	5'-ATC ATC ACA GCC TGT TGA ACT-3' 5'-CAA TCC CGA CCC TTC CCA G-3'	NM_000044.2
MyoD	5'-CGC CAC CGC CAG GAT ATG-3' 5'-GTC ATA GAA GTC GTC CGT TGT G-3'	X56677
Myogenin	5'-CTG GTG GCA GGA ACA AGC-3' 5'-GAT GGA CGG ACA GGT GGA G-3'	NM_002479
IGF-1Ea	5'-GTG GAT GAG TGC TGC TTC-3' 5'-GGT TCT GGG TCT TCC TTC-3'	X57025
p21-cip	5'-CAG CAT GAC AGA TTT CTA CC-3' 5'-GGA ATC AGA GTC AAA CAC AC-3'	L25610
β-Actin	5'-TAA GGA GAA GCT GTG CTA CGT-3' 5'-AGT TTC GTG GAT GCC ACA GG-3'	NM_001101

Two hundred ng of cDNA template was added to iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and each PCR reaction was amplified using real-time quantitative PCR (iCycler IQ Real-Time PCR Detection System, Bio-Rad, Hercules, CA, USA). The amplification profile was run for 40 cycles employing a denaturation step at 95°C for 30 s, primer annealing at 58°C for 30 s, and extension at 72°C for 30 s. The expression of mRNA was determined from the ratio of the C_T values relative to β-Actin.

Statistical Analysis

Statistical analysis for androgenic serum hormone concentrations, metabolite concentrations, and AR-DNA binding analyses were performed by utilizing separate 2 x 3 [Condition (Light Load, Heavy Load) x Time (Pre-, Immediately Post-, and 3-hours Post-exercise)] factorial analyses of variance (ANOVA) with repeated measures. If there were significant differences in baseline values an ANCOVA was used to account for these differences. Analysis of main effects for each condition were also be interpreted from the factorial analysis of variance tests respectively. Significant between-group differences and interactions of independent variables were then determined using the Tukey's Post Hoc Test.

CHAPTER FOUR

Results

Dietary Analysis

The mean (±SD) average total calories and macronutrient dietary intake relative to bodyweight (kg) recorded for the max testing and for each condition are displayed in Table 4.1. The results of the separate one way repeated measures ANOVAs indicated that there was no significant difference in total calories (F=3.323, p=0.061, η^2=0.270) carbohydrate intake (F=1.690, p=0.213, η^2=0.158), protein intake (F=0.805, p=0.462, η^2=0.082), fat intake (p=0.243, η^2=0.145, F=1.532), or fiber intake (F=0.395, p=0.680, η^2=0.047) between the max testing, high load, or low load conditions.

Table 4.1

Mean (±SD) total calories and macronutrient intake for all 3 visits.

Variable	Max Testing	Low Load	High Load
Total Calories (kcal/kg)	31.06 (±6.20)	31.02 (±5.5)	34.9 (±5.61)
Carbohydrate (g/kg)	3.1 (±0.78)	3.1 (±1.01)	3.4 (±1.04)
Protein (g/kg)	2.1 (±0.58)	2.1 (±0.57)	2.2 (±0.50)
Fat (g/kg)	1.1 (±0.42)	1.1 (±0.29)	1.23 (±0.41)
Fiber (g/kg)	0.24 (±0.08)	0.25 (±0.10)	0.27 (±0.10)

Resistance Exercise Volume and Rating of Perceived Exertion

The mean (±SD) exercise and session volume along with rating of perceived exertion of each condition is displayed in Table 4.2. The results of the separate pair samples t-tests indicated no significant differences in leg press volume (t=0.482, p=0.641), barbell bench press volume (t=-0.233, p=0.821), lat pulldown volume (t=1.297, p=0.227), single

leg extension volume (t=-0.860, p=0.412), total testing session volume (t=-0.482, p=0.641), or rating of perceived exertion (t=1.279, p=0.237) between conditions.

Table 4.2

Mean (±SD) exercise and session volume and RPE for each condition.

Variable	Low Load	High Load
Leg Press (kg)	19,709 (±4,282)	19,695 (±4,278)
Barbell Bench Press (kg)	3,303 (±788)	3306 (±783)
Lat Pulldown (kg)	2681 (±781)	2664 (±782)
Single Leg Extension (kg)	2173 (±431)	2249 (±636)
Total Volume (kg)	27,866 (±5528)	27,915 (±5744)
Rating of Perceived Exertion	7.9 (±1.0)	7.4 (±1.1)

Total Body Water Analysis

The mean (±SD) total body water recorded for the max testing and for each condition are displayed in Table 4.3. The results of the one way repeated measures ANOVA indicated no significant differences in total body water (F=1.521, p=0.285, η^2=0.276) between the max testing, high load, or low load conditions.

Table 4.3

Mean (±SD) total body water for all 3 visits.

Variable	Max Testing	Low Load	High Load
Total Body Water (%)	54.96 (±1.66)	54.36 (±1.33)	54.52 (±1.74)
Total Body Water (kg)	46.34 (±2.92)	46.71 (±2.96)	47.02 (±2.79)

Packed Cell Volume Analysis

The mean (±SD) packed cell volume (%) following each condition is indicated in Table 4.4. No significant main effect for time (F=1.257, p=0.299, η^2=0.077) or condition (F=0.242, p=0.630, η^2=0.016) was observed for packed cell volume between conditions.

Table 4.4

Mean (±SD) packed cell volume (%) for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	48.88 (±2.27)	48.87 (±2.80)	47.25 (±1.67)
High Load	48.22 (±0.83)	47.11 (±2.42)	48.44 (±1.81)

Delayed Onset Muscle Soreness Analysis

The mean (±SD) muscle soreness following each condition are indicated in Table 4.5 and Figure 4.1. No significant main effect for condition (F=0.813, p=0.380, η^2=0.046) or significant interaction for time and condition (F=0.396, p=0.676, η^2=0.023) for muscle soreness was observed. However, a significant main effect for time (F=10.983, p<0.001, η^2=0.392) was observed. Pairwise comparisons with a Bonferroni adjustment of the simple effects showed a significant increase in muscle soreness at 3h post- (p=0.003) and 24h post-exercise (p=0.001) compared to pre-exercise.

Table 4.5

Mean (±SD) for muscle soreness for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	1.98 (±1.89)	4.36 (±2.65)	4.68 (±3.11)
High Load	2.28 (±2.41)	4.88 (±2.99)	6.21 (±2.65)

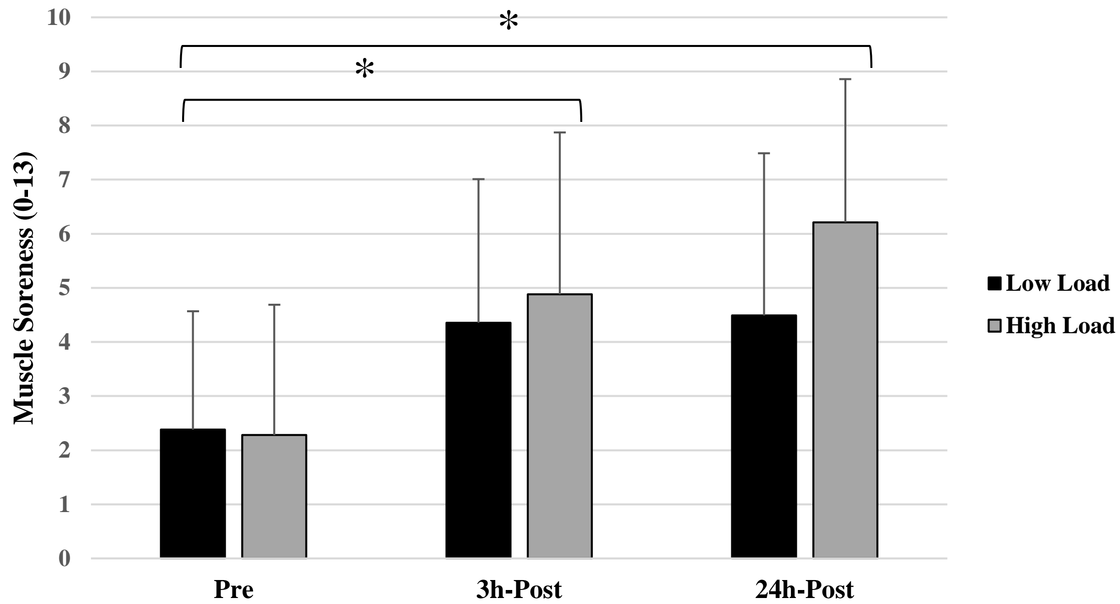

Figure 4.1: Mean (±SD) for changes in muscle soreness over time between conditions. There was a significant elevation in perceived soreness in both conditions at 3h post- and 24h post-exercise.

Intramuscular Androgen Receptor-DNA Binding

The mean (±SD) for intramuscular androgen receptor-DNA binding relative to total nucleoplasmic muscle protein content (Abs/mg) for each condition are indicated in Table 4.6 and Figure 4.2. No significant main effect of time ($F=1.554$, $p=0.225$, $\eta^2=0.079$) or condition ($F=1.697$, $p=0.209$, $\eta^2=0.086$) on AR-DNA binding activity was observed. However, a significant interaction for time and condition ($F=4.553$, $p=0.017$, $\eta^2=0.202$) was observed. Pairwise comparisons with a Bonferroni adjustment of the simple effects, revealed that there was a significant increase in AR-DNA binding at 3h post-exercise compared to pre-exercise in the high load condition ($p=0.030$).

