ABSTRACT

Effects of Arachidonic Acid Supplementation on Training Adaptations in Resistance-Trained Males

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The purpose of this study was to determine if 50 days of resistance training and arachidonic acid (AA) supplementation elicited changes in body composition, performance variables, hormonal/cytokine levels (i.e. prostaglandins, free and total testosterone, cortisol, and interleukin-6) and/or intramuscular markers [i.e. myosin heavy chain (MHC) I, -IIa, -IIx mRNA and protein levels] in resistance-trained males. Thirty-one subjects (22.1±5.0 yrs, 86.1±13.0 kg, 178.9±3.4 cm, 18.1±6.4 % body fat) were randomly assigned to a placebo (P: n=16; 1 g corn oil/day) or AA group (AA: n=15; 1 g AA/day) and were given supplemental protein in order attain an optimal protein intake of 2 g/kg/day while participating in a 2 d/wk upper body and 2 d/wk lower body resistance training regimen. Body composition, bench press one-repetition maximum (1-RM), leg press 1-RM and Wingate bike sprint tests were completed at 0, 25 and 50 days. Fasting blood was taken on days 0, 25, and 50 and muscle biopsies were taken from the vastus lateralis on days 0 and 50. Body composition, performance variables, hormonal levels and MHC mRNA and protein concentrations were analyzed by ANOVA with repeated measures while independent t-tests were used to assess changes in MHC mRNA

expression. A significant increase was observed in Wingate relative peak power in the AA group ($p=0.015$). Statistical trends were found for PGE_2 increases ($p=0.06$) and IL-6 decrements ($p=0.07$) in the AA group and a significant decrement in percent changes in MHC IIx mRNA expression was present in the AA group ($p=0.015$). Results suggest that AA supplementation in trained males may exert favorable alterations in training adaptations and fasting prostaglandin and IL-6 levels. However, additional research is needed to examine this hypothesis.

Effects of Arachidonic Acid Supplementation on Training Adaptations in Resistance-trained Males

by

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A Thesis

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CHAPTER ONE

Introduction and Rationale

Background

Arachidonic acid (AA) is a polyunsaturated essential fatty acid (PUFA) which is primarily found in fatty parts of red meat and fish and is normally consumed in small amounts in the regular diet (Li, Ng, Mann, & Sinclair, 1998). Much like omega-3 and omega-6 fatty acids, research has indicated a number of potential health benefits from AA supplementation (Driss, Vericel, Lagarde, Dechavanne, & Darcet, 1984; James, Gibson, & Cleland, 2000; Kelley, Taylor, Nelson, & Mackey, 1998; Nelson et al., 1997; Safayhi, Koopmann, & Ammon, 1993; Uauy, Hoffman, Peirano, Birch, & Birch, 2001). Of particular interest in this study is the purported role that AA appears to have on prostaglandin (PG) synthesis and regulation of protein synthesis (Kelley, 2001; Rodemann & Goldberg, 1982; Rodemann, Waxman, & Goldberg, 1982; Vandenburgh, Shansky, Karlisch, & Solerssi, 1993). Research has indicated that AA stimulates PG synthesis (Goldberg, Baracos, Rodemann, Waxman, & Dinarello, 1984; Rodemann & Goldberg, 1982; Rodemann et al., 1982), may have pro-inflammatory responses similar to that produced from exercise, and/or may inhibit immune response to stress (Calder, 1999; Kelley, 2001; Pompeia et al., 2000; Thies et al., 2001). Consequently, AA supplementation has been theorized to stimulate muscle growth during resistance training. Although a number of studies have evaluated the long-term effects of

polyunsaturated essential fatty acids [fish oil, Omega-3, Omega-6 fatty acids, conjugated linoleic acid (CLA)] and AA on a variety of health outcomes (Endres et al., 1989; Healy, Wallace, Miles, Calder, & Newsholm, 2000; Hoffman et al., 2000; Iijima et al., 2000; Kelley, 2001; SanGiovanni & Chew, 2005; Thies et al., 2001; Thies et al., 2001, 2001; Wong, 2005), the influence of AA supplementation on training adaptations has yet to be investigated.

Problem Statement

Does 50 days of AA supplementation affect body composition, training adaptations, hormonal adaptations, and molecular adaptations in experienced resistance-trained males?

Purpose

To examine the effects that AA supplementation (1 g/day for 50 days) has on body composition, training adaptations (e.g. muscular strength and anaerobic power), hormonal/cytokine adaptations (total testosterone, free testosterone, cortisol, IL-6, PGE_2, $PGF_{2\alpha}$), and molecular adaptations [i.e. complimentary PGE_2 (EP_3) & and $PGF_{2\alpha}$ (FP) receptors, MHC mRNA, and protein expression in skeletal muscle] in experienced resistance-trained males partaking in a periodized, full-body workout regimen (4-d/wk).

Hypotheses

H_1: There will be no difference between groups and changes in body composition.

H_2: There will be no difference between groups and muscular strength and/or anaerobic power.

H_3: There will be no difference between groups in whole blood values

H_4: There will be no difference between groups in PGE_2, and $PGF_{2\alpha}$ levels

H_5: There will be no difference between groups in other hormonal adaptations (i.e. total testosterone, free testosterone, cortisol, and IL-6).

H_6: There will be no difference between groups and molecular adaptations (e.g. EP_3 & FP receptors, MHC protein and mRNA and myofibrillar protein content in skeletal muscle).

Delimitations

The study was conducted within the following parameters:

1. Approximately thirty (30) healthy, resistance-trained males (ages 18-50, over 1 yr full-body training ≥ 2 days/wk) will participate in this study.
2. Subjects were recruited from the general public by radio and newspaper ads.
3. All familiarization and testing sessions were conducted in the Exercise & Sport Nutrition Laboratory (ESNL) in the Department of Health, Human Performance & Recreation at Baylor University.
4. A battery of tests were conducted pre-supplement intervention and throughout the course of study to determine the changes in metabolic variables being examined.
5. Each participant was randomly assigned to one of two supplement groups.

Limitations

1. Participants were responsible for consuming the supplement over the 50-day intervention.
2. Participants were responsible for complying with the periodized workout regimen over the 50-day intervention.
3. Participants were responsible for consuming an extra 500 kcals/day consisting of 2 grams/kg/day of protein.
4. Participants were expected to refrain from the regular consumption of foods known to be high in omega-3 fatty acids (fish oil, flax seed oil, cold water fish [e.g., salmon, cod], olive oil, sesame oil, peanut butter, N-acetyl-cysteine, conjugated linoleic acid, as well as anti-inflammatory medications (e.g.,

acetaminophen, ibuprofen, aspirin, and other non-steroidal anti-inflammatory drugs).

Assumptions

1. Subjects fasted for 12-hrs prior to each testing session.
2. Subjects maintained consistent levels of physical activity outside of the prescribed workout regimen.
3. Subjects abided by the supplement and dietary protocols over the course of the study.
4. Subjects reported adverse events to lab staff.

Definition of Terms

1. Activator Protein-1 (AP-1) – is a heterodimer transcription factor that binds to the enhancer regions on DNA in order to initiate gene transcription
2. Autocrine – the secretion of a hormone or growth factor that signals the secretory cell itself
3. Blood Urea Nitrogen (BUN) – a waste product present in the blood from the breakdown of protein; the kidneys filter blood and remove urea
4. Creatinine – a waste product from protein in the diet and from the muscles of the body that is removed from the body by the kidneys
5. Cyclooxygenase (COX-1, -2) – an intracellular endoperoxidase enzyme that catalyzes the production of PG intermediates from arachidonic acid precursors
6. Calcineurin – an intracellular enzyme that is activated by calcium and dephosphorylates downstream transcription factors to affect gene transcription patterns
7. Differentiation – a metabolic process during which unspecialized cells morphologically mature into tissue-specific cells
8. Eicosanoids – are lipid hormones including PGs, thromboxanes, and leukotrienes that contain 20 carbon atoms
9. Extracellular regulated kinase 1/2 (ERK1/2) – Ser/Thr MAPK proteins that are purported to affect gene expression patterns in response to resistance exercise

10. Fat Mass – the weight of the human body consisting of fat tissue

11. Fusion – is the formation of one hybrid cell from two separate cells; in the case of skeletal muscle it is the accretion of myoblasts onto muscle fibers

12. Focal adhesion kinase (FAK) – is a protein tyrosine kinase that is recruited to focal adhesions; these proteins mediate intracellular cascades which occur due to disturbances in the extracellular matrix

13. G-protein coupled receptors (GPCRs) – transmembrane receptors present on the extracellular membrane surface of cells that initiate hormonal signal transduction messages through coupled intracellular G-proteins

14. c-Jun N-terminal kinase (JNK) – is a stress activated protein kinase that has been found to be up-regulated an affects gene expression patterns in skeletal muscle following resistance training

15. Lean Body Mass (LBM) – also known as fat-free mass; the weight of the human body minus the fat content

16. Myofibrillar protein – is the protein content inside muscle fiber that is involved in mechanical contraction and/or the anchoring of contractile elements within the sarcoplasm

17. Myosin Heavy Chain (MHC) – constitutes a portion of the thick filament contractile protein that contains globular actin binding sites for mechanical contraction

18. Nuclear factors of Activated T-cells (NFAT) - is a family of calcium sensitive transcription factors activated by calcineurin; upon activation NFATs undergo nuclear translocation and bind to enhancer elements in order to initiate transcription

19. Paracrine – is the secretion of a hormone or growth factor that signals the cells adjacent to the secretory cell within the same tissue

20. Phospholipases – are enzymes that hydrolyze fatty acids, releasing them into the cytosol from membrane-bound phospholipids

21. Phospholipids – are membrane-bound compounds that are made up of a phosphate-linked hydrophilic molecule linked to a diacylglycerol (glycerol + 2 fatty acid molecules) compound

22. Polyunsaturated Fatty Acids (PUFAs) – are fatty acid molecules consisting of multiple double bonds

23. Proliferation – is the multiplication of unspecialized (precursor) cells

24. Prostaglandins (PGs) – are a subtype of eicosanoids secreted by most tissues involved in the intracellular signaling of inflammation

25. Protein kinases – intracellular signal transduction proteins that generally phophorylate Serine, threonine or tyrosine residues of downstream target proteins to induce alterations in the bioenergetics, ion homeostasis, gene expression and/or translational mechanisms within the cell

26. Transcription factors – proteins that participate in protein-nucleic acid interactions to induce changes in gene expression patterns

CHAPTER TWO

Literature Review

Introduction to Arachidonic Acid and Prostaglandin Formation

Polyunsaturated fatty acids are macronutrients found in various vegetable and animal foodstuffs that are composed of a multiple double bonded hydrocarbon chain with a delta (Δ) carboxyl group and omega (ω) methyl terminus. Specifically, a high content of ω-6 polyunsaturated fatty acids are found in vegetable oils and fatty portions of red meat (Li et al., 1998). In general, ω-6 polyunsaturated fatty acids are deemed essential fatty acids because human tissues do not have the enzymatic capabilities of synthesizing double bond carbons in these respective positions. Amongst many metabolic fates, ingested dietary fatty acids can undergo re-esterification with glycerol-3-phosphate molecules leading to the subsequent formation of membrane phospholipids. The aforementioned process is integral to the cellular functionality of all cells due to the large presence of phospholipids in organelle and extracellular membranes. Furthermore, the fatty acid profile of membrane phospholipids has been shown to modulate various cellular processes ranging from cellular proliferation and growth (Doi, Doi, Schroeder, Alberts, & Vagelos, 1978) to regulating immune function (Calder, Bond, Harvey, Gordon, & Newsholme, 1990; Miles & Calder, 1998) and insulin sensitivity (Borkman et al., 1993; Vessby, Tengblad, & Lithell, 1994). The fatty acid content in membrane phospholipids is dependent upon an individual's dietary habits (Ayre & Hulbert, 1996; Zhou & Nilsson, 2001) and activity levels (Andersson, Sjodin, Hedman, Olsson, & Vessby, 2000; Helge et al., 2001).

Arachidonic acid (AA, 20:4, ω-6) is a polyunsaturated fatty acid that is abundant in membrane phospholipids. Common membrane phospholipids include phosphatidylcholine, phospatidylethanolamine, phosphatidylserine, and phosphatidylinositol. However, phosphatidylcholine and phosphatidylethanolamine are the two most abundant phospholipids contained within eukaryotic cell membranes, comprising 50% and 25% of phospholipid mass, respectively (Henneberry, Wright, & McMaster, 2002). Interestingly, both phospholipids are involved in various cell signaling cascades due to their large makeup of AA which can be hydrolyzed and formed into a biologically active metabolites including leukotrienes, thromboxanes, and prostaglandins (Henneberry et al., 2002). Interestingly, both AA (Hii, Moghadammi, Dunbar, & Ferrante, 2001; Neeli, Yellaturu, & Rao, 2003; Verlengia et al., 2003) and its downstream metabolites (Adams, Sah, Henderson, & Brown, 1998; Bos, Richel, Ritsema, Peppelenbosch, & Versteeg, 2004; Guan et al., 2002; Horsley & Pavlath, 2003; Kunapuli, Lawson, Rokach, Meinkoth, & FitzGerald, 1998; Ohmichi et al., 1997) are involved in intracellular signal transduction pathways and are coupled to transcriptional/translational mechanisms that contribute to tissue growth.