Table 4.6

Mean (±SD) for androgen receptor-DNA binding (Abs/mg) for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	0.309 (±0.166)	0.259 (±0.143)	0.327 (±0.156)
High Load	0.298 (±0.111)	0.518 (±0.317)	0.359 (±0.251)

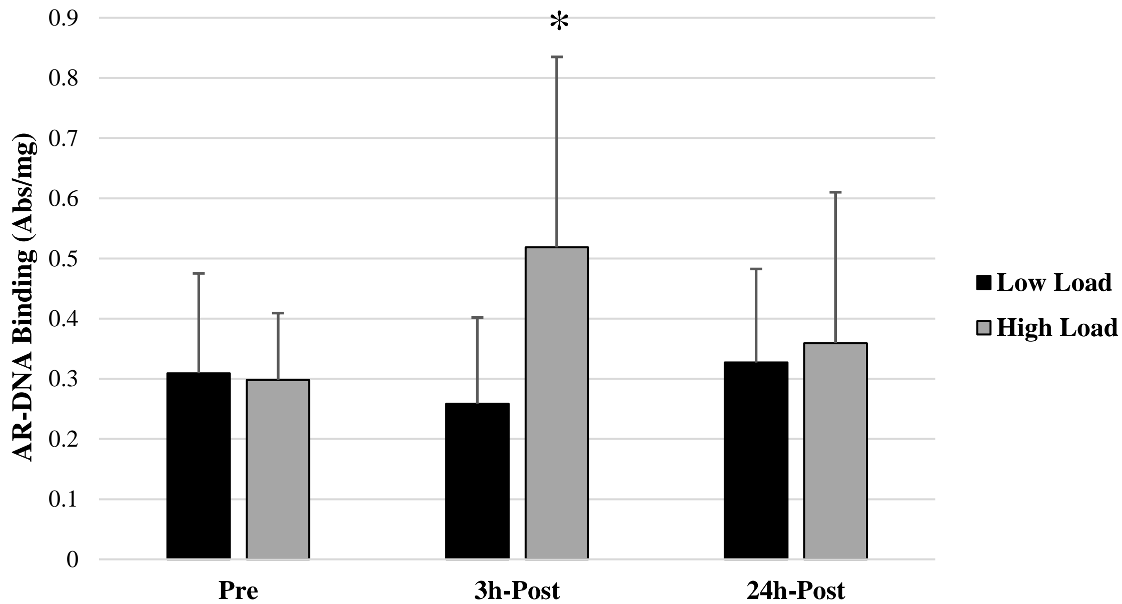

Figure 4.2: Mean (±SD) for changes in relative AR-DNA binding over time between conditions. There was no overall difference in volume or relative intensity between resistance exercise protocols. There was a significant elevation in relative AR-DNA binding activity in the high load condition at 3h post-exercise.

Intramuscular β-Catenin Content

The mean (±SD) for intramuscular β-catenin relative to total muscle protein content (ng/mg) for each condition are indicated in Table 4.7 and Figure 4.3. The Mauchly's Test of Sphericity indicated that there were violations in sphericity (p=0.018) of the data. Therefore, a Greenhouse-Geisser adjustment was used to meet the needed assumptions to run the appropriate statistical analysis. No significant main effect for time (F=2.578, p=0.109, η^2=0.125) or significant time and condition interaction (F=0.360, p=0.634, η^2=0.020) for β-catenin content was observed. However, a significant main effect for

condition where high load was significantly greater compared to low load when collapsed for time ($F=5.811$, $p=0.027$, $\eta^2=0.244$).

Table 4.7

Mean (±SD) for β-catenin content for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	8.768 (±8.836)	14.382 (±13.841)	14.618 (±12.519)
High Load	16.351 (±11.460)	27.090 (±14.614)	29.833 (±28.145)

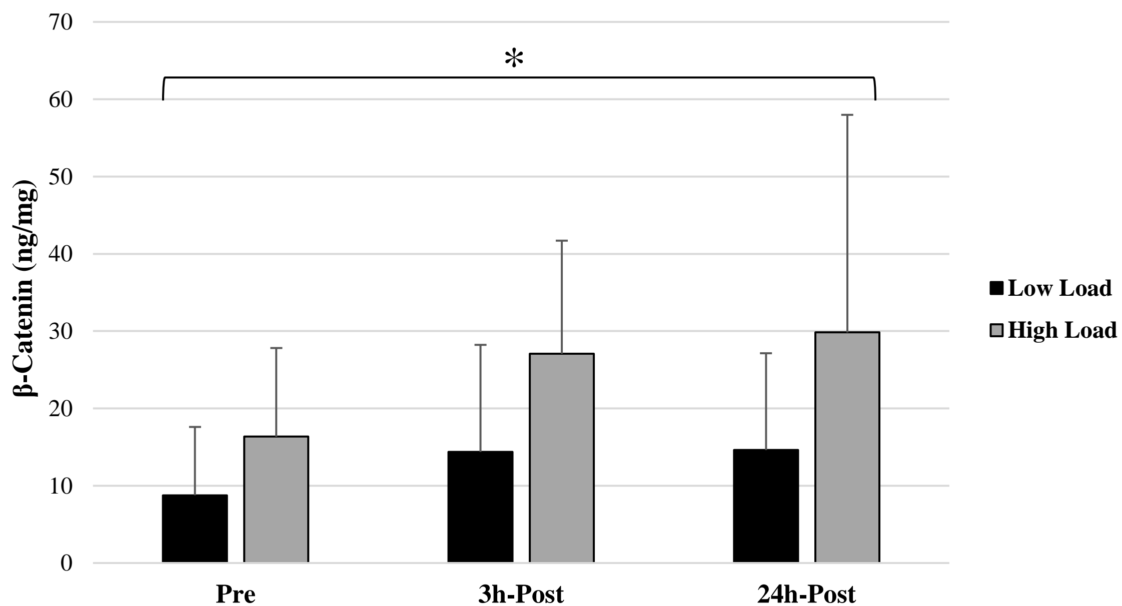

Figure 4.3 Mean (±SD) for changes in β-catenin over time between conditions. There were no significant changes over time or significant time and condition interactions between conditions. There was a main effect for condition where high load was significantly greater low load when collapsed for time.

Intramuscular Androgen Receptor Protein Content

The mean (±SD) for total intramuscular androgen receptor protein content relative to total muscle protein content (ng/mg) for each condition are indicated in Table 4.8 and Figure 4.4. A 2 x 2 [Condition (Low Load, High Load) x Time (3h Post-, 24h Post-Exercise)], with pre-exercise as a covariate, factorial ANCOVA with repeated measures

was used to account for differences in baseline values. The analysis indicated that there was no significant main effect for time ($F=1.058$, $p=0.318$, $\eta^2=0.059$) or condition ($F=1.427$, $p=0.249$, $\eta^2=0.077$). However, while not statistically significant, a moderate trend for a time and condition interaction was observed ($F=3.404$, $p=0.083$, $\eta^2=0.167$). Pairwise comparisons with a Bonferroni adjustment of the simple effects showed high load was elevated from baseline at 3h post-exercise ($p=0.048$).

Table 4.8

Mean (±SD) for total androgen receptor protein content (ng/mg) for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	102.847 (±29.918)	74.212 (±30.763)	102.917 (±60.731)
High Load	76.202 (±20.723)	113.022 (±64.653)	98.713 (±43.504)

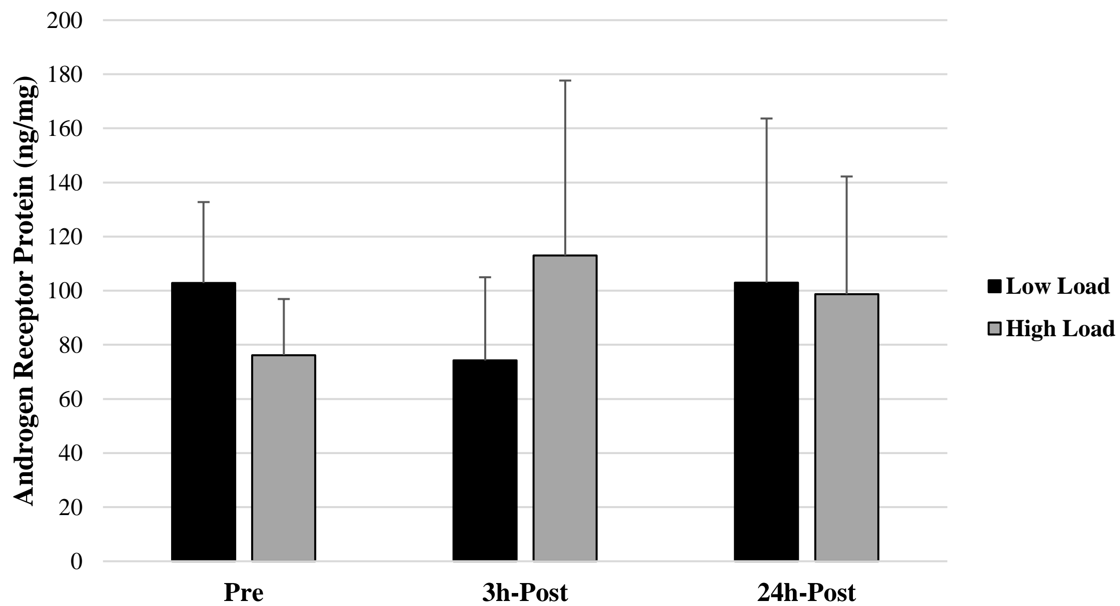

Figure 4.4: Mean (±SD) for changes in total androgen receptor protein content over time between conditions. There were no significant changes over time or differences between conditions. There was a moderate trend for a time and condition interaction where high load was elevated from baseline at 3h post-exercise.

Intramuscular Testosterone Concentration

The mean (±SD) for intramuscular testosterone relative to total muscle protein content (pg/mg) for each condition are indicated in Table 4.9 and Figure 4.5. No significant main effect of time (F=0.477, p= 0.624, η^2= 0.026) or condition (F=0.339, p=0.568, η^2=0.018) for intramuscular testosterone concentrations was observed. Moreover, no significant interaction for time and condition (F=0.731, p=0.488, η^2= 0.0.39) was observed.