Prostaglandins (PGs) are classified as lipid hormones known as eicosanoids which act as autocrine/paracrine mediators formed within most tissues in the body (Funk, 2001). Specifically, PGs are synthesized from the ω-6 fatty acid AA. Again, AA is a polyunsaturated fatty acid that is liberated from membrane phospholipids by phospholipases in response to cytokine/growth factor signaling and/or mechanical trauma. The release of PGs from cells is facilitated by prostaglandin transporters and subsequent PG cell-signaling cascades are initiated by the binding of PGs to various G-

protein-coupled prostaglandin receptors (GPCRs) (Narumiya & FitzGerald, 2001). PG formation and release from skeletal muscle is known to be caused by the mechanical loading incurred during resistance training (Vandenburgh, Shansky, Solerssi, & Chromiak, 1995). It has been well established that PGs are involved in specific cell-signaling processes including the initiation of inflammation as well as protein degradation within skeletal muscle (Rodemann *et al.*, 1982). Cytosolic phospholipase A_2 ($cPLA_2$) catalyzes AA release into the cytosol where cyclooxygenase-2 (COX-2) catalyzes the formation of prostaglandin intermediates. These intermediates then undergo a tissue-specific conversion to specific prostaglandin isoforms (PGE_2, $PGF_{2\alpha}$, PGI_2, and PGD_2) via PG synthases (Funk, 2001). Studies examining the inhibition of the COX-2 pathway have found that COX-2 is essential in protein degradation within skeletal muscle leading to myofibrillar regeneration (Bondesen, Mills, Kegley, & Pavlath, 2004; Goldberg et al., 1984). One investigation (Trappe *et al.*, 2002) studied the effects of whole-body protein synthesis in twenty-four males receiving either the maximal over-the-counter doses of COX-2-specific NSAIDS including ibuprofen (1,200 mg/day), acetaminophen (4,000 mg/day), or a placebo after completing 10-14 sets of 10 eccentric leg extensions at 120% of concentric one-repetition maximum. Post-exercise (24 hrs) skeletal muscle fractional synthesis rates increased 76 ± 19% ($P < 0.05$) in the placebo groups whereas protein synthetic rates in the two groups receiving ibuprofen or acetaminophen remained unaltered. The authors noted that neither drug had measurable influences on whole body protein breakdown. The COX-2 pathway and PG cell-signaling have also been implicated in myogenesis via satellite cell proliferation

(McArdle, Edwards, & Jackson, 1994; McLennan, 1991), differentiation (Zalin, 1987) and fusion (David & Higginbotham, 1981; Entwistle, Curtis, & Zalin, 1986).

Mitogenic Signaling Pathways Initiated by Prostaglandins

The formation of prostaglandins within skeletal muscle is known to occur via mechanical stretch and/or loading (Palmer, Reeds, Atkinson, & Smith, 1983; Vandenburgh et al., 1995). For instance, interstitial levels of the prostaglandins appear almost immediately in response to dynamic exercise training (Karamouzis *et al.*, 2001). $PGF_{2\alpha}$ is a prostaglandin isomer that mediates interleukin synthesis, uterine contraction, luteolysis, and cellular hypertrophy in various tissues (Bos et al., 2004). In skeletal muscle, increases of $PGF_{2\alpha}$ levels have been shown to increase protein synthesis by nearly 50% (Rodemann & Goldberg, 1982). One study (Palmer et al., 1983) demonstrated that rabbit skeletal muscle incubated under constant tension with intermittent stretching increased protein synthetic rates by 70% in concert with a 105% elevation in $PGF_{2\alpha}$ secretion. Interestingly, the addition of COX-2-specific NSAIDs or the omission of Ca^{2+} in the incubation medium completely ceased the production of $PGF_{2\alpha}$ as well as the associated increases in protein synthesis. Another investigation (Trappe, Fluckey, White, Lambert, & Evans, 2001) studied the effects of whole-body protein synthesis in twenty-four males receiving either the maximal over-the-counter doses of COX-2-specific NSAIDS including ibuprofen (1,200 mg/day), acetaminophen (4,000 mg/day), or a placebo after completing 10-14 sets of 10 eccentric leg extensions at 120% of concentric one-repetition maximum and found that there was a 24-hr post-exercise elevation in intramuscular $PGF_{2\alpha}$ levels in the placebo group in comparison to the ibuprofen and acetaminophen groups. There was also an exercise-induced change in

PGE_2 levels in the placebo group (64%) that was significantly different from that in the acetaminophen group (-16%) and exercise-induced changes in PGE_2 and $PGF_{2\alpha}$ were not different between the ibuprofen and acetaminophen groups. These authors concluded that these two NSAIDs have similar effects in diminishing $PGF_{2\alpha}$ levels in human skeletal muscle after resistance training, which seems to be deleterious to protein synthesis. A related investigation observed that during periods of coronary ischemia ventricular myocytes in neonatal rats have been found to secrete $PGF_{2\alpha}$ which act in an paracrine manner to induce compensatory hypertrophy of the myocardial cells (Adams et al., 1998). Hence, there scientific evidence that $PGF_{2\alpha}$ is produced as a result of mechanical stimulation and/or alterations in the intracellular environment and that $PGF_{2\alpha}$ acts in an autocrine/paracrine manner to induce cell growth in different muscle tissues.

The distinct intracellular signal transduction cascades that are proposed to result from $PGF_{2\alpha}$ binding to its complimentary G-protein coupled receptor (FP receptor) involve the G_q-mediated activation of phospholipase C followed by production of inositol triphosphate ($Ins(1,4,5)P_3$) and phosphorylation of protein kinase C. These events lead to the subsequent activation MAP kinase pathways including the Ras-Raf-MEK-extracellular regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK) pathways (Adams et al., 1998). Although one study found a 4-fold increase in ERK activation via FP receptor activation, employing an ERK activator inhibitor did not disrupt the hypertrophic responses. Interestingly, $PGF_{2\alpha}$-treated bovine luteal cells demonstrated an increase in the protein kinase C-dependent ERK pathway which up-regulated the expression of c-*fos* and c-*jun* mRNA. It should be noted that these two genes have been previously identified as early response genes which are induced during skeletal muscle

hypertrophy (Aronson, Dufresne, & Goodyear, 1997). More recent data has implicated AA as being a stimulator of vascular smooth muscle cell growth through an increase in phosphoinositol-3 kinase (PI-3K) activation and subsequent phosphorylation of protein kinase B, ribosomal protein S6 kinase 1, ribosomal protein S6, and eukaryotic initiation factor-4E due to its metabolic conversion through the COX and LOX/MOX pathways (Neeli et al., 2003); all of these aforementioned kinases being integral to the initiation of mRNA translation in skeletal muscle. These authors also reported an AA-induced global protein synthesis in these cells and this action was rate-limited by the conversion of AA into its downstream metabolites. A similar study (Rao *et al.*, 1999) found that FP receptor signaling induces a phosphoinositol 3 kinase-dependent and independent activation of the ERK pathway and a subsequent phosphorylation of eukaryotic initiation factor-4E and 4E-binding protein which greatly attribute to global protein synthesis in vascular smooth muscle cells. Activation of phosphoinositol 3 kinase in a other cell types has also been shown to be stimulated by AA (Hii et al., 2001) and is thought to be caused by the conversion of AA to $PGF_{2\alpha}$ and the associated signaling of the FP receptor.

As mentioned previously, $PGF_{2\alpha}$ has been found to activate the phospholipase C, stimulating a significant formation of $Ins(1,4,5)P_3$ in murine ventricular myocytes via the FP receptor (Kunapuli et al., 1998; Ohmichi et al., 1997). Likewise, more recent data demonstrates that FP receptors cloned from the cDNA libraries of humans and mammals elicit a high turnover in $Ins(1,4,5)P_3$ seen in the detection of radiolabelled inositol phosphates and the notable increases of intracellular calcium (Yousufzai, Gao, & Abdel-Latif, 2000). The nuclear factors of activated T cells (NFATs) are a family of ubiquitous calcium sensitive transcription factors activated by calcineurin, a calcium-activated

phosphatase enzyme, that dephosphorylates cytosolic NFAT proteins (Horsley *et al.*, 2001). Upon activation, NFAT transcription factors undergo nuclear translocation and coalesce with activator protein-1 transcription factors (c-Fos-c-Fos homodimers or c-Fos-c-Jun heterodimers) to bind to a purine-rich enhancer elements on specific genes in order to initiate transcription (Abbott, Friday, Thaloor, Murphy, & Pavlath, 1998). Much of the research examining NFAT signaling has examined its role in cardiomyocyte hypertrophy. One study (Wilkins *et al.*, 2002) revealed that NFATc3-null mice showed a significant impairment in calcineurin-induced cardiac hypertrophy up to 10 weeks of age. Moreover, these mice also demonstrated a diminished pressure overload as well as a reduction in angiotensin II-mediated cardiac hypertrophy (Wilkins *et al.*, 2002). Synergistic interactions also exist between calcineurin-NFAT and MEK1-ERK1/2 MAP kinase signal transduction pathways in mediating myocardial cell hypertrophy (Sanna, Bueno, Dai, Wilkins, & Molkentin, 2005). Furthermore, numerous related investigations have also concluded that multiple NFAT isoforms are expressed in cardiomyocytes where they function as necessary transducers of calcineurin in facilitating cardiomyocyte hypertrophy (Sanna et al., 2005; van Rooij *et al.*, 2002)

Evidence suggests that NFAT signaling assumes a role in the expression of genes responsible for fiber-type transition during mechanical loading in skeletal muscle and conflicting data exists demonstrating its importance in skeletal muscle hypertrophy. Unspecified NFAT-dependent pathways and/or activated calcineurin over-expression has been shown to preferentially activate the MHC-IIa promoter, suggesting a distinct mechanism where NFAT elicits the transcription of hypertrophic genes (Allen & Leinwand, 2002; Allen, Sartorius, Sycuro, & Leinwand, 2001). Dunn and colleagues

(Dunn, Burns, & Michel, 1999) have found that the administration of cyclosporine A, which is a calcineurin inhibitor, prevents compensatory skeletal muscle hypertrophy induced by mechanical overload in mice during a 4-week period. Dunn also reported that CsA treatment inhibited the rapid doubling of muscle mass and individual fiber size as well as the expected 4- to 20-fold increases in oxidative muscle fiber number seen in the compensatory hypertrophy of the plantaris muscle subsequent to the removal of the soleus and gastrocnemius muscles. The phenomenon of fiber type transition via NFAT activation within skeletal muscle was further studied by Parsons and colleagues (Parsons *et al.*, 2004) who investigated the ability of calcineurin-deficient mice to undergo skeletal muscle hypertrophy following mechanical overload or insulin-like growth factor-1 (IGF-1) stimulation. These authors found that a fast to slow fiber-type conversion in overloaded skeletal muscle was dramatically reduced with impaired calcineurin activity, but concluded that calcineurin is integral during the fiber-type transition seen with resistance training but not skeletal muscle hypertrophy.

A landmark study (Horsley & Pavlath, 2003) deemed $PGF_{2\alpha}$ as being an important regulator of NFAT activation by demonstrating its role in recruiting the fusion of multinucleated cells to myotubes through the NFATc2 pathway. As mentioned previously, the translocation of NFATc2 proteins can be likely attributed to the production of $Ins(1,4,5)P_3$ resulting from $PGF_{2\alpha}$ binding to the FP receptor and the subsequent elevations of cytosolic calcium seen from $Ins(1,4,5)P_3$ stimulating the release of calcium from intracellular calcium stores. Although the investigators claimed that $PGF_{2\alpha}$ may mediate myonuclear accretion to pre-existing muscle fibers by affecting cell motility, alignment, adhesion, or membrane union, they did not elucidate the

hypertrophic genes regulated by the $PGF_{2\alpha}$-dependednt activation of NFATc2. Nonetheless, this finding does provide a model linking FP receptor activation to an increase in skeletal muscle fiber nuclei which is necessary for the upkeep of cellular processes during perpetuated muscle growth over chronic periods resistance training (Mozdziak, Schultz, & Cassens, 1997).

Dietary AA Supplementation in Humans

Past research has shown that short-term AA supplementation in young, healthy men elicits a significant increase in PG production (Kelley *et al.*, 1998). Although numerous studies have evaluated the long-term effects that PUFA and AA supplementation exert on numerous health variables, the influence that AA supplementation has on resistance-training adaptations has yet to be studied. Consequently, this study will investigate the effects of AA supplementation (1 gram/day for 50 days) on body composition, training adaptations (e.g. strength and muscular endurance), hormonal adaptations (PGE_2, $PGF_{2\alpha}$, IL-6, total testosterone, free testosterone, cortisol), and molecular adaptations (FP and EP_3 receptors, MHC and myofibrillar protein content in skeletal muscle) in experienced resistance-trained males. Furthermore, the data obtained from this study can be used to further elucidate the prostaglandin-dependent myogenic pathways that occur in resistance-trained individuals during 50 days of AA supplementation and concomitant resistance training.