Table 4.9

Mean (±SD) for intramuscular testosterone (pg/mg) for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	0.710 (±0.478)	0.571 (±0.279)	0.645 (±0.247)
High Load	0.554 (±0.173)	0.573 (±0.211)	0.608 (±0.323)

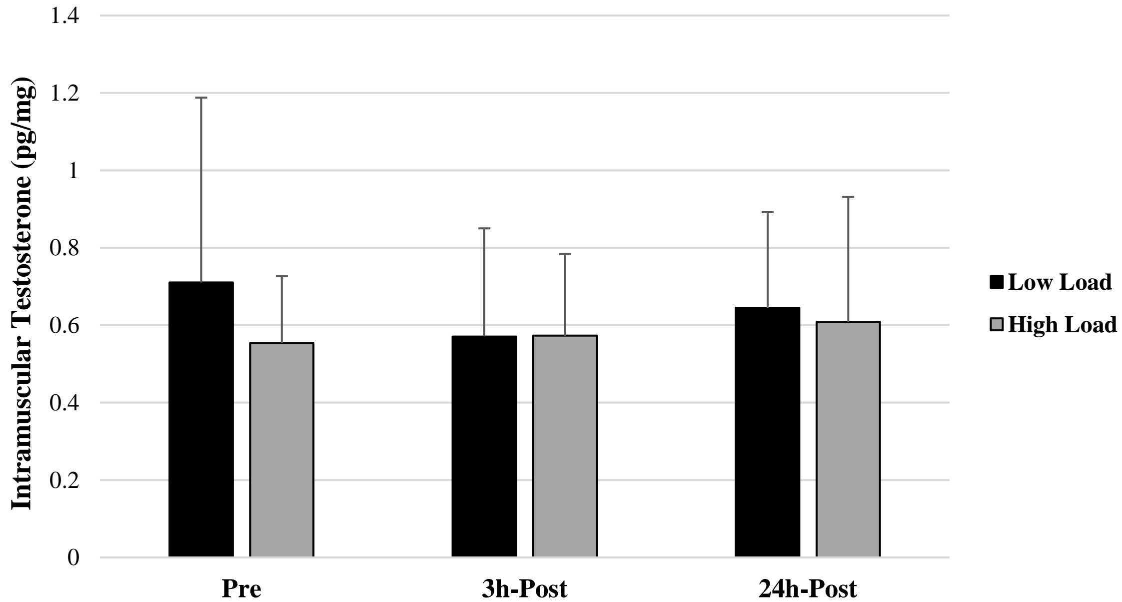

Figure 4.5: Mean (±SD) for changes in intramuscular testosterone concentrations over time between conditions. There were no significant changes over time or differences between conditions.

Intramuscular Dihydrotestosterone Concentration

The mean (±SD) for intramuscular dihydrotestosterone relative to total muscle protein content (pg/mg) for each condition are indicated in Table 4.10 and Figure 4.6. No significant main effect of time ($F=2.067$, $p=0.141$, $\eta^2=0.103$) or condition ($F=1.547$, $p=0.230$, $\eta^2=0.079$) for intramuscular dihydrotestosterone concentrations was observed. Moreover, no significant interaction for time and condition ($F=0.053$, $p=0.948$, $\eta^2=0.003$) was observed.

Table 4.10

Mean (±SD) for intramuscular dihydrotestosterone (pg/mg) for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	928.317 (±242.202)	975.603 (±228.746)	1047.053 (±357.399)
High Load	1048.111 (±258.654)	1137.315 (±352.988)	1190.964 (±354.099)

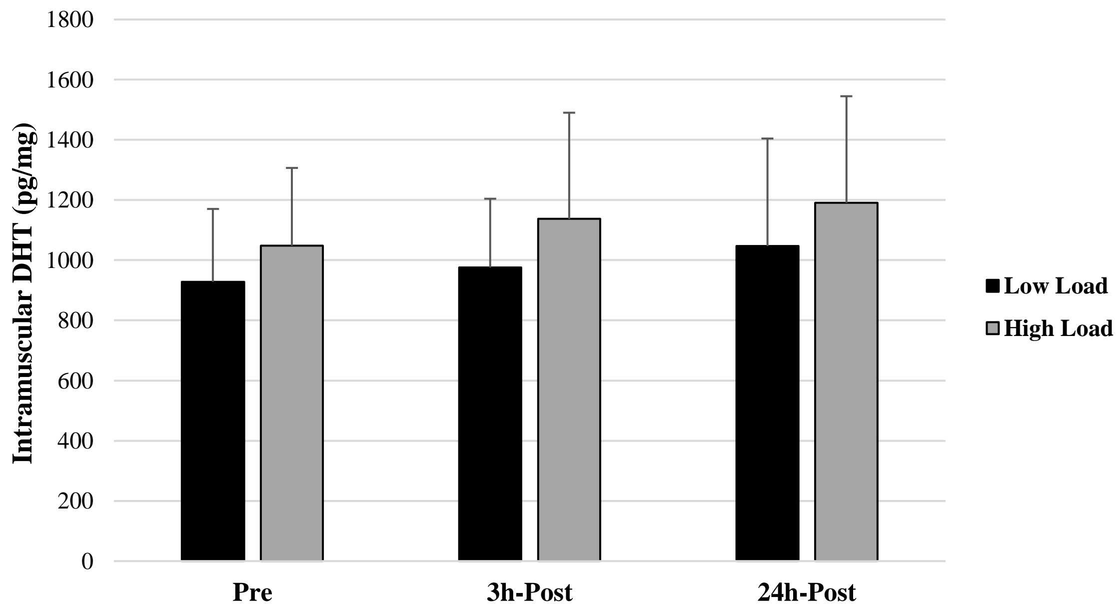

Figure 4.6: Mean (±SD) for changes in intramuscular dihydrotestosterone concentrations over time between conditions. There were no significant changes over time or differences between conditions.

Serum Total Testosterone Concentration

The mean (±SD) for serum total testosterone relative (pg/ml) for each condition are indicated in Table 4.11 and Figure 4.7. No significant main effect of condition (F=1.190, p=0.301, η^2=0.106) or significant interaction for time and condition (F=0.114, p=0.892, η^2=0.011) for serum total testosterone concentrations was observed. However, a significant main effect for time (F=14.904, p<0.001, η^2=0.598) was observed. Pairwise comparisons with a Bonferroni adjustment of the simple effects, revealed that there was a significant decrease in serum total testosterone concentrations at 3h post-exercise compared to pre-exercise (p=0.006) and returns to baseline at 24h post-exercise.

Table 4.11

Mean (±SD) for serum total testosterone (pg/ml) for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	10.753 (±3.960)	7.690 (±2.348)	10.386 (±2.015)
High Load	12.953 (±4.322)	9.255 (±2.976)	12.228 (±3.263)

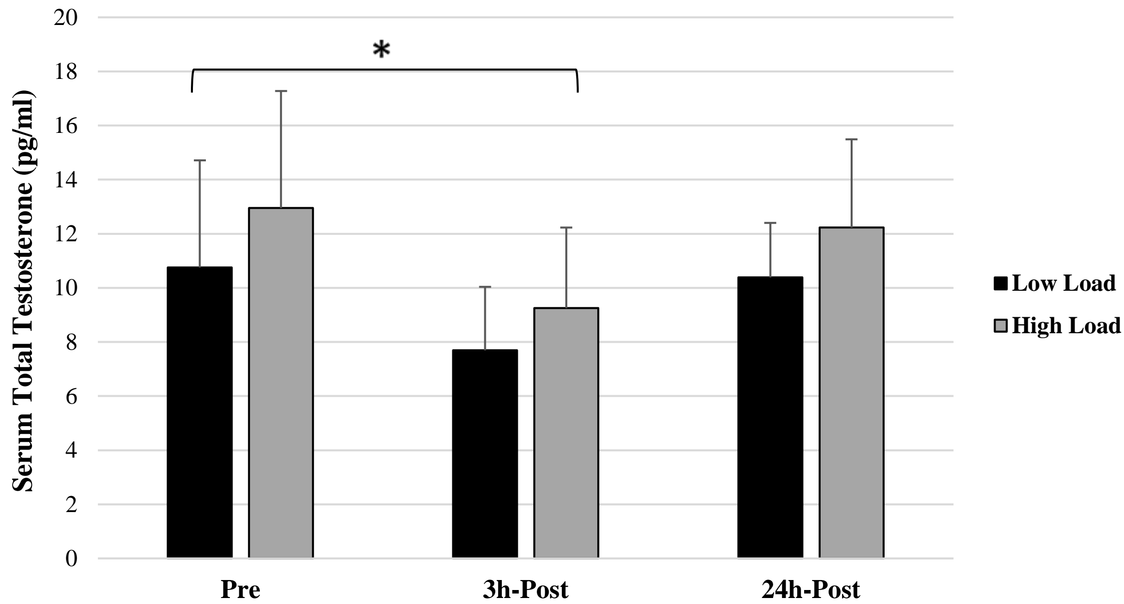

Figure 4.7: Mean (±SD) for changes in serum total testosterone concentrations over time between conditions. There were no significant differences between conditions. There was a significant decrease at 3h post-exercise compared to pre-exercise values and returns to baseline values at 24h post-exercise.

Serum Free Testosterone Concentration

The mean (±SD) for serum free testosterone relative (pg/ml) for each condition are indicated in Table 4.12 and Figure 4.8. No significant main effect of condition (F=0.008, p=0.929, η^2=.000) or significant interaction for time and condition (F=0.501, p=.610, η^2=0.027) for serum free testosterone concentrations was observed. However, a significant main effect for time (F=25.650, p<0.001, η^2=0.588) was observed. Pairwise comparisons with a Bonferroni adjustment of the simple effects, revealed that there was a significant decrease in serum free testosterone concentrations at 3h post-exercise compared to pre-exercise (p<0.001) in both conditions. Additionally, 24h post-exercise was significantly greater than 3h post-exercise (p=0.005) but did not return to pre-exercise baseline values (p=0.023) in either condition.

Table 4.12

Mean (±SD) for serum free testosterone (pg/ml) for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	23.030 (±10.286)	18.052 (±10.369)	20.836 (±8.170)
High Load	22.671 (±6.722)	18.864 (±8.642)	21.407 (±6.883)

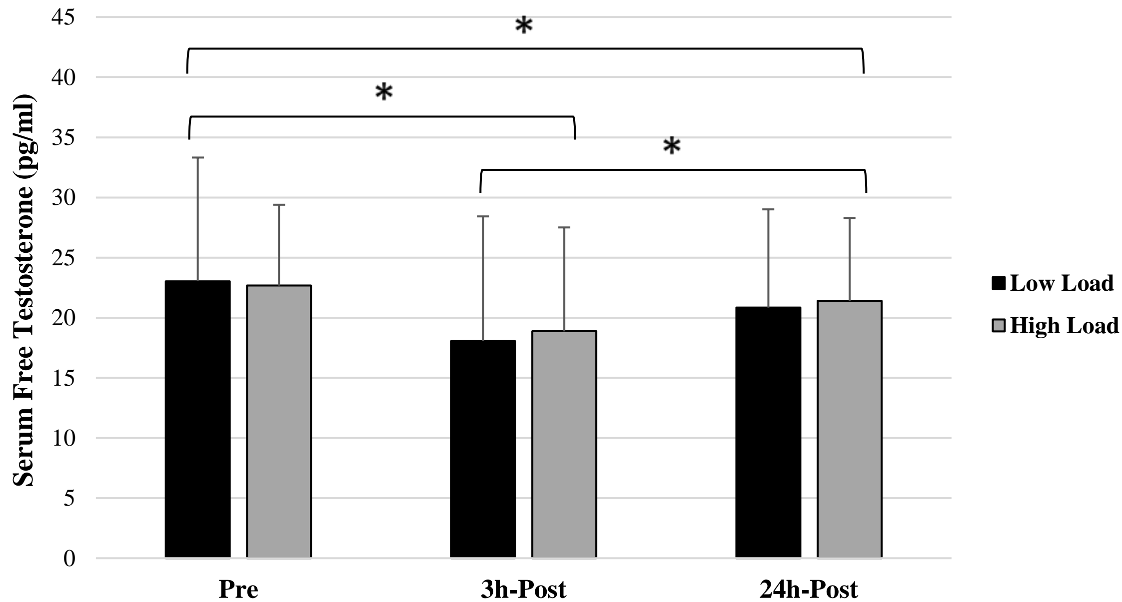

Figure 4.8: Mean (±SD) for changes in serum free testosterone concentrations over time between conditions. There were no significant differences between conditions. There was a significant decrease at 3h post-exercise compared to pre-exercise values. Also, 24h post-exercise was significantly greater than 3h post-exercise but did not return to pre-exercise baseline values in either condition.