CHAPTER THREE

Methods

Subjects

Approximately 30 healthy, resistance trained males (≥ 1 year; whole-body training ≥ 2 days/wk) between the ages 18 to 50 participated in this study. Subjects were not allowed to participate in this study if they have any metabolic disorder including known electrolyte abnormalities; heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism; a history of hypertension, hepatorenal, musculoskeletal, autoimmune, or neurologic disease; if they took thyroid, antihyperlipidemic, hypoglycemic, anti-hypertensive, or androgenic medications; and, if they had taken ergogenic levels of nutritional supplements that may affect muscle mass [e.g., creatine, hydroxy-beta-methylbutyrate (HMB)] or anabolic/catabolic hormone levels (androstenedione, dihydroepiandrosterone (DHEA), etc.] within three months prior to the start of the study. The only exception was if the prospective subject had a medical condition or history that the subject's personal physician felt is controlled and therefore would not be a limitation for them to participate in the study. In this case, the subject had to obtain a letter signed by his physician approving his participation in this study.

Study Site

All familiarization and testing assessments were conducted in the Exercise & Sport Nutrition Laboratory (ESNL) in the Department of Healthy, Human Performance, and Recreation at Baylor University.

Study Design

The study included baseline testing followed by 50 days of AA supplementation during a hypercaloric state while subjects participated in a prescribed resistance-training regimen. Participants reported to the ESNL for testing three times over the 50 day study. The independent variable was nutritional supplementation. Dependent variables included: estimated dietary energy intake; Quality of Life questionnaires, body weight, body water, body composition, resting hemodynamic measures (heart rate and blood pressure), upper and lower body isotonic muscle strength, anaerobic power, fasting clinical blood profiles (substrates, electrolytes, muscle and liver enzymes, red cells, white cells), hormonal profiles (total and free testosterone, cortisol, interleukin-6, PGE_2, $PGF_{2\alpha}$), and molecular attributes within skeletal muscle (i.e. EP_3 and FP receptors, myofibrillar protein content, MHC mRNA and protein content).

Entry and Medical Screening Session

Subjects were recruited by ads in Waco, Texas, and surrounding communities. The ads will briefly describe the study, outline qualifications, and instruct participants to call the ESNL. Subjects expressing interest in participating in this study were interviewed on the phone to determine whether they appear to qualify to participate in this study. Subjects believed to meet eligibility criteria were then invited to attend an entry/familiarization session. Any subject who did not meet entry criteria were required to obtain medical clearance from their personal physician prior to participating in baseline assessments.

Familiarization Session

Subjects eligible to participate in the study were familiarized to the study protocol via a verbal and written explanation outlining the study design. During this session, subjects signed an Informed Consent Statement and completed personal and medical histories. This session also included describing the dietary program and familiarizing the subjects to the tests to be performed. Subjects were then given an appointment time to perform baseline assessments.

Baseline Testing

Following the familiarization session, the subjects recorded dietary histories on dietary record forms four days prior to the first testing session. Subjects refrained from exercise for 48-hrs and fast for 12-hrs prior to baseline testing. Subjects then reported to the ESNL for clinical assessments. Once reporting to the lab, subjects completed questionnaires regarding quality of life (QOL) and health status, were weighed, had total body water determined by bioelectrical impedance (BIA), and had body composition determined using a Hologic Discovery W Dual Energy X-ray Absorptiometer (DEXA). Subjects then had blood pressure and resting heart rate determined using standard procedures. Subjects donated approximately 20 ml of fasting blood using venipuncture techniques of an antecubital vein in the forearm according to standard procedures. Blood samples were analyzed in the Exercise & Biochemical Nutrition lab for standard clinical chemistry profiles [glucose, total protein, blood urea nitrogen (BUN), creatinine, BUN/creatinine ratio, uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), lactate dehydrogenase (LDH), gamma-glutamyl transpeptidase (GGT), albumin, globulin, sodium, chloride, calcium, carbon

dioxide, total bilirubin, alkaline phosphatase, triglycerides, cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) and whole blood cell counts including hemoglobin, hematocrit, red blood cell counts, white blood cell counts, neutrophils, lymphocytes, monocytes, eosinophils, baosphils]. In addition, serum samples were assayed to quantify the influence that AA exerts on selected hormonal markers (e.g. cortisol, total and free testosterone, IL-6, PGE_2 and $PGF_{2\alpha}$). Muscle samples taken from the vastus lateralis were frozen at -70°C and were later assayed in the EBNL where myofibrillar protein content, MHC isoform mRNA, and total protein content was determined from the samples. Immunoblots were also performed to evaluate EP_3 and FP receptor content for PGE_2 and $PGF_{2\alpha}$, respectively. Subjects performed a one repetition maximum (1-RM) test on the isotonic bench press after performing two warm-up sets of 10 repetitions (approximately 50% 1-RM). The subject rested 2-min between sets until a 1-RM was reached. Subjects then rested 10-min before performing the same structured protocol for lower body strength assessment on the Nebula 45° leg/hip sled. Subjects rested 10-min after the completion of the isotonic 1-RM assessment on the leg/hip sled and performed a 30-sec Wingate anaerobic capacity test on a computerized Lode cycle ergometer to assess anaerobic capacity and power. This test consisted of having each participant sprint in an all out fashion on the bicycle ergometer for 30-sec against a standard workload of 0.075 kg/kg of body weight. Biopsies were taken 10-min after the Wingate test under the supervision of Dr. Darryn Willoughby who has widespread experience performing biopsy related research (Willoughby & Rosene, 2001; Willoughby & Rosene, 2003).

Randomization and Supplementation Protocol

Subjects were placed into one of two groups according to age, fat free mass and training history. Subjects were randomly assigned in two groups ingesting, in a double blind manner, capsules containing a corn oil placebo or X-Factor (*Molecular Nutrition, Jupiter FL*). Subjects were instructed to ingest one capsule (250 mg) four times per day for 50 days. The supplements were prepared in capsule form and packaged in generic bottles for double blind administration by Molecular Nutrition. Supplementation compliance was monitored by having the subjects return empty bottles of the supplement at the end of 25 and 50 days of supplementation. Subjects were instructed to eat a slightly hypercaloric diet relative to baseline dietary analysis (an extra 500 kcals/day) consisting of 2 grams/kg/day of protein which corresponds to the recommended amounts for resistance-trained individuals (Lemon, 2000). Subjects were provided a commercial protein powder (Lean Body, Labrada Nutrition, Houston, TX) containing approximately 45 grams of protein per serving in an attempt to accommodate the protein requirements for resistance training. Each subject was told to ingest at least one packet of protein powder immediately following each workout in order to optimize the nutrient timing known to exist with protein supplementation. Additionally, subjects were told to avoid regular consumption of foods known to be high in omega-3 fatty acids (fish oil, flaxseed oil, cold water fish [e.g., salmon, cod], olive oil, sesame oil, peanut butter, N-acetyl-cysteine, conjugated linoleic acid, as well as anti-inflammatory medications (e.g., acetaminophen, ibuprofen, aspirin and other non-steroidal anti-inflammatory drugs).

Resistance-training Protocol

Subjects performed a standardized series of stretching exercises before each workout and participated in a periodized 4-day per week resistance-training program divided into two upper and two lower body workouts for 50 days. The upper body resistance-training program contained eight exercises including bench press, lat pull, shoulder press, seated rows, shoulder shrugs, chest flies, biceps curls, and triceps press-downs. The lower body resistance training program contained seven exercises including leg press or squat, back extension, step ups, leg curls, leg extension, heel raises, and abdominal crunches performed twice per week. Subjects performed 3 sets of 10 repetitions with as much weight as they could lift per set (typically 60 – 80% of 1-RM). The rest periods between exercises lasted no longer than 3-min and the rest between sets lasted no longer than 2-min. Training was conducted at the Student Life Center (SLC) at Baylor University or at Gold's Gym (Golds Gym, Inc, Waco, TX), documented in training logs, and signed off by a designated staff member to verify compliance and monitor progress.

Medical Monitoring

Interested subjects attended a familiarization session where they completed consent forms and medical history information. A trained researcher evaluated the medical and training history questionnaires to determine whether the subject met entry criteria and may therefore participate in the study. Trained, non-physician exercise specialists certified in CPR supervised subjects that performed exercise assessments. A telephone and an automated electronic defibrillator were located in the laboratory in case of any emergencies and there were no less than two researchers working with each

subject during testing sessions. In the event of any unlikely emergency one researcher was instructed to check for vital signs and begin any necessary interventions while the other researcher would contact Baylor's campus police, which is standard university policy. Instructions for emergencies were posted above the phone in the ESNL in the event that any other research investigators were available for assistance. Subjects were informed to report any unexpected problems or adverse events that they encountered during the course of the study to Richard B. Kreider, PhD, EPC or Chris Rasmussen, MS, MX, EPC, CSCS. If clinically significant side effects were reported, the subjects were referred to discuss the problem with the research nurse (currently Melyn Galbreath, FNP) or Lori Greenwood, PhD, ATC who is an Associate Professor of Athletic Training at Baylor University. If deemed necessary, Dr. Greenwood referred the subject to Ron Wilson, MD for medical follow-up. Dr. Wilson is one of the Sports Medicine physicians for Baylor University and is an adjunct Professor in the Department of HHPR. He has agreed to provide medical support and consultation for this study and to our lab.

Analytical Methods

Dietary Inventories

Subjects recorded all food and fluid intake on dietary record forms for 4-days prior to baseline testing. The record included three week days and one weekend day of food consumption. Dietary intake was assessed using the Food Processor III Nutrition Software (ESHA Nutrition Research, Salem, OR).

Body Composition Assessments

Subjects participated in body composition tests in the ESNL. Prior to each assessment, height was measured using standard anthropometry and total body weight was measured using a calibrated electronic scale with a precision of +/-0.02 kg. Total body water was then estimated using a Xitron 4200 Bioelectrical Impedance Analyzer (*San Diego, CA*) which measures bio-resistance of water and body tissues based on a minute low energy, high frequency current (500 micro-amps at a frequency of 50 kHz) transmitted through the body. This analyzer is commercially available and has been used in the health care/fitness industry as a means to assess body composition and body water for over 20 years. The use of this device has been approved by the Food and Drug Administration (FDA) to assess total body water and the current to be used has been deemed safe (NIH, 1996). Body composition and water using this method is measured through four electrodes placed on the body. One electrode was specifically placed on the posterior surface of the right wrist, in between the radial and ulna styloid processes (wrist bones). Another electrode was placed on the posterior surface of the right hand at the distal base of the second metacarpal. The third and fourth electrodes were placed on the anterior surface at the base of the right foot and at the distal end of the first metatarsals. Subjects lay flat on a table in the supine position and electrodes were connected to the analyzer. After the subject was connected, age, gender, weight, height, and activity level were entered into the unit by the technician. After the unit measured resistance, which takes approximately 30-sec, the unit calculated total body water and body water percent.

Body composition/bone density was then determined using a calibrated Hologic 4500W dual-energy x-ray absorptiometry (DEXA) by qualified personnel with limited x-

ray technology training under the supervision of Richard B. Kreider, PhD, MX. The DEXA body composition test involved having the subject lie down on their back in a standardized position in a pair of shorts/t-shirt or a gown. A low dose of radiation then scanned their entire body for approximately 6-min. The DEXA segmented regions of the body (right arm, left arm, trunk, right leg, and left leg) into three compartments for determination of fat, soft tissue (muscle), and bone mass. Radiation exposure from DEXA for the whole body scan was approximately 1.5 mR per scan. This is similar to the amount of natural background radiation a person would receive in one month while living in Texas. The maximal permissible x-ray dose for non-occupational exposure is 500 mR per year. Total radiation dose was approximately 15 mR for the entire study.

Resting Heart Rate & Blood Pressure

Heart rate was determined by palpitation of the radial artery using standard procedures (ACSM, 2000). Blood pressure was assessed in the supine position after resting for 5-min using a mercurial sphygmomanometer using standard procedures (ACSM, 2000).

Isotonic Upper Body and Lower Body Strength Testing

Subjects then performed a 1-RM test on the isotonic bench press after performing two warm-up sets of 10 repetitions (approximately 50% 1-RM). Subjects rest 3-min between sets until a 1-RM was determined. Subjects rested 10-min performing the same structured protocol for lower body strength assessment on the Nebula 45° leg/hip sled. All strength testing was supervised by a Certified Strength and Conditioning Specialist (CSCS). Test to test reliability of performing these strength tests on resistance-trained

subjects in our laboratory have yielded low mean coefficients of variation and high reliability for the bench press (1.9%, intraclass $r = 0.94$) and leg press/hip sled (0.7%, intraclass $r = 0.91$).

Anaerobic Wingate Test

Subjects then rested 10-min after the completion of the isotonic 1-RM assessment on the leg/hip sled and performed a 30-sec Wingate anaerobic capacity test on a computerized Lode cycle ergometer to assess anaerobic capacity and power. This test consisted of having each participant warm up at 30 rpm for 120-sec followed by a maximal sprint in on a bicycle ergometer for 30-sec against a standard workload of 0.075 kg/kg of body weight. Correlation coefficients of test-retest reliability for absolute peak power and mean power has been found to be r = 0.692 and r = 0.950, respectively.

Blood and Biopsy Samples

Subjects fasted overnight for 12-hrs and then donated approximately 4 teaspoons of fasting venous blood (20 milliliters). Blood samples were obtained through standard phlebotomy procedures using standard sterile venipuncture of an antecubital vein by a laboratory technician trained in phlebotomy in compliance with guidelines established by the Texas Department of Health and Human Services. The phlebotomists and lab technicians wore personal protective clothing (gloves, lab coats, etc.) when handling blood samples. Subjects were seated in a phlebotomy chair during the blood draw. Their arm was cleaned with a sterile alcohol wipe and sterile gauze. A standard rubber tourniquet was then placed on the brachium. The antecubital vein of either arm was palpated and then a 23 gauge sterile needle attached to a plastic vacutainer holder was

inserted into the vein using standard procedures. Three serum separation vacutainer tubes (red tops) and one EDTA vacutainer tubes (purple top) were inserted into the vacutainer holder for blood collection in succession using multiple sample phlebotomy techniques. Once samples were obtained, the vacutainer holder and needle was removed. The needle was discarded as hazardous waste in a plastic sharps container. The site of the blood draw was then be cleaned with a sterile alcohol wipe and gauze and a sterile Band-Aid was placed on the site. The blood collection tubes was labeled and placed in a test tube rack. Laboratory technicians (who have received blood borne pathogen training and were wearing personal protective clothing) centrifuged the serum samples for 15-min, transferred serum into labeled serum storage containers, and prepared samples for storage a -20° Celsius freezer for subsequent analysis. Serum and whole blood samples were analyzed in the EBNL at Baylor University. Samples were assayed for standard clinical chemistry profiles (glucose, total protein, blood urea nitrogen, creatinine, BUN/creatinine ratio, uric acid, AST, ALT, CK, LDH, GGT, albumin, globulin, sodium, chloride, calcium, carbon dioxide, total bilirubin, alkaline phosphatase, triglycerides, cholesterol, HDL, LDL) and whole blood cell counts (including hemoglobin, hematocrit, red blood cell counts, MCV, MCH, MCHC, RDW, white blood cell counts, neutrophils, lymphocytes, monocytes, eosinophils, baosphils) in order to evaluate markers of catabolism and clinical safety of the supplementation protocols. The blood samples were also assayed to quantify the influence that AA exerts on anabolic/catabolic hormones (e.g. cortisol, total and free testosterone, IL-6, PGE_2 and $PGF_{2\alpha}$). Muscle samples taken from the vastus lateralis were frozen at -80°C until assayed in the EBNL where

myofibrillar protein content, MHC isoform mRNA and protein content, and content for EP_3 and FP receptors were determined.

Serum Hormone Detection

Serum $PGF_{2\alpha}$, PGE_2, and IL-6 were determined with an immunoabsorbent assay (EIA) (Cayman Chemical, Ann Arbor, MI). Serum cortisol (CORT), free testosterone (fTEST), and total testosterone (tTEST) were determined with an enzyme immunoabsorbent assay (EIA) (Cayman Chemical, AnnArbor, MI; Diagnostic Systems Laboratories, Webster, TX). $PGF_{2\alpha}$, PGE_2, IL-6,CORT, fTEST, and tTEST were expressed relative to changes in plasma volume (Dill & Costill, 1974). EIA kits use the competitive binding biotin immunoassay format. In the assay, standards, controls and unknowns containing CORT/ fTEST /tTEST were incubated with biotin-labeled CORT/ fTEST/ tTEST and rabbit anti-CORT/ anti-fTEST/ anti-tTEST antiserum in microtitration wells coated with goat anti-rabbit gamma globulin where the unlabeled and biotin-labeled antigens compete for a limited number of anti-CORT/ anti-fTEST/ anti-tTEST binding sites. After incubation and washing, the wells were incubated with streptavidin-HRPO, which binds to the biotinylated anti-CORT/ anti-fTEST/ anti-tTEST. The unbound streptavidin-HRPO was washed, followed by incubation with the substrate tetramethylbenzidine (TMB). An acidic stopping solution (0.5 N HCL) was then added and the degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance at 405 or 450 nm.

Percutaneous Muscle Biopsy Extraction

Percutaneous muscle biopsy procedures using suction have proven to be clinically safe and very reliable in providing a specimen sample for histochemical and metabolite analysis when multiple samples are taken from the same sample foci on the muscle (Blomstrand & Ekblom, 1982; Greig, Askanazi, & Kinney, 1985). The specimens were taken under the supervision of Dr. Darryn Willoughby who has widespread experience performing biopsy related research (Willoughby & Rosene, 2001; Willoughby & Rosene, 2003).

FP and EP_3 Receptor Quantitation

FP and EP_3 receptor quantitation was performed by extracting total muscle protein from the homogenate and slot-blotting 50 µg of total protein onto nitrocellulose membranes using a Bio-Dot (Bio-Rad, Hercules, CA). The blotted membranes were incubated with blocking solution for 1-hr on an orbital rocker at room temperature, the blocking solution was then decanted and the membranes were incubated with a TTBS wash solution for 5-min for a total of three washes. The membranes were then incubated with specific anti-FP receptor and anti-EP_3 receptor polyclonal antibodies (Cayman Chemical, Ann Arbor, MI), diluted to 4 µg/ml, for 1-2-hrs on an orbital rocker at room temperature. The primary antibody solutions were then decanted and the membranes washed with TTBS solution for 5-min on an orbital rocker at room temperature for a total of three washes. The TTBS wash solution was then decanted and the membranes were incubated with a secondary biotinylated goat anti-rabbit antibody solution (Bio-Rad, Hercules, CA) for 1-hr on an orbital rocker at room temperature. The secondary biotinylated goat anti-rabbit antibody solution was then decanted and the membranes

incubated in TTBS wash solution for a total of three washes at 5-min per wash. The membranes were then incubated with a streptavidin-biotinylated alkaline phosphatase complex solution (Bio-Rad, Hercules, CA) for 1-hr on an orbital rocker at room temperature. The streptavidin-biotinylated alkaline phosphatase complex solution was then decanted and the membranes washed three times with TTBS solution at 5-min per wash on an orbital shaker at room temperature. Color development solution containing BCIP/NBT (Bio-Rad, Hercules, CA) was added and color development was monitored over a 30-60-min period. The color development was stopped by incubating the membrane in ddH_2O for 10-min on an orbital rocker at room temperature. Blotted membranes were digitized by way of densitometry using a Chemi-Doc XRS imaging system (Bio-Rad, Hercules, CA) and band density was expressed in integrated density units relative to muscle weight.

Total RNA Isolation

Total cellular RNA was extracted from the homogenate of biopsy samples with a monophasic solution of phenol and guanidine isothiocyanate (Willoughby et al., 2000, 2002, 2003, 2004) contained within the TRI-reagent (Sigma Chemical Co., St. Louis, MO). RNA concentrations from each sample were determined spectrophotometrically with an optical density (OD) of 280 nm, and the final concentration adjusted to 1 mg/ml by diluting the crude total RNA extracts into nuclease-free H_2O (Willoughby et al., 2000, 2002, 2003, 2004). Previous work performed in our laboratory has demonstrated (Willoughby et al., 2000, 2002, 2003, 2004) that this procedure yields un-degraded RNA, free of DNA and proteins as indicated by prominent 28s and 18s ribosomal RNA bands,

as well as an OD_{260}/OD_{280} ratio of approximately 2.0 (data not shown). The RNA samples were stored at -80°C until later analyses.

Reverse Transcription and cDNA Synthesis

The standardized solutions (1 μg/ml) of total cellular RNA were reverse transcribed to synthesize cDNA (Willoughby et al., 2000, 2002, 2003, 2004). A reverse transcription (RT) reaction mixture [1 μL of total cellular RNA, 4 μL 5x reverse transcription buffer, a dNTP mixture containing dATP, dCTP, dGTP, and dTTP, $MgCl_2$, RNase inhibitor, an oligo$(dT)_{15}$ primer, 10 μL of nuclease-free H_2O and 1u/μL MMLV reverse transcriptase enzyme (Bio-Rad, Hercules, CA)] were incubated at 42°C for 40 min, heated to 85°C for 5 min, and then quick-chilled on ice. Starting cDNA template concentrations were standardized by adjusting the RT reactions for all samples to 200 ng prior to real time RT-PCR amplification by detecting crude cDNA synthesized products spectrophotometrically at a wavelength of 260 nm and diluting them in nuclease-free H_2O (Willoughby et al., 2000, 2002, 2003, 2004). The standardized cDNA solutions were frozen at -80°C until real-time RT-PCR was performed.

Oligonucleotide Primers for MHC mRNA

Antisense and sense oligonucleotide primer pairs were constructed using commercially available Primer Express software (Bio-Rad, Hercules, CA) from known mRNA sequences published in the GenBank nucleotide database (www.ncbi.nlm.nih.gov) and commercially synthesized (Integrated DNA Technologies, Coralville, IA). The following 5′ sense and 3′ antisense oligonucleotide primers were used to isolate the three adult MHC isoforms (Type I, IIa, and IIx): Type I MHC mRNA

(5′ primer: bases 776-796, 3′ primer: bases 1398-1378, GenEMBL AC X06976), Type IIa MHC mRNA (5′ primer: bases 1785-1805, 3′ primer: bases 2440-2420, GenEMBL AC AF111784), Type IIx MHC mRNA (5′ primer: bases 1138-1158, 3′ primer: bases 1746-1726, GenEMBL AC AF111785). These primers were shown to amplify fragments of 141, 145, and 148 bp, respectively, for Type I, IIa, IIx MHC. 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 real-time PCR (Mahoney et al., 2004), β-actin was used as an external reference standard for detecting the relative change in the quantity of target mRNA using PCR. For β-actin, these primers were shown to amplify a PCR fragment of 135 bp.

Real-Time PCR Amplification of MHC mRNA

Two hundred ng of cDNA was added to each of the four PCR reactions for MHC Type I, -IIa, and -IIx, and an internal control using a constitutively expressed housekeeping gene (β-actin). Specifically, each PCR reaction contained the following mixtures: 12.5 μL of 2X SYBR Green Supermix (Bio-Rad, Hercules, CA) [100 mM KCl mixture, 40 mM Tris-HCl, 0.4 mM of each dNTP, 50 units/ml of iTaq DNA polymerase, 6.0 mM $MgCl_2$, SYBR Green I, 20 nM flourescein], 1.5 μL of sense and anti-sense primers and 7.5 μL nuclease-free dH_2O]. Each PCR reaction was amplified with a thermal cycler (Bio Rad, Hercules, CA) and the amplification sequence involved a denaturation step at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 60 s (Willoughby & Nelson, 2002; Willoughby & Pelsue, 2000; Willoughby & Rosene, 2001). Real-time PCR was performed over 40 cycles and fluorescence emitted from the SYBR green fluorophore was measured after each cycle. An emission of

fluorescence occurs due to the integration of the SYBR green into the double-stranded cDNA produced during the PCR reaction.

Quantitation of MHC I, IIa, and IIx mRNA

The C_T values were assessed in the linear portion of amplification and a DNA melting curve analysis was performed after amplification to assure that the single gene products were amplified in absence of primer dimers. The quantity of mRNA for each gene product was expressed relative to the constitutive expression of β-actin. A comparison of C_T value ratios [Day 1 (MHC mRNA C_T / β-actin mRNA C_T) versus Day 50 (MHC mRNA C_T / β-actin mRNA C_T)] were used to compare basal gene expression between the AA group and placebo group.

Agarose Gel Electrophoresis and MHC mRNA Quantitation

Agarose gel electrophoresis using 25 μL aliquots of the finalized PCR reaction mixtures were performed in 1.5% agarose gels using 1X Tris-Boric acid-EDTA (TBE) buffer (Willoughby & Nelson, 2002; Willoughby & Pelsue, 2000) to verify positive amplification of target mRNA. The oligonucleotides within the gels were stained with ethidium bromide (present in the TAE buffer at 1 μg/mL) and illuminated with a UV transilluminator.

Myofibrillar Protein Quantitation

Total protein remaining from the total RNA isolation procedure was isolated with isopropanol, ethanol, and 0.3 M guanidine hydrochloride. Myofibrillar protein was isolated with 0.1% SDS (Wing & Banville, 1994). Based on a previous study myofibrillar protein content was determined spectrophotometrically (Bradford, 1976) at a

wavelength of 595 nm using bovine serum albumin as the standard and quantified relative to muscle wet weight.