Intramuscular Androgen Receptor (AR) mRNA Expression

The mean (±SD) for intramuscular *AR* expression for each condition are indicated in Table 4.13 (ΔΔct) and Figure 4.9 (fold change). No significant main effect of condition (F=0.016, p=0.899, η^2=0.001) or significant interaction for time and condition (F=0.583, p=0.563, η^2=0.031) for *AR* expression was observed. However, a significant main effect for time (F=6.030, p=0.006, η^2=0.251) was observed. Pairwise comparisons with a Bonferroni adjustment of the simple effects, revealed that there was a significant increase

in *AR* expression at 24h post-exercise compared to pre-exercise (p=0.010) in both conditions.

Table 4.13

Mean (±SD) for AR expression (ΔΔct) for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	22.257 (±3.402)	23.922 (±2.875)	24.489 (±4.266)
High Load	23.966 (±4.116)	24.568 (±3.638)	26.682 (±3.262)

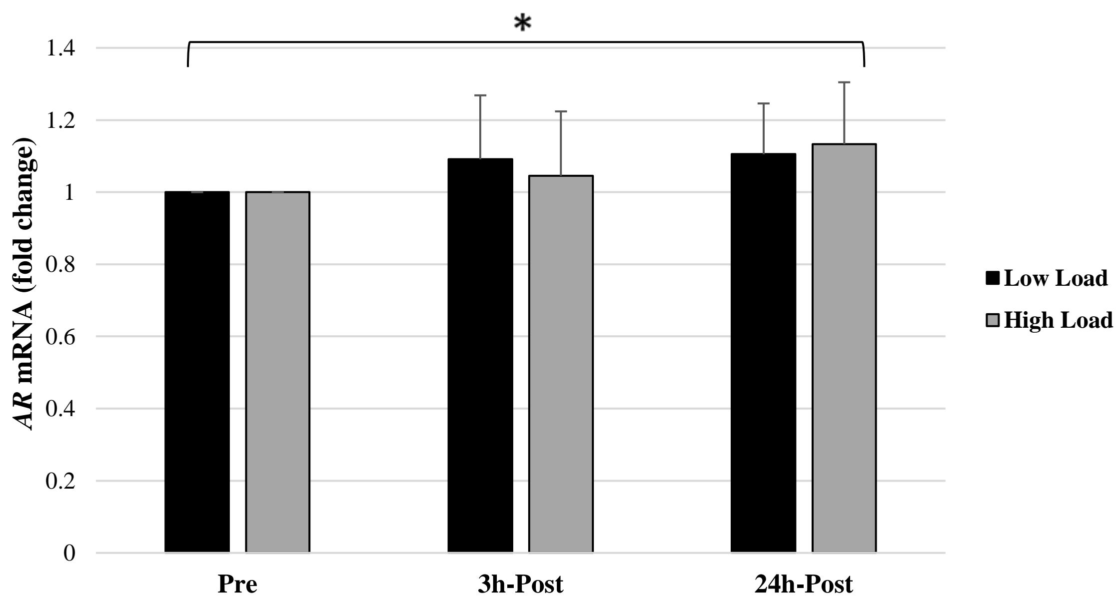

Figure 4.9: Mean (±SD) for changes in *AR* expression over time between conditions. There were no significant differences between conditions. There was a significant increase at 24h post-exercise compared to pre-exercise values.

Intramuscular Myoblast Determination Protein 1 (MyoD) mRNA Expression

The mean (±SD) for intramuscular *MyoD* expression for each condition are indicated in Table 4.14 (ΔΔct) and Figure 4.10 (fold change). No significant main effect of condition (F=0.103, p=0.752, η^2=0.006) or significant interaction for time and condition (F=0.124, p=0.884, η^2=0.007) for *MyoD* expression was observed. However, a significant

main effect for time ($F=4.583$, $p=0.017$, $\eta^2=0.203$) was observed. Pairwise comparisons with a Bonferroni adjustment of the simple effects, revealed that there was a significant increase in *MyoD* expression at 24h post-exercise compared to 3h post-exercise ($p=0.012$) but not statistically different from pre-exercise ($p=.382$) in both conditions.

Table 4.14

Mean (±SD) for MyoD expression (ΔΔct) for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	18.878 (±3.270)	17.899 (±5.809)	20.530 (±3.919)
High Load	20.765 (±4.587)	18.976 (±4.067)	21.331 (±4.731)

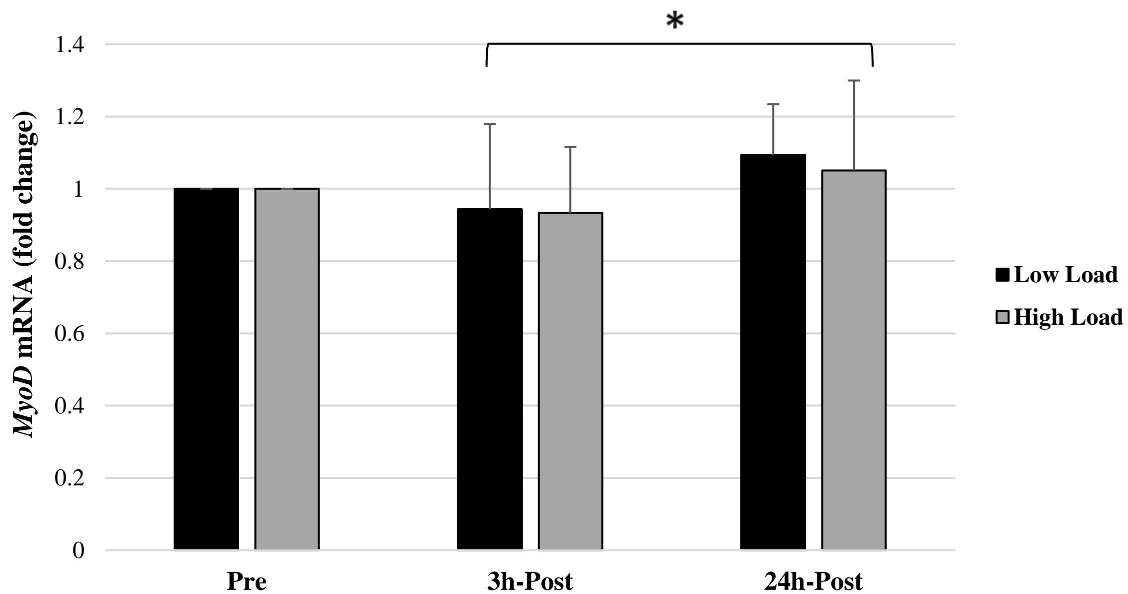

Figure 4.10: Mean (±SD) for changes in *MyoD* expression over time between conditions. There were no significant differences between conditions. There was a significant increase at 24h post-exercise compared to 3h post-exercise but not pre-exercise values.

Intramuscular Myogenin (MYOG) mRNA Expression

The mean (±SD) for intramuscular *MYOG* expression for each condition are indicated in Table 4.15 (ΔΔct) and Figure 4.11 (fold change). No significant main effect of

time (F=0.434, p=0.651, η^2=0.024), condition (p=0.503, η^2=0.025, F=0.468), or time and condition interaction (F=1.489, p=0.239, η^2=0.076) for *MYOG* expression was observed.

Table 4.15

Mean (±SD) for MYOG expression (ΔΔct) for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	19.288 (±4.724)	20.695 (±4.420)	18.806 (±3.893)
High Load	21.355 (±4.118)	20.055 (±4.415)	20.980 (±3.06)

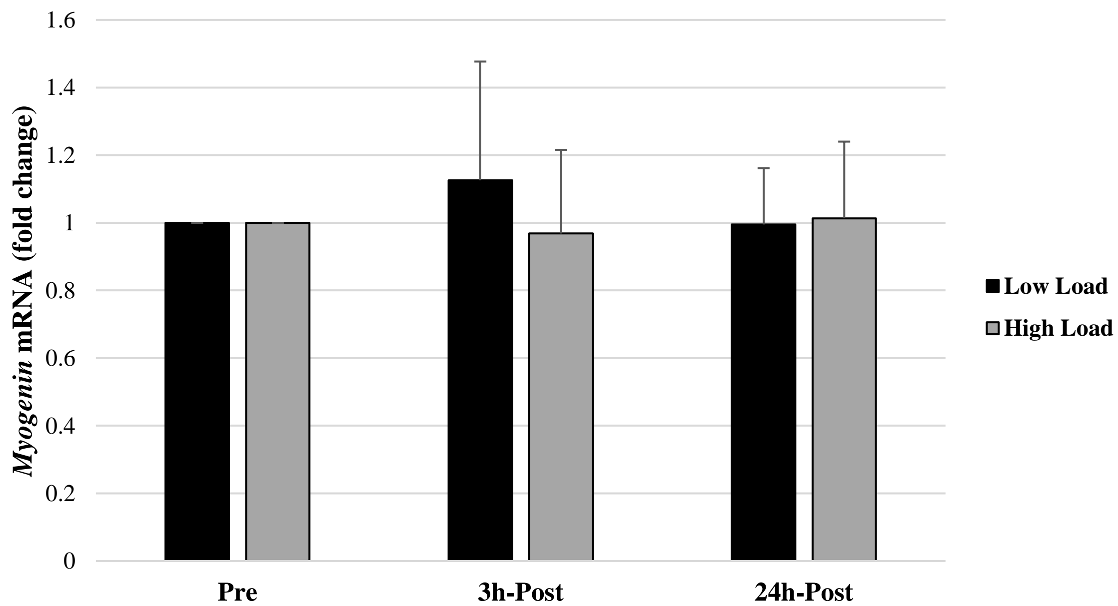

Figure 4.11: Mean (±SD) for changes in *MYOG* expression over time between conditions. There were no significant differences over time or between conditions.