MHC Protein Isoform Quantitation

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is an analytical technique that has been used extensively in research to separate and analyze proteins from a specimen that differ in molecular weight and/or structure. The composition of MHC protein isoforms within each muscle homogenate sample was determined by automated SDS-PAGE using Experion™ Pro260 chips (Bio-Rad, Hercules, CA). Each sample well on the microchip contained 6 μL of unknown that was prepared from 4 μL of the protein dilution from each subject or the molecular weight ladder, 2 μL of sample buffer with β-mercaptoethanol, and 84 μL of de-ionized water. The gels were digitally visualized by the Experion™ software and MHC concentrations in each sample were determined from relative protein dilutions at 50 μg total protein/30 μl. Based upon the novel findings from Gazith and colleagues (Gazith, Himmelfarb, & Harrington, 1970), it was expected that the MHC isoforms migrate in the 200 kD region within the PAGE gel relative to the molecular weight ladder. Scion Imaging software was used (Scion Corporation, Frederick, MD) to extrapolate MHC concentrations by comparing the band density of each sample to the band density from the known molecular weight ladder protein concentrations and these concentrations were expressed in ng/ml.

Statistical Analysis

A two-way (2 x 3) ANOVA with repeated measures were used to analyze all data over progressing time points. If a significant group, treatment and/or interaction alpha level was observed, least significant differences (LSD) post-hoc analyses was performed to determine where significance was obtained. Effect-size calculations (using Cohen's d) were also performed to quantify the size and significance that may exist between groups independent of group size. Finally, partial correlations were performed to determine if there were significant relationships between dietary fat intake and circulating prostaglandins when controlling for supplement group. All data was considered statistically significant when the probability of type I error is 0.05 or less. Power analysis of the design indicates that an n-size of 15 per group yields high power (>0.8) for delta values of 0.75 to 1.25.

CHAPTER FOUR

Results

Subject Demographics

Thirty-seven subjects were recruited for the study and completed informed consent statements in compliance with the university's Institutional Review Board out of which thirty-one subjects (AA = 15, P = 16) finished the study. Table 1 summarizes the average age, height, initial (Day 1) weight, and self-reported training age for each group. Statistical analysis revealed that there was no statistical significance between the group means for age ($p = 0.81$), weight ($p = 0.67$), and training age ($p = 0.13$).

Table 1

Subject Demographics

Variable	AA Group (± SD)	P Group (± SD)	Group p-level
Age (yr)	21.9 (5.3)	22.3 (4.8)	0.81
Height (m)	1.79 (0.08)	1.79 (0.09)	0.89
Weight (kg)	85.1 (11.5)	87.1 (14.5)	0.67
Training age (yr)	3.6 (2.9)	6.2 (5.6)	0.13

Note: This data represents the average demographics of all subjects that completed the study. Data are expressed as means ± SD.

Dietary Intake and Lifting Volume

Caloric intake values over the course of the study were assessed by ESHA Nutritional software (ESHA Nutritional Research, Salem, OR) from 4-day self-reported dietary food logs prior to each testing session. Table 2 represents the average dietary/supplement intake levels for both groups over the course of the study. Statistical analysis revealed that there was a significant increase in protein intake for both groups ($p < 0.001$) but there was not a significant increase in protein intake between groups ($p = 0.22$) over the course of the study. The main effect for time in the increase of dietary protein is most likely due to the supplemental protein (Labrada Lean Body, Labrada Nutrition, Houston, TX) that each subject was required to ingest over the course of the study. Furthermore, the intention was to provide each subject with enough supplemental protein to attain close to 2.0 g/kg/day. One average, both groups increased dietary protein intake by 67.9 ± 33.4 g/d thus elevating relative protein intake by Day 50 to 2.01 ± 0.53 g/kg/day in comparison with day 1 levels of 1.25 ± 0.37 g/kg/day. Although subjects did not reach the intended protein intake goal of 2.0 g/kg/day, relative protein intake levels were elevated to a level slightly exceeding a general range that is prescribed to a resistance-trained athlete in order to ensure the each subject received an optimal amount of protein to build muscle regardless of the supplement assignment (Lemon, 2000).

Lifting volumes were measured by calculating total repetitions multiplied by weight lifted for all exercises over the course of the study. The upper body lifting volume for the AA group was 166,737 ± 46,985 kg versus 163,573 ± 63,349 kg for the P group with no significant difference ($p = 0.89$) existing between groups. The lower

body lifting volume performed by the AA group was 200,420 ± 70,323 kg versus 186,769 ± 40,035 kg for the P group with no significant difference (p = 0.51) existing between groups. The total lifting volume performed by the AA group was 367,156 ± 110,142 kg versus 350,341 ± 54,679 kg performed by the P group with no significant difference (p = 0.59) existing between groups.

Table 2

Dietary/Supplement Intake Levels over the Course of the Study

Variable	Session	AA Group (± SD)	P Group (± SD)	Time effect p-level	Group x Time p-level
Calories (kcal/d)	Day 1	2,602 (867)	2,049 (587)	-	-
	Day 25	2,845 (898)	2,273 (432)	-	-
	Day 50	2,796 (1,008)	2,437 (850)	0.24	0.50
Protein (g/d)	Day 1	115.1 (42.3)	98.5 (21.3)	-	-
	Day 25	188.4 (46.8)	165.7 (25.8)	-	-
	Day 50	190.9 (64.2)	158.9 (26.2)	* <0.001	0.22
Dietary Fat (g/d)	Day 1	96.0 (33.4)	81.0 (30.1)	-	-
	Day 25	94.2 (30.7)	72.3 (24.5)	-	-
	Day 50	91.0 (38.2)	67.7 (27.7)	0.46	0.11
Carbohydrates (g/d)	Day 1	298.9 (93.5)	229.9 (108.5)	-	-
	Day 25	297.6 (92.1)	243.1 (59.3)	-	-
	Day 50	294.4 (108.0)	218.3 (65.1)	0.58	0.81
Pills ingested	Day 50	196.5 (5.2)	196.3 (4.1)	-	0.90

Note: This data represents dietary/supplement intake levels over the course of the study. Data are expressed as means ± SD. Significant time and/or group x time interactions contain an asterisk.

Body Composition Variables

The data in Table 3 demonstrates the alterations in body composition observed over the course of the study while the illustrations in Figures 1 and 2 depict changes in bodyweight (Δkg) and fat-free mass (Δkg), respectively. Consistent with our H_1 hypothesis, there were no significant differences between groups for bodyweight ($p = 0.45$), fat-free mass ($p = 0.71$), fat mass ($p = 0.22$), and body fat percentage ($p = 0.43$). Thus, based upon our data we accept hypothesis H_1 stating that there would be no significant changes in the aforementioned body composition variables between groups. There was, however, a significant time effect increase in bodyweight ($p = 0.003$) and fat-free mass ($p = 0.001$) over the course of the study for both groups indicating possible increases in muscle mass.

Table 3

Body Composition Variables

Variable	Session	AA Group (± SD)	P Group (± SD)	Time effect p-level	Group x Time p-level
Weight (kg)	Day 1	85.1 (11.5)	87.1 (14.5)	-	-
	Day 25	85.6 (11.6)	87.9 (14.4)	-	-
	Day 50	86.6 (12.2)	88.1 (13.0)	* 0.003	0.45
LBM (kg)	Day 1	59.9 (8.2)	63.9 (8.4)	-	-
	Day 25	60.9 (8.1)	64.8 (8.2)	-	-
	Day 50	61.1 (8.5)	64.9 (8.0)	* 0.001	0.71
Fat mass (kg)	Day 1	15.8 (5.6)	13.8 (7.3)	-	-
	Day 25	15.5 (5.7)	13.5 (6.9)	-	-
	Day 50	16.3 (5.9)	13.8 (7.2)	0.24	0.22

(table continues)

Variable	Session	AA Group (± SD)	P Group (± SD)	Time effect p-level	Group x Time p-level
	Day 25	19.3 (6.1)	15.9 (5.9)	-	-
	Day 50	20.0 (6.0)	16.3 (6.2)	0.97	0.43

Note: This data represents body composition variables over the course of the study. Data are expressed as means ± SD. Significant time effects and/or group x time effects contain an asterisk.

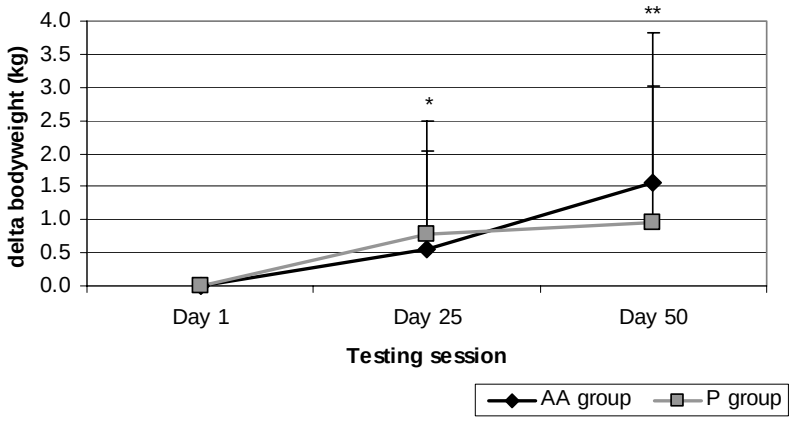

Figure 1. Changes in bodyweight (Δkg) over the course of the study. A significant time interaction was present for fat-free mass increases in both groups at day 25 *($p = 0.001$) and day 50 **($p = 0.001$).

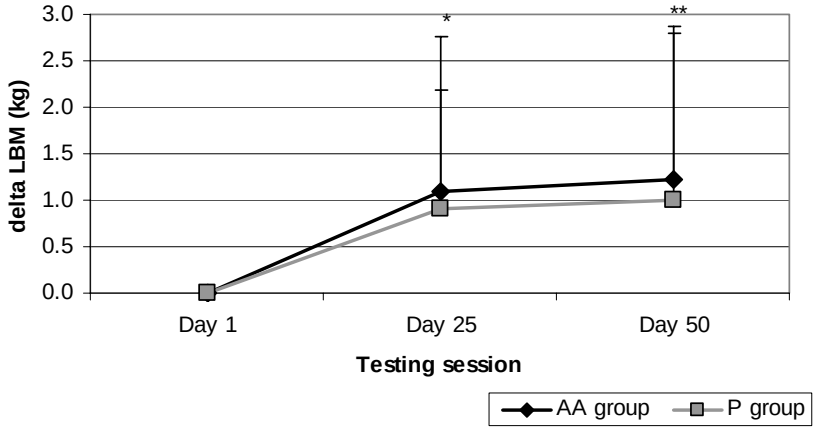

Figure 2. Changes in fat-free mass (Δkg) over the course of the study. A significant time effect was present for fat-free mass increases in both groups at day 25 *($p = 0.027$) and day 50 **($p < 0.003$).

Strength and Power Variables

Table 4 presents the alterations in strength and performance variables that were observed over the course of the study, while the illustrations in Figures 3 and 4 depict changes in bench press strength (Δkg) and leg press strength (Δkg), respectively. Figure 5 demonstrates changes in relative anaerobic peak power (ΔW/kg). There were no significant differences between groups for bench press strength ($p = 0.20$); although, statistical analysis revealed that a low to moderate effect-size increase in bench press strength in the AA group ($d = 0.47$). No significant group x time interactions existed for leg press strength ($p = 0.83$), however, although there were significant time effect increases in both groups for bench press strength ($p < 0.001$) and leg press strength ($p < 0.001$).

Contrary to hypothesis H_2, a significant group x time interaction increase existed in relative peak power during the 30-sec Wingate anaerobic bike sprint in the AA group ($p = 0.015$). Statistical analysis also revealed that there was a statistical trend for the time effect in the increase in relative peak power in both groups ($p = 0.06$), and there was a significant time effect ($p = 0.007$) for the increase in relative mean power during the 30-sec Wingate anaerobic bike sprint in both groups, but there was no significant group x time effect ($p = 0.28$). Finally, there was a significant time effect ($p < 0.001$) as well as a statistical group x time interaction trend for the increase in total work for the AA group ($p = 0.09$). There also existed a moderate effect size for total work performed between groups ($d = 0.62$). Therefore, the H_2 hypothesis stating that there would be no significant changes in all of the aforementioned performance variables between groups is rejected due to the fact that there was a significant group x time interaction in the increase in relative peak power with AA supplementation.