Intramuscular Insulin-Like Growth Factor-1Ea (IGF-1Ea) mRNA Expression

The mean (±SD) for intramuscular *IGF-1Ea* expression for each condition are indicated in Table 4.16 (ΔΔct) and Figure 4.12 (fold change). No significant main effect of time (F=0.348, p=0.709, η^2=0.019), condition (F=0.044, p=0.836, η^2=0.002), or time and condition interaction (F=0.910, p=0.412, η^2=0.048) for *IGF-1Ea* expression was observed.

Table 4.16

Mean (±SD) for IGF-1Ea expression (ΔΔct) for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	31.389 (±4.276)	31.319 (±3.316)	30.914 (±4.054)
High Load	30.978 (±3.446)	30.306 (±3.865)	32.175 (±2.886)

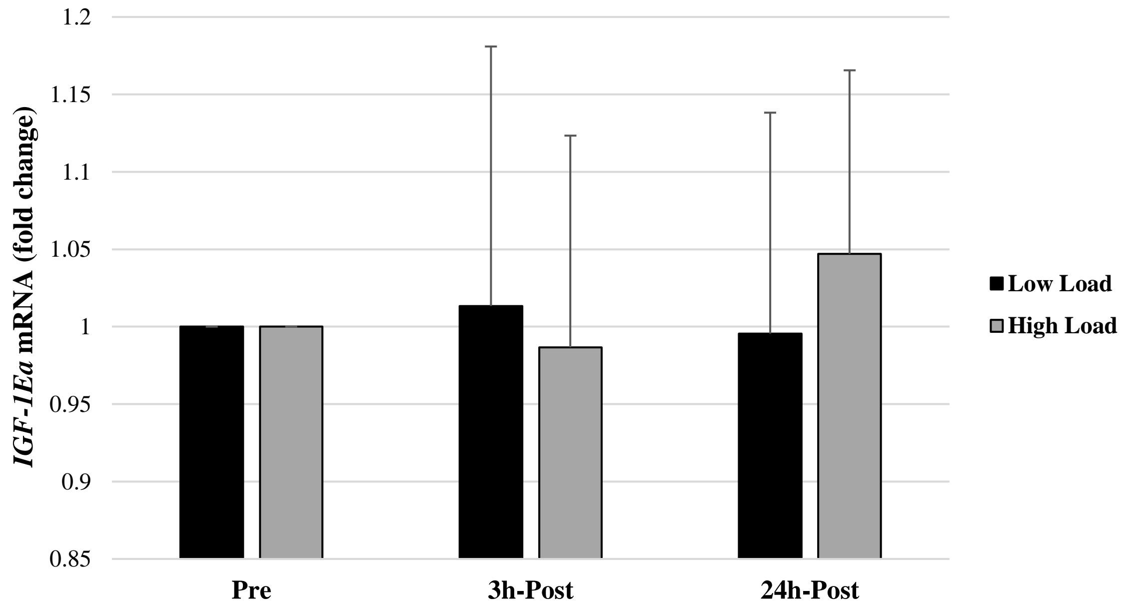

Figure 4.12: Mean (±SD) for changes in *IGF-1Ea* expression over time between conditions. There were no significant differences over time or between conditions.

Intramuscular Cyclin-Dependent Kinase Inhibitor 1 (p21-cip1) mRNA Expression

The mean (±SD) for intramuscular p21-cip1 expression for each condition are indicated in Table 4.17 (ΔΔct) and Figure 4.14 (fold-change). No significant main effect of time (F=0.124, p=0.884, η^2=0.007), condition (F=0.028, p=0.870, η^2=0.002), or time and condition interaction (F=0.081, p=0.922, η^2=0.004) for *p21-cip1* expression was observed.

Table 4.17

Mean (±SD) for p21-cip1 expression (ΔΔct) for each condition.

Condition	Pre-Exercise	3h Post	24h Post
Low Load	37.570 (±4.739)	37.328 (±3.382)	37.387 (±2.697)
High Load	37.594 (±4.171)	38.025 (±3.327)	37.469 (±4.723)

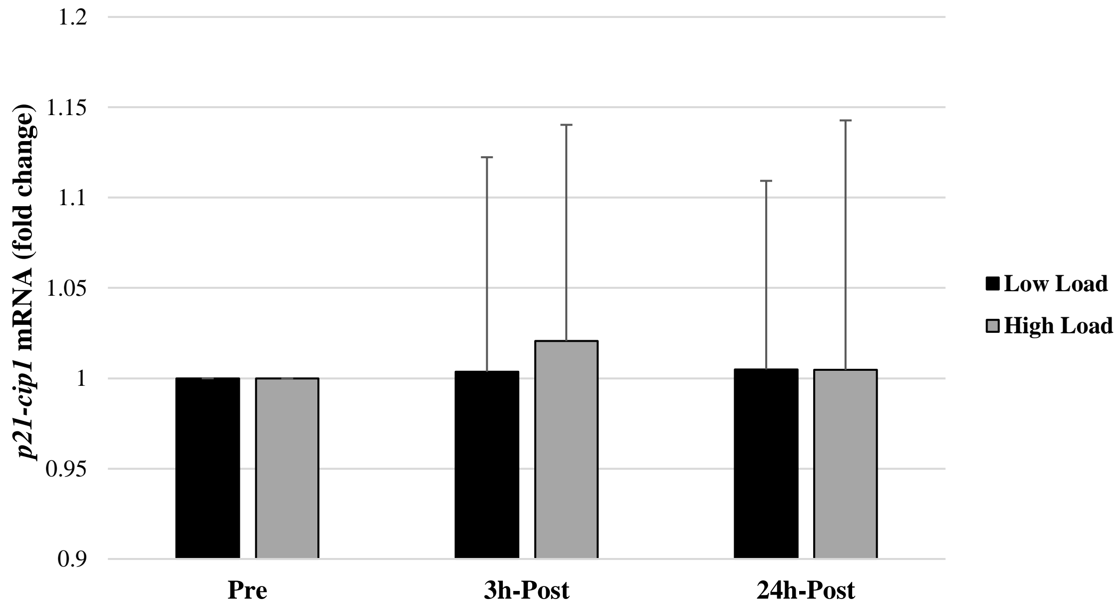

Figure 4.13: Mean (±SD) for changes in *p21-cip1* expression over time between conditions. There were no significant differences over time or between conditions.

CHAPTER FIVE

Discussion

Baseline Testing Measures

Participants did not have significantly different caloric intakes, macronutrient intakes, or hydration status across all scheduled testing visits. Moreover, all participants underwent similar fasting procedures and performed the testing visit at the same time of day (upon waking between 7am-10am) to account for diurnal responses in testosterone secretion. Lastly, equating resistance exercise intensity (volitional muscular failure of all sets) and volume of the testing visits allowed for changes in our dependent variables to be due to resistance exercise load and not other program design variables that may have confounded results. Equivalent ratings of perceived exertion and soreness of each testing condition further support our findings to be primarily due to alterations in resistance exercise load. Additionally, all baseline concentrations of AR-DNA binding, AR protein, β-catenin, and serum/muscle androgens were not statistically different and were, therefore, statistically accounted for between testing conditions. This indicates that participants complied with the regulations outlined in the study and to the best of our knowledge the changes seen in each dependent variable were altered as a result of the independent variables.

Resistance Exercise Load-Mediated AR Signaling

It is generally thought that low (≤60% 1RM) and high (>60% 1RM) load resistance exercise has similar outcomes on skeletal muscle hypertrophy when performed to volitional

muscular failure (Schoenfeld et al., 2017). Therefore, the molecular mechanisms which regulate skeletal muscle hypertrophy are also suggested to be identical in both low and high load scenarios. As one of the primary signaling pathways that contribute to up-regulating muscle specific gene and protein expression, understanding the AR signaling response to low and high load resistance exercise can provide valuable insight into the molecular outcomes of these different stimuli as well as challenge this idea. This appears to be the first study to investigate the effects of resistance exercise load, in an intensity- and volume- equated manner, on AR-DNA binding activity, AR-regulated gene expression, and the influence of the androgenic hormones, AR protein content, and β-catenin concentrations on AR signaling. Our data shows a significant increase (~74%) in AR-DNA binding in response to high load (80% 1RM) full-body resistance exercise at 3h post-exercise compared to pre-exercise values. Moreover, a lack of significant change from the low load resistance exercise condition across all sampling times indicates a potential load dependence in AR activation, translocation, and DNA binding. Interestingly, in response to both low and high load conditions, respectively, we also observed a significant decrease in serum total testosterone concentrations at 3h post-exercise (~40% vs. ~40%) as well as a significant decrease in serum free testosterone concentrations at 3h post- (~28% vs. ~20%) and 24h post-exercise (~11% vs. ~6%) compared to pre-exercise. However, muscle testosterone and dihydrotestosterone concentrations did not significantly change suggesting the decrease in circulating free and total testosterone concentrations did not influence the skeletal muscle testosterone and dihydrotestosterone concentrations. Therefore, the observed increase in AR-DNA binding in the high load condition at 3h post-

exercise does not appear to be driven by load-mediated changes in either circulating or skeletal muscle androgen concentrations concentration.

As an integral role in facilitating increases in AR signaling, the AR protein response itself may conceivably be mediating this load dependent increase shown in the high load condition. However, there were no significant changes in AR protein content across all time points in either condition. While not significant, there was a moderate trend ($p=0.083$; $\eta^2=0.167$) for increases in AR protein content at 3h post-exercise in the high load condition, consistent with the only sampling time and condition we observed increases in AR-DNA binding. Lack of significance limits speculation of a load dependent AR protein response, or its ability in facilitating the increase in AR-DNA binding, but it should not be disregarded. Lastly, we also found significantly greater concentrations of the AR co-activating protein, β-catenin in the high load condition compared to the low load condition regardless of time. Specifically, skeletal muscle β-catenin protein concentrations were ~94% greater in the high load condition versus the low load condition. As a multifunctional protein that has been shown to positively influence a number of processes involved in cell cycle progression, cell-to-cell adhesion activity, and ribosome biogenesis, in addition to its alleged potent ability to co-activate the AR, the load-mediated response provides further evidence of superior anabolic signaling activity in high load compared to low load resistance exercise (Mulholland et al., 2005; Chaillou et al., 2014; Newmire & Willoughby, 2015).