Table 4

Performance Variables

Variable	Session	AA Group (± SD)	P Group (± SD)	Time effect p-level	Group x Time p-level
1-RM bench press (kg)	Day 1	103.0 (24.9)	113.5 (24.6)	-	-
	Day 25	107.0 (23.4)	118.5 (23.0)	-	-
	Day 50	114.4 (25.8)	121.5 (21.5)	* <0.001	0.20
1-RM leg press (kg)	Day 1	370.8 (113.5)	413.4 (80.4)	-	-
	Day 25	379.2 (109.5)	416.8 (77.2)	-	-
	Day 50	395.8 (123.8)	436.1 (78.9)	* <0.001	0.83
Peak power/mass (W/kg)	Day 1	16.9 (3.1)	18.3 (2.6)	-	-
	Day 25	17.8 (2.6)	18.3 (2.9)	-	-
	Day 50	18.1 (2.6)	18.1 (2.8)	0.06	* 0.015
Mean power/mass (W/kg)	Day 1	8.3 (1.2)	8.9 (0.7)	-	-
	Day 25	8.5 (1.1)	8.9 (0.8)	-	-
	Day 50	8.6 (1.2)	9.1 (0.7)	* 0.007	0.28
Total Work (J)	Day 1	21,102 (3,619)	23,389 (4,220)	-	-
	Day 25	21,658 (3,307)	23,340 (3,909)	-	-
	Day 50	22,394 (4,905)	23,899 (3,492)	* <0.001	0.09

Note: This data represents the performance variable values over the course of the study. Data are expressed as means ± SD. Significant time effects and/or group x time effects contain an asterisk.

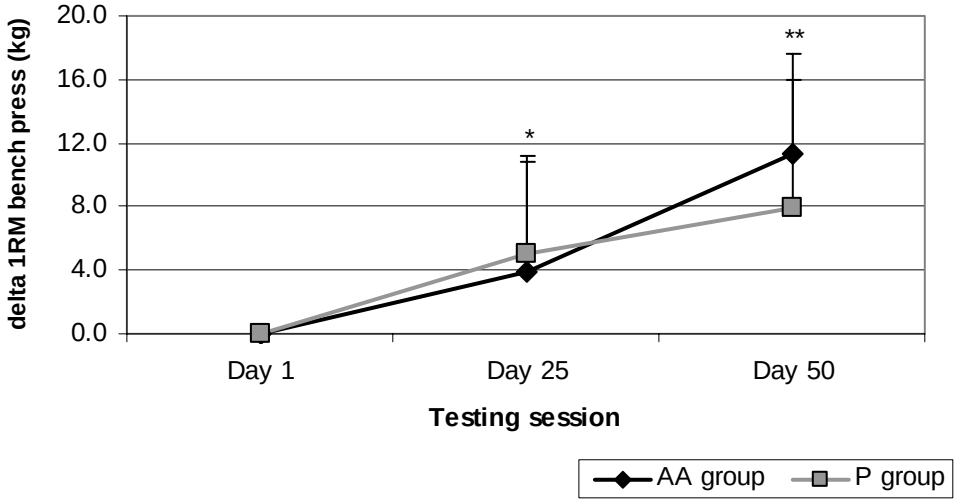

Figure 3. Changes in bench press strength (Δkg) over the course of the study. A significant time effect for both groups was observed at day 25 *($p = 0.001$) and day 50 **($p < 0.001$).

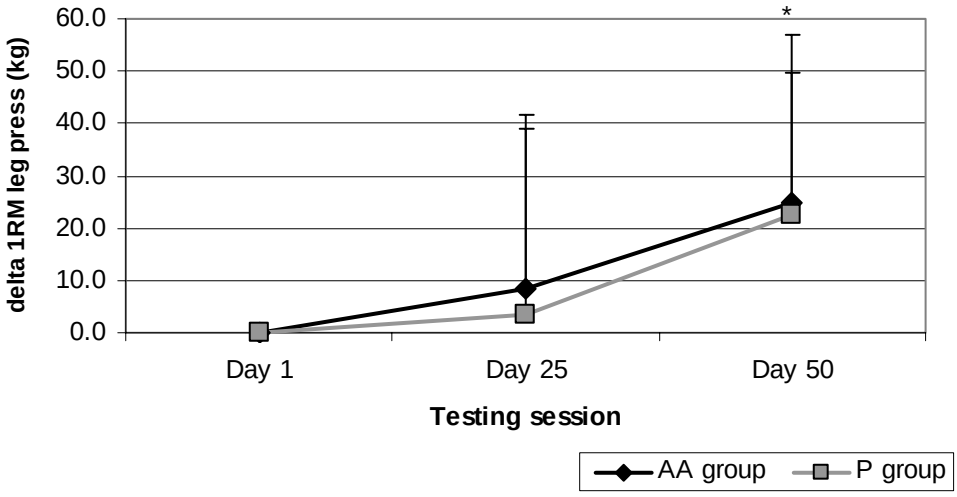

Figure 4. Changes in leg press strength (Δkg) over the course of the study. A significant time effect was observed at day 50 *($p < 0.001$).

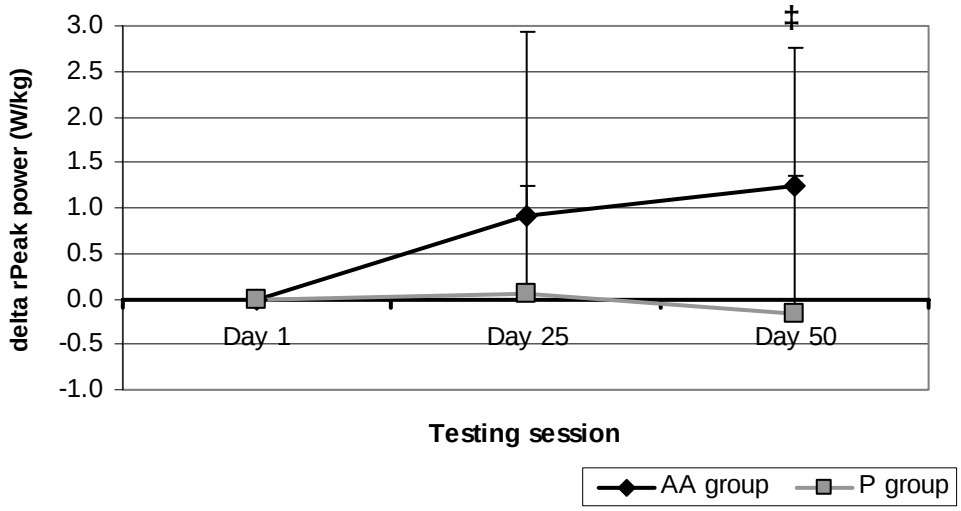

Figure 5. Changes in relative anaerobic peak power (ΔW/kg) performed during the 30-sec Wingate anaerobic bike sprint. A significant increase was observed in the AA group ‡($p = 0.017$).

Whole Blood Variables

The data in Table 3 demonstrates the alterations in differential white blood cell counts that were observed over the course of the study. There were no statistical differences existed between groups for total white blood cell count ($p = 0.12$), neutrophils levels ($p = 0.13$), lymphocyte levels ($p = 0.20$), circulating monocyte levels ($p = 0.14$), eosinophil levels ($p = 0.58$) and basophil levels ($p = 0.10$) over the course of the study. Statistical analysis revealed a group x time interaction trend for increases in basophil level increases in the P group ($p = 0.09$); although, these levels remained within normal clinical ranges. A significant time effect trend also existed for the increase in neutrophil levels for both groups ($p = 0.09$); although, these levels remained within normal clinical ranges. Statistical analysis also revealed a significant time effect for the increase of neutrophil: lymphocyte ratios in both groups over the course of the study ($p = 0.014$); although, there was no group x time effect ($p = 0.84$). Statistical analysis also revealed a

significant time effect for the decrement of RBC levels (p = 0.035) and hemoglobin concentrations (p = 0.002); although, these levels still remained within a normal clinical range. Therefore, AA supplementation appears to have no detrimental effects on whole blood levels.

Table 5

Differential White Blood Cell Values

Variable	Session	AA group (± SD)	P group (± SD)	Time effect p-level	Group x Time p-level
WBC (K/μl)	Day 1	5.6 (0.3)	5.3 (1.1)	-	-
	Day 25	5.5 (1.2)	5.1 (1.3)	-	-
	Day 50	6.1 (1.4)	5.1 (1.1)	0.49	0.12
Neutrophils (K/μl)	Day 1	3.1 (0.9)	2.8 (0.8)	-	-
	Day 25	3.2 (1.0)	2.9 (1.0)	-	-
	Day 50	3.6 (0.9)	2.9 (0.8)	0.09	0.13
Lymphocytes (K/μl)	Day 1	1.9 (0.6)	1.9 (0.5)	-	-
	Day 25	1.7 (0.4)	1.6 (0.4)	-	-
	Day 50	1.8 (0.6)	1.6 (0.4)	0.10	0.20
Neutrophil: lymphocyte	Day 1	1.7 (0.6)	1.5 (0.5)	-	-
	Day 25	2.0 (0.6)	1.9 (0.7)	-	-
	Day 50	2.1 (0.8)	1.9 (0.6)	* 0.014	0.84
Monocytes (K/μl)	Day 1	0.5 (0.1)	0.4 (0.1)	-	-
	Day 25	0.5 (0.1)	0.4 (0.1)	-	-
	Day 50	0.5 (0.2)	0.4 (0.1)	0.27	0.14

(table continues)

Variable	Session	AA group (± SD)	P group (± SD)	Time effect p-level	Group x Time p-level
Eosinophils (K/µl)	Day 1	0.07 (0.02)	0.07 (0.04)	-	-
	Day 25	0.11 (0.18)	0.07 (0.04)	-	-
	Day 50	0.08 (0.03)	0.07 (0.04)	0.52	0.58
RBC (M/µl)	Day 1	5.3 (0.3)	5.4 (0.4)	-	-
	Day 25	5.0 (0.5)	5.2 (0.4)	-	-
	Day 50	5.2 (0.5)	5.3 (0.4)	0.49	* 0.035
Hemoglobin (g/dl)	Day 1	16.2 (1.1)	16.3 (1.0)	-	-
	Day 25	15.7 (1.2)	15.7 (0.9)	-	-
	Day 50	15.7 (1.1)	15.7 (1.0)	0.65	* 0.002
Hematocrit (%)	Day 1	46.0 (2.6)	46.5 (2.8)	-	-
	Day 25	44.3 (3.5)	44.8 (2.6)	-	-
	Day 50	45.4 (3.5)	45.6 (3.6)	0.17	0.79

Note: This data represents differential white blood cell levels over the course of the study. Data are means ± SD. Significant time and/or group x time interactions contain an asterisk.

Serum Chemistry Variables

The data in Table 4 demonstrates the alterations in serum chemistry values that were observed over the course of the study. There was also significant time effect for both groups in the increase of LDL levels ($p = 0.046$) and GGT levels ($p = 0.005$), as well as a decrease in ALT levels ($p = 0.022$), but these levels were within a normal clinical range. There was a statistical time interaction trend in the increase in serum triglyceride levels in the AA group ($p = 0.09$); although, these levels remained within normal clinical ranges. There was also a statistical time interaction trend in the decrement in the liver enzyme aspartate aminotransferase (ASP) in the P group ($p = 0.07$), but again these levels remained within normal ranges over the course of the study.

Table 6

Serum Chemistry Values

Variable	Session	AA group (± SD)	P group (± SD)	Time effect p-level	Group x Time p-level
Triglycerides (mg/dl)	Day 1	78.9 (44.2)	99.1 (55.5)	-	-
	Day 25	74.3 (46.4)	86.6 (29.9)	-	-
	Day 50	92.3 (45.9)	90.8 (40.3)	0.68	0.09
Total cholesterol (mg/dl)	Day 1	152.0 (53.3)	174.6 (53.9)	-	-
	Day 25	149.1 (57.3)	161.3 (42.5)	-	-
	Day 50	173.1 (32.7)	185.4 (43.2)	0.11	0.59
LDL cholesterol (mg/dl)	Day 1	90.7 (38.8)	101.4 (36.2)	-	-
	Day 25	90.7 (42.3)	96.3 (33.0)	-	-
	Day 50	108.1 (35.3)	113.8 (35.8)	* 0.046	0.73
HDL cholesterol (mg/dl)	Day 1	40.3 (15.3)	49.1 (19.9)	-	-
	Day 25	37.4 (15.1)	43.8 (14.8)	-	-
	Day 50	42.2 (17.4)	50.8 (16.5)	0.57	0.96
Glucose (mg/dl)	Day 1	86.9 (12.7)	98.4 (19.3)	-	-
	Day 25	81.7 (17.9)	91.8 (16.8)	-	-
	Day 50	89.9 (10.0)	95.6 (14.0)	0.98	0.24
GGT (U/L)	Day 1	30.1 (15.0)	28.3 (8.4)	-	-
	Day 25	33.5 (28.2)	29.3 (7.2)	-	-
	Day 50	34.4 (12.3)	36.0 (8.5)	* 0.005	0.40

(table continues)

Variable	Session	AA group (± SD)	P group (± SD)	Time effect p-level	Group x Time p-level
ALT (U/L)	Day 1	36.3 (16.3)	34.9 (11.8)	-	-
	Day 25	27.5 (13.1)	30.5 (11.8)	-	-
	Day 50	31.5 (15.8)	26.8 (5.4)	* 0.022	0.53
ASP (U/L)	Day 1	25.9 (12.5)	36.6 (29.7)	-	-
	Day 25	25.9 (16.7)	38.3 (32.2)	-	-
	Day 50	29.9 (9.2)	26.1 (6.7)	0.41	0.07
ALP (U/L)	Day 1	68.9 (25.2)	82.1 (25.6)	-	-
	Day 25	62.7 (23.0)	76.4 (16.5)	-	-
	Day 50	76.3 (23.7)	90.9 (21.4)	* 0.028	0.84
Total protein (g/dl)	Day 1	6.8 (1.7)	7.7 (2.0)	-	-
	Day 25	6.5 (2.0)	7.2 (1.3)	-	-
	Day 50	7.8 (0.7)	8.1 (0.8)	0.06	0.46
Albumin (g/dl)	Day 1	4.2 (1.0)	4.7 (1.1)	-	-
	Day 25	4.0 (1.2)	4.3 (0.8)	-	-
	Day 50	4.6 (0.4)	4.8 (0.5)	0.21	0.43
BUN (mg/dl)	Day 1	13.1 (3.4)	15.6 (4.0)	-	-
	Day 25	13.9 (4.0)	16.3 (5.3)	-	-
	Day 50	15.8 (5.5)	15.8 (5.5)	0.16	0.21
Creatinine (mg/dl)	Day 1	1.0 (0.2)	1.1 (0.2)	-	-
	Day 25	0.9 (0.3)	1.0 (0.2)	-	-
	Day 50	1.0 (0.2)	1.1 (0.2)	0.72	0.41
BUN: creatinine	Day 1	13.2 (3.4)	14.3 (5.0)	-	-
	Day 25	16.3 (3.8)	16.6 (4.9)	-	-
	Day 50	15.4 (5.0)	15.0 (3.7)	0.13	0.41

Note: This data represents serum metabolite/enzyme levels over the course of the study. Data are means ± SD. Significant time and/or group x time interactions contain an asterisk.