Despite this preferential high load response in our markers of AR signaling activity, transcriptional activity of AR regulated or responsive genes did not appear to be significantly elevated. *MyoD* and *myogenin* expression was not significantly elevated from

baseline values in either condition at any time point. As AR responsive genes, these two genes are reported to be significantly increased in response to elevations in androgenic hormone concentrations (Montano et al. 2007; Lee, 2002). Consistent with this mechanism, our lack of increases in serum total and free testosterone or muscle testosterone and dihydrotestosterone did not result in elevated mRNA expression of these genes. However, *AR*, *IGF-1Ea*, and *p21-cip1* have all been previously reported to contain AREs (Roberts et al., 2009; Grad et al., 1999; Lu et al., 2002; Wu et al., 2007). Thus, theoretically, significant elevations in AR-DNA binding should result in escalations in transcriptional activity and subsequent mRNA expression of these genes. Our data does not reflect this scenario as we did not observe any meaningful elevations in mRNA expression of *AR*, *IGF-1Ea*, or *p21-cip1*. Moreover, it is important to note that while our statistical analysis unveiled a significant time effect where *AR* mRNA expression was increased at 24h post-exercise in both conditions, a 1.11- and 1.13-fold increase in low and high load, respectively, does not necessarily indicate meaningful elevations in mRNA expression. Therefore, these data demonstrate no load-mediated increases in transcriptional activity were found due to absence of changes in AR regulated or responsive genes. Collectively, our findings reject the current theory that low and high load resistance exercise, when performed to muscular failure, are equivalently effective in influencing the molecular mechanisms that regulate skeletal muscle anabolism and potentially hypertrophic outcomes.

Contrary to our findings, previous research lends support to the notion of load not dictating hypertrophy or the AR signaling response in skeletal muscle. In a recent study by Morton et al. (2016), they investigated androgenic hormone responses to low load (30-50% 1RM) and high load (75-90% 1RM) full-body resistance exercise acutely as well as

following 12 weeks of training. It is important to note every set was performed to volitional failure (or 100% intensity). Interestingly, they found a significant acute increase in serum free and total testosterone in both load conditions. Furthermore, both load conditions did not preferentially increase skeletal muscle hypertrophy over the 12-week training period. Thus, they concluded resistance exercise load did not preferentially influence androgen hormone concentrations or dictate hypertrophy in skeletal muscle. Similarly, our study shows serum free and total testosterone as well as muscle testosterone and dihydrotestosterone to have no preferential load response. Furthermore, our data demonstrate systemic and local androgen concentrations may not be significantly impacting AR signaling responses since the observed increase in AR-DNA binding was associated with decreases in androgen content in serum and no change in skeletal muscle. Practically, our model cannot directly conclude hypertrophic outcomes of different loads due to the acute nature of our design. However, the observed load-mediated increase in AR-DNA binding activity suggests a potential preferential anabolic response to high load resistance exercise. We speculate that due to the overwhelming role volume and intensity have on resistance exercise mediated hypertrophy, Morton et al.'s study design does not allow for determining a preferential load response or lack thereof (Schoenfeld et al., 2019; Hayes et al., 2013; Kraemer & Ratamass, 2005; Gotshalk et al., 1997). Specifically, the high load condition only completed ~62% of the total volume completed by the low load condition. Dissimilarly, our design equated both volume and intensity of the different load conditions allowing for the acute effects of load to be carefully disseminated in the context of AR signaling and potential implications in skeletal muscle hypertrophy. Nevertheless, while this study does not demonstrate the effects of varying loads on practical hypertrophic

outcomes, it corroborates our data and many others (West et al., 2010; West and Phillips, 2012; Mitchell et al., 2013; Mobley et al., 2018; Morton et al., 2018) providing evidence that systemic hormones are neither related to nor predictive of resistance exercise induced changes in skeletal muscle mass in healthy young male participants.

At present, it appears that research investigating the acute AR protein response to varying resistance exercise loads has yet to be examined. However, extrapolation from multiple studies provides critical insight into the effects of high load resistance on this highly relevant marker. In a study by Ratamass et al. (2005), performing 6 sets of 10 repetitions of squats at a high load (80-85% 1RM) resulted in significant decreases in AR protein content at 1h post-exercise compared to pre-exercise values. Another study (Spiering et al., 2009) showed a nonsignificant (p=0.17) increase in AR protein content 3h following a full body bout of resistance exercise consisting of 5 sets of 5 repetitions of bilateral knee extensions and 4 sets of 10 repetitions of bench press, seated row, and overhead press all at a high load (80-90% 1RM). Lastly, Ahtiainen et al. (2011) found no significant differences in AR protein content at 1h post- or 48h post-exercise when compared to pre-exercise values in response to 5 sets of 10 repetitions performed to muscular failure (or 100% intensity). While no previous research has been conducted investigating low load resistance exercise's (≤60% 1RM) influence on AR protein content, our data offers insight about the AR protein response observed in response to high load mechanical loading in healthy resistance trained males. Similar to Spiering et al. (2009), we did not observe significant increases in AR protein content at 3h post-exercise. However, our "moderate trend" for AR protein elevations at 3h post-exercise (p=0.083) in the high load condition mirrors Spiering et al.'s (2009) nonsignificant (p=0.17) increase at

3h post-exercise. To reiterate, lack of significance limits speculation of AR protein facilitating this load dependent increase in AR-DNA binding but it should not be disregarded. Furthermore, due to the dissimilar study designs and failure of this study to have participants perform to muscular failure (although theoretically 5-10 repetitions at 80-90% should be nearing 100% intensity) our findings should be independently considered as participants experienced different stimuli between these two protocols. On the contrary, Ahtiainen et al. (2011) had subjects perform all sets to muscular failure. In this context, the identical intensity between our study designs allows for further speculations to be drawn. Thus, similar to our findings, in this scenario they observed no significant increases in AR protein content at 1h post- or 48h-post exercise. Furthermore, lack of increases in our low load condition (with volume and intensity equated) suggests this response is not load dependent. While more research is certainly warranted, this provides further evidence along with others (Lee et al., 2003; Vingren et al., 2009; Spiering et al., 2009; Ratamass et al., 2005) that AR protein content does not appear to acutely increase in response to resistance exercise and provides preliminary evidence that it may not be suggestive of upregulations in AR signaling or predictive of hypertrophic outcomes [AR protein response to chronic resistance training may be more indicative of hypertrophic outcomes; see Morton et al., 2018 for clarification].

In resistance exercise research, rarely is the effect of load on muscular adaptations examined without being affected by confounding variables such as volume and intensity. Previous research has clearly demonstrated the overwhelming influence volume and intensity have on skeletal muscle hypertrophy and the molecular responses that regulate these adaptations (Schoenfeld et al., 2019; Kraemer & Ratamass, 2004; Gotshalk et al.,

1997). Moreover, the majority of the literature fails to properly define load as well as differentiate resistance exercise load from intensity (Schwab et al., 1993; Raastad et al., 2000; Lima et al., 2011; Fry, 2004; McGuigan et al., 2004). As previously stated, intensity is commonly defined as a percent of one repetition maximum (Haff & Triplett, 2016). However, this is more appropriately termed load (Cintineo et al. 2018). Therefore, future research is needed to account for these semantic differences and should be carefully considered in future study design. At this point, it appears that this is the first study to control for these variables in order to accurately disseminate the effects of resistance exercise load on anabolic signaling pathways suggested to mediate skeletal muscle hypertrophy. The novelty in our study design limits our ability to speculate since no data seem to exist investigating the effects of resistance exercise load, in a volume- and intensity-equated manner, on markers of AR signaling. Nevertheless, our findings suggest a preferential load dependent increase in AR signaling activity. Furthermore, the absence of significant elevations in serum free and total testosterone, skeletal muscle testosterone and dihydrotestosterone, and AR protein content, with concomitant increase in AR-DNA binding in response to high load resistance exercise, insinuates this phenomenon may be governed by other factors. Rather, our data provides evidence that the upregulation in AR-DNA binding activity in response to high load resistance exercise may be due to the AR co-activating protein, β-catenin. To date, there is extremely limited data investigating the effects of resistance exercise-induced elevations in β-catenin and its impacts on AR signaling. This multifunctional AR-interacting protein may be one of the driving factors in facilitating increases in AR signaling and by extension, skeletal muscle hypertrophy.

β-Catenin and AR Signaling

The multifunctional Wnt-signaling protein, β-catenin, has been shown to robustly impact stability, activation, and transcriptional activity of the AR (Mulholland et al., 2005; Yang et al., 2002; Truica et al., 2000). Theoretically, elevations in this AR co-activating protein directly increase AR activation, translocation, DNA binding, and result in upregulations in muscle specific gene and eventual protein expression. As previously discussed, our data showed a ~74% significant increase in AR-DNA binding in the high load condition at 3h post-exercise compared to baseline values. This elevation in AR signaling activity occurred in the absence of significant elevations in serum free and total testosterone, skeletal muscle testosterone and dihydrotestosterone, or AR protein content. However, consistent with this notion, we observed a load-specific response for β-catenin where cytoplasmic β-catenin concentrations were significantly greater (~94%) in the high versus low load condition. Our data provides compelling evidence that β-catenin may be playing a key regulatory role in encouraging AR-androgen interactions or activating the AR in a ligand-independent manner. Furthermore, the load-dependent elevation in β-catenin suggests a novel mechanism in which high amounts of mechanical load results in the accumulation of β-catenin in the cytoplasm, ultimately interacting with the AR and increasing AR-DNA binding within the nucleus.