Muscle Damage Markers

Table 5 represents muscle damage markers assessed from the blood collected during each testing session. Statistical analysis revealed a significant time effect for lactate dehydrogenase levels ($p = 0.050$), but there was no significant group x time interaction ($p = 0.21$). Statistical analysis also revealed no significant time interaction in creatine kinase levels ($p = 0.138$), nor a significant group x time interaction ($p = 0.29$). There was no time effect over the course of the study ($p = 0.138$), nor was there a group x time effect ($p = 0.286$). Therefore, based upon the aforementioned data the H_3 hypothesis stating that there would be no difference between groups in serum and whole blood values is accepted.

Table 7

Serum Muscle Damage Markers

Variable	Session	AA group (± SD)	P group (± SD)	Time effect p-level	Group x Time p-level
LDH (mg/dl)	Day 1	133.1 (42.2)	128.8 (40.9)	-	-
	Day 25	122.1 (53.9)	134.9 (39.9)	-	-
	Day 50	161.9 (56.8)	135.4 (26.2)	* 0.05	0.21
Creatine kinase (mg/dl)	Day 1	224.3 ± 251.7	458.6 (706.1)	-	-
	Day 25	224.3 ± 251.7	487.1 (775.6)	-	-
	Day 50	182.5 ± 169.6	210.9 (176.6)	0.13	0.29

Note: This data represents muscle damage markers assessed from the blood collected during each testing session. Data are means ± SD. Significant time and/or group x time interactions contain an asterisk.

Serum $PGF_{2\alpha}$ and PGE_2 Levels

Table 6 represents the raw mean values for PGE_2 and $PGF_{2\alpha}$ over the course of the study and Figures 6 and 7 depict changes in PGE_2 (Δpg/ml) and $PGF_{2\alpha}$ (Δpg/ml) concentrations over the supplement intervention, respectively. There was no group x time effect ($p = 0.06$), or time interaction in PGE_2 levels ($p = 0.78$) although there was a moderate to strong effect-size increase in PGE_2 levels in the AA group ($d = 0.68$). There was also no time interaction ($p = 0.83$) nor was there a significant difference between groups in $PGF_{2\alpha}$ levels ($p = 0.14$) although there was a moderate effect size for $PGF_{2\alpha}$ levels ($d = 0.53$). Thus, the H_5 hypothesis stating that there would be no significant changes in PGE_2 and/or $PGF_{2\alpha}$ concentrations is accepted.

Table 8

Prostaglandin Levels

Variable	Session	AA group (± SD)	P group (± SD)	Time effect p-level	Group x Time p-level
$PGF_{2\alpha}$ (pg/ml)	Day 1	164.6 ± 104.4	183.6 ± 96.0	-	-
	Day 25	221.5 ± 153.2	281.5 ± 248.2	-	-
	Day 50	209.7 ± 226.3	150.0 ± 104.5	0.83	0.14
PGE_2 (pg/ml)	Day 1	224.3 (145.7)	293.6 (256.7)	-	-
	Day 25	288.5 (191.2)	314.8 (236.1)	-	-
	Day 50	322.8 (281.2)	219.9 (163.6)	0.78	0.06

Note: This data represents prostaglandin levels assessed from collected serum during each testing session. Data are means ± SD.

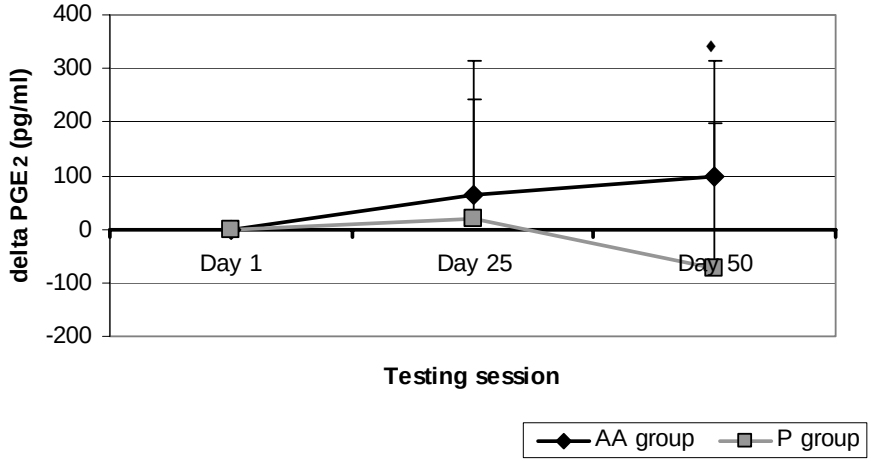

Figure 6. Changes in PGE_2 levels (Δpg/ml) over the course of the study. Data are expressed as means ± SD, AA: n = 15, P: n = 16. ♦A statistical trend was observed in an increase in PGE_2 levels in the AA group (p = 0.06).

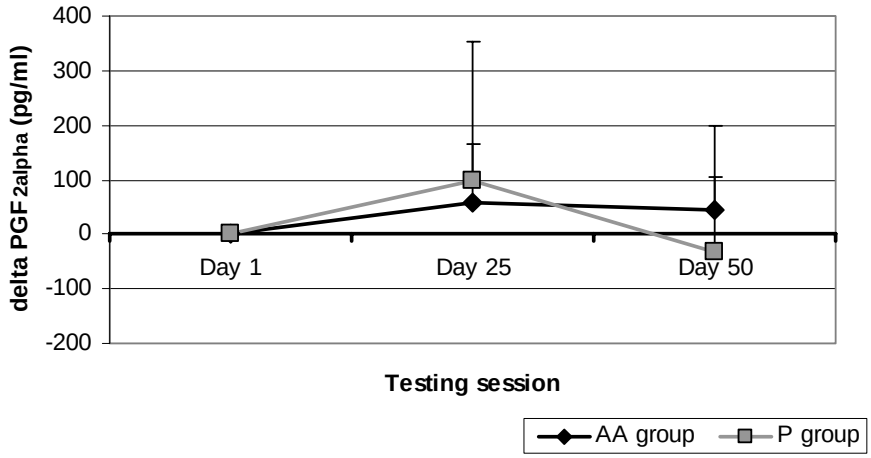

Figure 7. Changes in $PGF_{2\alpha}$ levels (Δpg/ml) over the course of the study. Data are expressed as means ± SD. Non-significant increases were observed in $PGF_{2\alpha}$ levels in AA group (p = 0.14).

Hormonal Alterations

The data in Table 7 demonstrates the hormonal levels that were observed over the course of the study while figure 8 depicts baseline changes in IL-6 (Δpg/ml). There was a significant time effect in fTEST values over the 50 day trial (p = 0.031). Statistical analysis also revealed that there was no time effect (p = 0.25), nor was there a group x

time interaction in IL-6 levels ($p = 0.06$); although, there was a moderate to strong effect-size in IL-6 decrements in the AA group ($d = 0.68$). Thus, the H_5 hypothesis stating that there would be no significant changes in IL-6, CORT, fTEST and tTEST concentrations is accepted.

Table 9

Hormonal Variables

Variable	Session	AA group (± SD)	P group (± SD)	Time effect p-level	Group x Time p-level
IL-6 (pg/ml)	Day 1	137.2 (69.6)	120.1 (92.1)	-	-
	Day 25	138.0 (83.1)	172.6 (90.5)	-	-
	Day 50	108.4 (48.6)	127.0 (56.8)	0.25	0.06
CORT (μg/dl)	Day 1	34.0 (24.1)	26.2 (10.6)	-	-
	Day 25	28.2 (20.0)	23.1 (7.8)	-	-
	Day 50	36.8 (22.9)	34.2 (35.8)	0.23	0.57
tTEST (ng/ml)	Day 1	21.4 (24.8)	14.0 (12.1)	-	-
	Day 25	18.7 (22.4)	12.2 (7.1)	-	-
	Day 50	18.7 (18.3)	12.2 (7.7)	0.26	0.83
fTEST (pg/ml)	Day 1	25.3 (14.7)	24.9 (19.6)	-	-
	Day 25	23.1 (12.1)	20.0 (12.1)	-	-
	Day 50	22.3 (13.6)	22.3 (15.0)	* 0.031	0.88
tTEST: CORT ratio	Day 1	0.069 (0.063)	0.050 (0.026)	-	-
	Day 25	0.066 (0.051)	0.052 (0.022)	-	-
	Day 50	0.058 (0.049)	0.048 (0.030)	0.43	0.63

Note: This data represents hormonal variables over the course of the study. Data are expressed as means ± SD. Significant time and/or group x time interactions contain an asterisk.

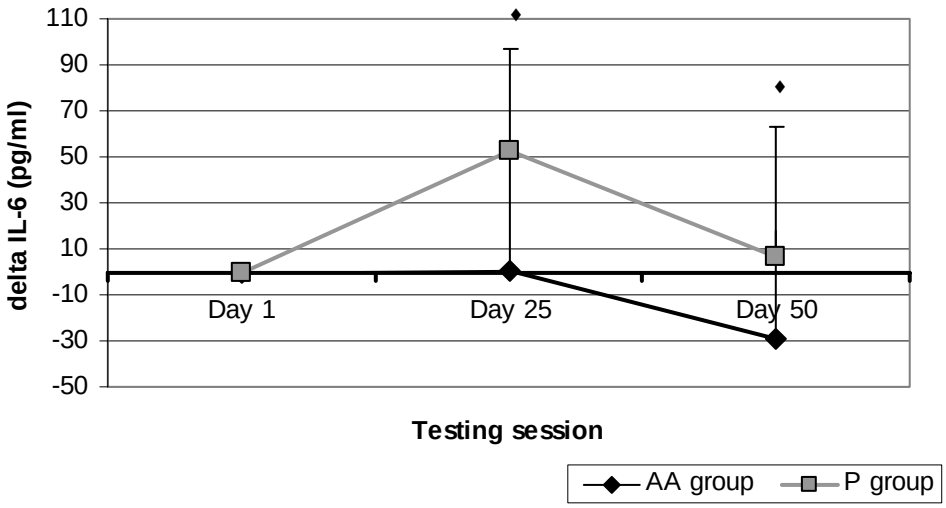

Figure 8. Changes in IL-6 levels (Δpg/ml) over the course of the study. Data are expressed as means ± SD. A statistical trend was observed in IL-6 decrements in the AA group at days 25 and 50 in the AA group ♦(p = 0.063).

FP and EP_3 Receptor Levels

The data in Table 10 represents the FP and EP_3 receptor density values in both groups over the course of the study. Figures 9 and 10 depict the changes in FP and EP_3 receptor density values in both groups, respectively. Appendix E shows the nitrocellulose membrane used during the immunoblotting procedure for PG receptor semi-quantitation. Corresponding FP receptor values for the P group were 2,253.4 ± 1,309.9 INT*mm^2/mg and 2,518.2 ± 992.2 INT* mm^2/mg. Consistent with the H_6 hypothesis, there was no significant time effect (p = 0.13) nor was there a significant group x time interaction (p = 0.64) concerning changes in FP receptor density values over the course of the study. Consistent with the H_6 hypothesis, there was also no significant time effect (p = 0.11) nor was there a significant group x time interaction (p = 0.66) concerning changes in EP_3 receptor density values.