Cytoplasmic β-catenin concentrations increase in response to a series of regulatory steps. As previously discussed, this process involves 1) the binding of a Wnt ligand (there are 19 known Wnt ligands) to the extracellular cysteine rich domain (termed the "Wnt binding domain") on the transmembrane Frizzled receptor, 2) phosphorylation/activation of the protein disheveled by low-density lipoprotein receptor-related protein-5 or -6 co-

receptors, 3) blocking of glycogen synthase kinase-3β activity (GSK-3β) by sequestering GSK-3β via the inhibitory protein frequently rearranged in advanced T cell lymphomas (Frat), and 4) inactivation of the "destruction complex" resulting in decreased phosphorylation and down-regulating proteasomal degradation of β-catenin (Armstrong & Esser, 2005; Mumford et al., 2018; Newmire & Willoughby, 2015; Zhou et al., 2011). Another documented mechanism of β-catenin accumulation in the cell is upregulation in PI3K/Akt signaling. This increases GSK-3β phosphorylation and results in the down-regulation of destruction complex activity (Armstrong & Esser, 2005). Lastly, β-catenin's dual ability to accumulate in the cytoplasm and translocate to the nucleus by nuclear-cytoplasmic shuttling proteins (such as adenomatous polyposis coli) is important to note (Armstrong & Esser, 2005; Henderson, 2000). Theoretically, this occurrence may allow for an increase in β-catenin-protein interactions in both of these cell compartments. Although our study did not directly analyze changes in many of these markers, we observed greater β-catenin in response to high load resistance exercise which suggests upregulations in these mechanisms likely occurred given their documented responsiveness to mechanical loading (Armstrong & Esser, 2005; Leal et al., 2011; Spillane et al., 2015). Consistent with previous research, this provides further evidence Wnt/β-catenin signaling is responsive to mechanical loading (Armstrong & Esser, 2005; Leal et al., 2011; Spillane et et al., 2015). However, this is the first study to propose this response may be dependent on the resistance or load placed on skeletal muscle itself.

There is considerable evidence of crosstalk between Wnt/β-catenin and AR signaling in the literature (Wang et al., 2008; Mulholland et al., 2002; Newmire & Willoughby, 2015; Pawlowski et al., 2002; Truica et al., 2000; Zhou et al., 2011).

Moreover, β-catenin's documented ability to act as an AR co-activator suggests it may play a substantial role in facilitating muscle specific gene expression (and subsequent protein expression) ultimately influencing resistance exercise induced skeletal muscle adaptations. Kinetically, armadillo repeats 1-6 of β-catenin interact with the AF-2 region (located within the LBD) of the AR to facilitate LBD-NTD interactions and the ensuing formation of a "ligand-binding pocket" and/or stabilization of the bound androgen (Pratt & Toft, 1997; Zhou et al., 2011). Once activated, dissociation of heat shock proteins (and other co-chaperones) and dimerization of the AR allows for translocation into the nucleus where it binds to the ARE on the target gene up-regulating muscle specific gene expression. This mechanism is greatly relevant in interpreting our findings and divulging the protein kinetics of β-catenin and the AR in human skeletal muscle in response to mechanical loading. While our findings cannot provide direct evidence of these interactions, the elevations in β-catenin paired with the observed increases in AR-DNA binding activity lend further evidence of crosstalk between β-catenin and AR signaling and the aforementioned protein-protein interactions.

Armadillo (ARM) repeats 1-6 of β-catenin bind to the leucine-rich binding site [LXXLL (L=leucine, X=any other amino acid)] within the AF-2 region (in the LBD) of the AR. These ARM repeats are similarly reported to interact with TCF/LEF (downstream transcription factors of Wnt/β-catenin signaling), E-cadherin (cell adhesion protein), and the retinoic acid receptor α (vitamin A metabolite receptor protein) (Mulholland et al., 2002; Song et al., 2003; Yang et al., 2002). Therefore, competitive binding of β-catenin between the AR and these proteins has proposed to occur. While we did not analyze these proteins, our data provides further context when disseminating a potential mechanism of

our findings. Therefore, our data can limit speculation of the binding kinetics and competitive nature between β-catenin and these proteins. Nevertheless, our observed elevations in AR-DNA binding activity imply that β-catenin-AR interactions may have occurred, despite the described competitive binding of β-catenin.

β-catenin has been reported to activate the AR in both an androgen-dependent and androgen-independent manner (Trucia et al., 2000; Yang et al., 2002; Wang et al., 2008). Previous *in vitro* and *in vivo* animal models offer inconclusive support in determining this potential androgen dependence of β-catenin-AR activity (Trucia et al., 2000; Yang et al., 2002). Trucia et al. (2000) and Yang et al. (2002) provide evidence of AR-β-catenin signaling to be androgen-dependent due to the enhanced AR-β-catenin interaction observed upon the addition of androgen in cell cultures. Contrarily, in a study by Wang et al. (2008), they found androgen-independent AR activation by β-catenin in castrate-resistant prostate cancer samples from castrated mice, observing increases in AR co-localization and protein-protein interactions between the AR and β-catenin without the presence of androgens. Lack of significant changes in muscle testosterone, dihydrotestosterone, or AR protein content with concomitant elevations in AR-DNA binding activity and greater β-catenin content alone does not provide sufficient evidence to make definitive mechanistic inferences about our data. Nevertheless, since we have demonstrated β-catenin's ability to accumulate in the cytoplasm and nucleoplasm this implies our findings may be due to a number of scenarios or a combination thereof including: 1) cytoplasmic stabilization and co-activation of the AR-bound androgen by β-catenin (Figure 5.1), 2) nucleoplasmic AR stabilization and co-activation of the AR-bound androgen by β-catenin (Figure 5.2), and/or 3) androgen-independent activation of the AR by β-catenin within the cytoplasm (Figure 5.3) (Trucia et

al., 2000; Yang et al., 2002; Wang et al., 2008; Armstrong & Esser, 2005; Henderson, 2000).

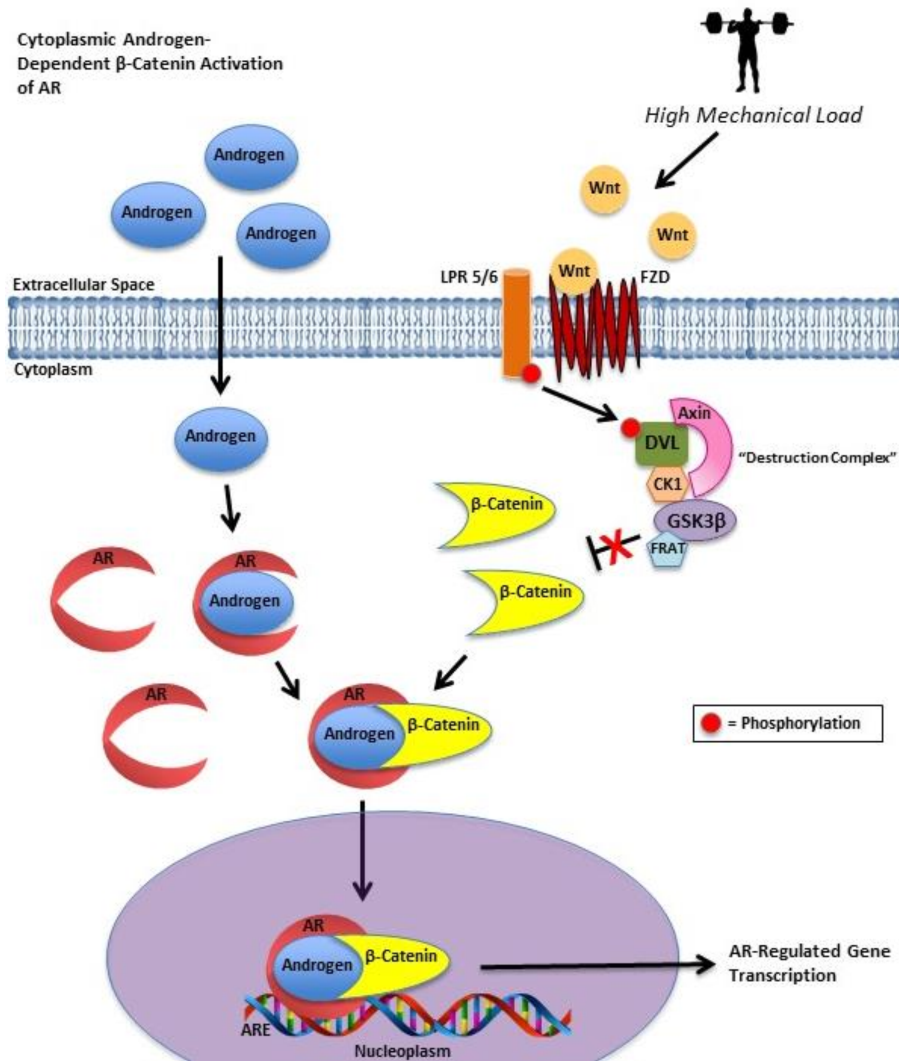

Figure 5.1: Above shows the proposed cytoplasmic androgen-dependent actions of β-catenin on AR signaling. (AR = androgen receptor; LPR 5/6 = low-density lipoprotein receptor-related protein 5 or 6; FZD = frizzled receptor; Wnt = wingless-type MMTV integration site (Wnt) protein; DVL = disheveled; CK1 = casein kinase 1; GSK3β = glycogen synthase kinase 3β; FRAT = frequently rearranged in advanced T cell lymphomas; ARE = Androgen Response Element)

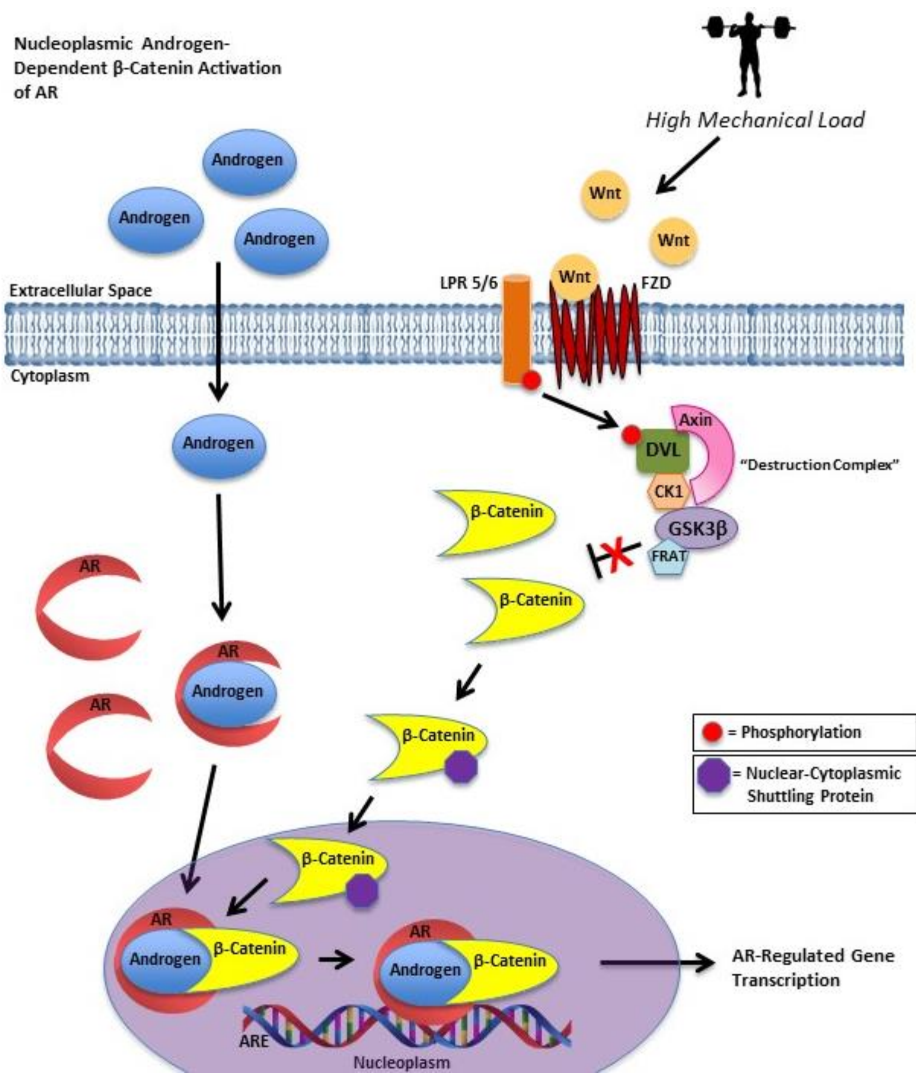

Figure 5.2: Above shows the proposed nucleoplasmic androgen-dependent actions of β-catenin on AR signaling. (AR = androgen receptor; LPR 5/6 = low-density lipoprotein receptor-related protein 5 or 6; FZD = frizzled receptor; Wnt = in wingless-type MMTV integration site (Wnt) protein; DVL = disheveled; CK1 = casein kinase 1; GSK3β = glycogen synthase kinase 3β; FRAT = frequently rearranged in advanced T cell lymphomas; ARE = Androgen Response Element)

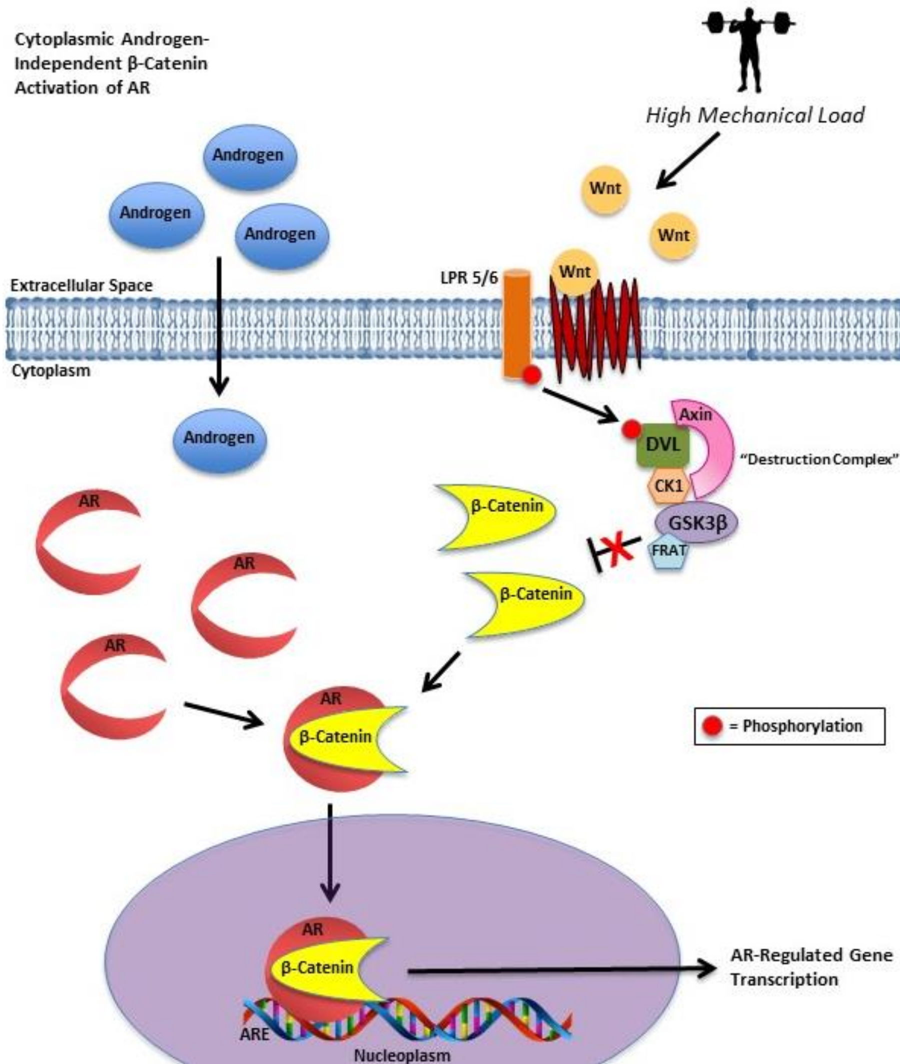

Figure 5.3: Above shows the proposed cytoplasmic androgen-independent actions of β-catenin on AR signaling. (AR = androgen receptor; LPR 5/6 = low-density lipoprotein receptor-related protein 5 or 6; FZD = frizzled receptor; Wnt = in wingless-type MMTV integration site (Wnt) protein; DVL = disheveled; CK1 = casein kinase 1; GSK3β = glycogen synthase kinase 3β; FRAT = frequently rearranged in advanced T cell lymphomas; ARE = Androgen Response Element)

While *in vitro* and *in vivo* animal models provide valuable insight into the potential molecular mechanisms governing this response, human models are more comparable given our study design. In a recent study by Spillane et al. (2015), they investigated the β-catenin and AR signaling response to an acute bout of lower- and full-body resistance training. Similar to Spiering et al. (2009), participants performed 5 sets of 5 repetitions of bilateral

knee extensions and 4 sets of 10 repetitions of bench press, seated row, and overhead press at high load (80-90% 1RM) during the full-body condition and 5 sets of 5 repetitions of bilateral knee extensions at high load (80-90% 1RM) during the lower-body condition. It is also important to note this study design did not equate resistance exercise volume between the two conditions. Therefore, the full-body condition performed significantly more volume than the lower-body condition. Similar to our findings, they found increased AR-DNA binding activity and β-catenin content at 3h post- and 24h post-exercise, in addition to elevated serum Wnt4 concentrations at 30min post-, 1h post-, and 2h post-exercise, following the full-body resistance exercise bout. Consistent with our observations, no significant changes for serum free and total testosterone or muscle testosterone and dihydrotestosterone were observed at any time point or condition. However, they did witness a significant increase in AR protein content at 3h post- and a significant decrease at 24h post-full body resistance exercise. Being an increase in AR-DNA binding activity occurred at these time points, it further begs the question of whether the AR protein response is facilitating this acute increase in AR signaling activity. Given the incongruent AR protein and AR-DNA binding response in our study and that of both Spillane et al. (2015), we propose these observed acute elevations in AR signaling are not driven by changes in AR content. Rather, this data indicates up-regulations in Wnt/β-catenin signaling are causing increases in AR-DNA binding which appears reflective of elevations in AR signaling and potentially transcriptional activity. Furthermore, these data collectively suggest this response appears to be sensitive to load as well as the volume of mechanical work placed on skeletal muscle.

As discussed, AR-regulated gene expression should be reflexive of AR-DNA binding activity upregulation. However, our study did not observe any meaningful increases in mRNA expression of any AR-regulated genes at 3h post- or 24h post-exercise despite elevations in AR-DNA binding activity 3h post-exercise following high load resistance exercise. In a similar study by Hulmi et al. (2008), they found no significant change in *AR* expression (an AR-regulated gene) at 1h post-resistance exercise [although a "trending" increase (p=0.09)] but significant elevations at 48h post-resistance exercise following 5 sets of 10 repetitions. Moreover, they reported no changes in *IGF1-Ea* expression (an AR-regulated gene) at all time points. Another study by Roberts et al. (2009) investigated mRNA expression of a number of AR-regulated and -responsive genes that we evaluated. In this study, they reported no significant changes in *AR*, *IGF1-Ea*, *p21-cip1*, or *myogenin* expression at 24h post-exercise in untrained men following 3 sets of 10 repetitions at high load (80%1RM) of smith machine squats, leg press, and leg extensions. Consistent with these findings, we did not observe practical increases in any of our genes of interest at 3h post- or 24h post-exercise. We speculate this may have been due to our sampling window. Our findings and conclusions are only reflexive of the AR signaling-related genes at these limited times in which a sample was collected. Although pure conjecture, based on the protein kinetics and signaling response of this pathway, we suspect elevations in mRNA expression of these genes may have occurred at another time.

Conclusion

This appears to be the first study to date investigating the impacts of resistance exercise load, in a volume- and intensity-equated manner, on AR-DNA binding activity, AR-regulated/-responsive gene expression, serum and muscle androgen concentrations,

AR protein content, and β-catenin concentrations. No significant changes were observed in response to low load full-body resistance exercise across all time points. However, following high load full-body resistance exercise, we observed a significant ~74% increase in AR-DNA binding activity, compared to baseline values, without any significant elevations in serum or muscle androgen concentrations or AR protein content. Furthermore, skeletal muscle β-catenin content was ~94% significantly greater when comparing the high load versus low load conditions regardless of time. Collectively, our findings provide evidence that when volume and intensity are equated, the acute AR signaling response to mechanical loading on skeletal muscle appears to be load-mediated. Moreover, the observed up-regulations in AR-DNA binding activity at 3h post-exercise and greater β-catenin content suggest a preferential AR signaling response to high load resistance exercise. Mechanistically, our data further supports previous evidence of acute increases in AR signaling not being driven by changes in serum or muscle androgen concentrations nor AR protein content. Rather, AR co-activating proteins, such as β-catenin, may be responsible for mediating this response. However, a relatively small sample size and the specific participant demographic (resistance trained males) in our study is a clear limitation and must be considered when interpreting our findings. Based on these data, future research is needed to better understand the impacts of resistance exercise load on molecular and practical outcomes in skeletal muscle whilst controlling for other confounding variables such as volume and intensity. Furthermore, many questions still remain about a variety of factors driving the acute AR signaling response to resistance exercise. Better understanding molecular mechanisms in which AR co-activating proteins, such as β-catenin, interact with the AR may allow for more clarity and provide the

necessary context to better explain these occurrences. Therefore, future research should investigate this phenomenon and determine if manipulation of other program design variables (i.e. rest intervals, volume, intensity, time under tension, etc.) differentially impact the acute AR signaling response and by extension, hypertrophic outcomes in skeletal muscle.

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