Table 10

Prostaglandin Receptor Densities

Variable	Session	AA group (± SD)	P group (± SD)	Time effect p-level	Group x Time p-level
FP receptor (INT*mm^2/mg)	Day 1	1,929.4 ± 1,395.2	2,253.4 ± 1,309.9	-	-
	Day 50	2,425.2 ± 1,746.2	2,518.2 ± 992.2	0.14	0.64
EP_3 receptor (INT*mm^2/mg)	Day 1	5,990.1 ± 3,563.3	6,854.3 ± 3,287.2	-	-
	Day 50	7,149.6 ± 3,383.0	7,528.7 ± 2,790.8	0.11	0.66

Note: This data represents prostaglandin receptor densities assessed from collected biopsies during the first and last testing sessions. Data are means ± SD.

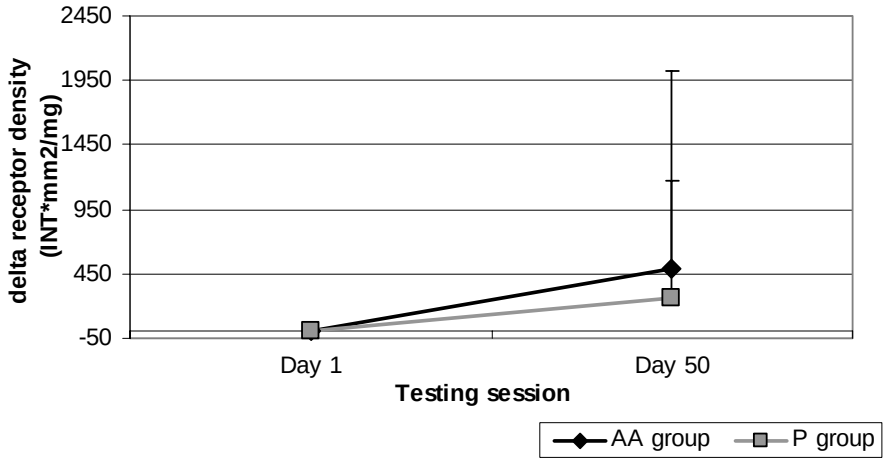

Figure 9. Changes in FP receptor density values (INT*mm^2/mg) over the study. Data are expressed as means ± SD. There was no significant increases in muscular FP receptor density over the course of the study (p = 0.64).

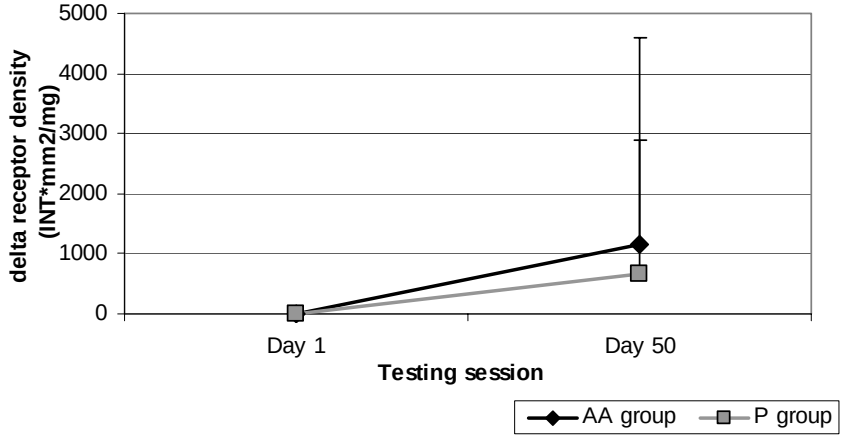

Figure 10. Changes in EP_3 receptor density values (INT*mm^2/mg) over the study. Data are expressed as means ± SD. There was no significant increases in muscular EP_3 receptor density over the course of the study ($p = 0.66$).

Myosin Heavy Chain Isoform and mRNA Content

The data in Table 11 demonstrates the MHC mRNA and protein alterations that were observed over the course of the study. Figure 11, figure 12, and figure 13 represent changes in MHC I, -IIa, and -IIx mRNA expression standardized to β-actin levels. Appendices B and C represent the real-time RT-PCR amplification curve for MHC mRNA gene expression and agarose gel electrophoresis, respectively. Appendix D visualizes MHC isoform band density in various subjects. There was a significant main effect ($p = 0.009$) for the increase of MHC IIa protein levels. However, there were no significant group x time interactions present for changes in MHC I, -IIa, and -IIx protein levels between groups.

There were no significant changes in the expression of all of the MHC mRNA isoforms. There were, however, statistical trends for the decrement in MHC I ($p = 0.14$), MHC IIa ($p = 0.08$), and MHC IIx ($p = 0.10$) mRNA expression; although, there were moderate effect sizes for the decrements in these variables (MHC I: $d = 0.55$, MHC IIa: d

= 0.66, MHC IIx: d = 0.61). Therefore, the H_6 hypothesis stating that there would be no significant alterations in intramuscular adaptations with AA supplementation is accepted due to the observed non-significant findings in gene expression between groups.

Table 11

MHC mRNA and Protein Content

Variable	Session	AA group (± SD)	P group (± SD)	Time effect p-level	Group x Time p-level
MHC I mRNA/β-actin mRNA	Day 1	0.69 (0.16)	0.68 (0.14)	-	-
	Day 50	0.64 (0.07)	0.71 (0.15)	0.69	0.14
MHC IIa mRNA/β-actin mRNA	Day 1	0.66 (0.12)	0.66 (0.14)	-	-
	Day 50	0.63 (0.07)	0.71 (0.13)	0.55	0.08
MHC IIx mRNA/β-actin mRNA	Day 1	0.66 (0.14)	0.65 (0.15)	-	-
	Day 50	0.62 (0.07)	0.69 (0.14)	0.97	0.10
MHC I (ng/ml)	Day 1	187.8 (180.7)	172.8 (123.0)	-	-
	Day 50	176.3 (104.4)	164.3 (126.6)	0.64	0.96
MHC IIa (ng/ml)	Day 1	184.35 (217.1)	156.9 (84.5)	-	-
	Day 50	305.02 (366.3)	295.8 (328.5)	* 0.009	0.84
MHC IIx (ng/ml)	Day 1	128.7 (79.1)	133.6 (83.5)	-	-
	Day 50	103.9 (68.2)	131.9 (81.7)	0.23	0.29
MHC I %	Day 1	34.9 (21.6)	34.5 (13.6)	-	-
	Day 50	32.5 (10.6)	28.9 (13.5)	0.16	0.57
MHC IIa %	Day 1	35.5 (20.0)	36.1 (10.5)	-	
	Day 50	44.3 (18.7)	46.7 (13.6)	* 0.003	- 0.77
MHC IIx %	Day 1	29.7 (15.8)	29.4 (10.9)	-	-
	Day 50	23.2 (14.8)	24.4 (8.2)	* 0.035	0.78

Note: Relative MHC mRNA changes and protein levels over the course of the study. Data are means ± SD. Significant time and/or group x time interactions contain an asterisk.

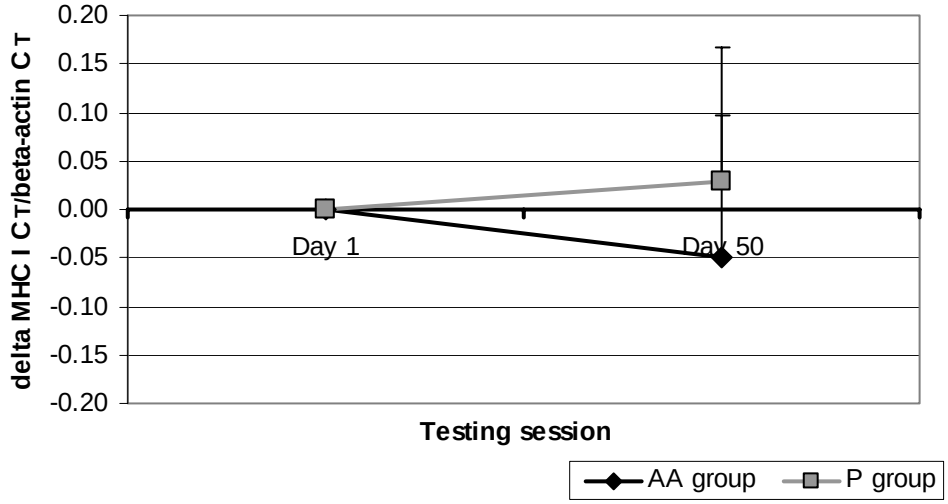

Figure 11. Changes in MHC I mRNA expression relative to β-actin over the study. Data are expressed as means ± SD. There was a statistical trend in the decrement in IIx expression at day 50 in the AA group versus the P group ($p = 0.14$).

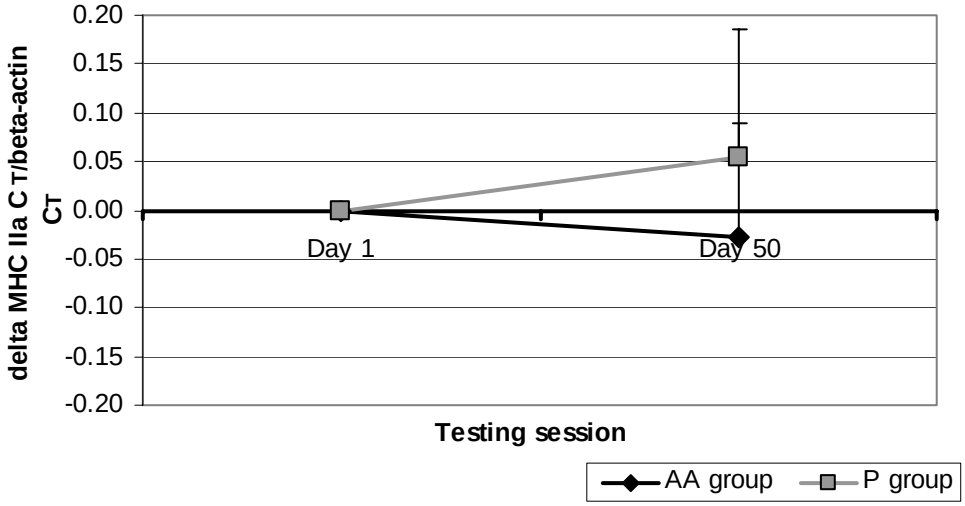

Figure 12. Changes in MHC IIa mRNA expression relative to β-actin over the study. Data are expressed as means ± SD. There was a statistical trend in the decrement in IIx expression at day 50 in the AA group versus the P group ($p = 0.08$).

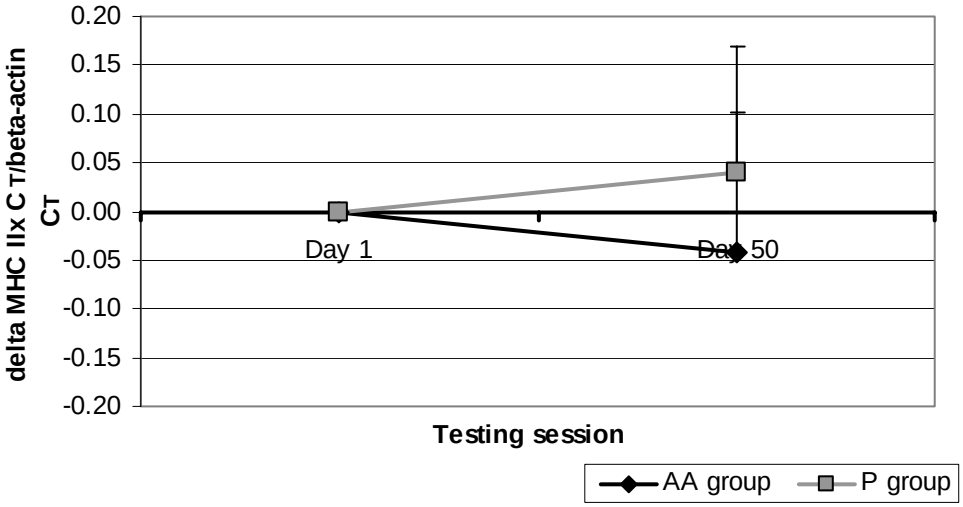

Figure 13. Changes in MHC IIx mRNA expression relative to β-actin over the study. Data are expressed as means ± SD. There was a statistical trend in the decrement in IIx expression at day 50 in the AA group versus the P group ($p = 0.10$).

Correlations

Partial correlations were performed in order to determine if a relationship existed between dietary fat intake and prostaglandin synthesis when controlling for groups. Statistical analysis revealed that there were weak correlations between overall fat intake and $PGF_{2\alpha}$ levels ($d = -0.29$, $p = 0.13$) and/or PGE_2 levels ($d = -0.24$, $p = 0.22$).

CHAPTER FIVE

Discussion

Non-significant Changes in Body Composition and Strength

The purpose of our investigation was to examine the effects that AA supplementation (1 g/day for 50 days) had on body composition, training adaptations (i.e. muscular strength and anaerobic power), hormonal/cytokine adaptations (i.e. CORT, fTEST, tTEST, and IL-6), and intramuscular adaptations (i.e. MHC mRNA and protein levels) in healthy resistance-trained males partaking in a periodized, full-body workout regimen. Post eccentric leg extensor exercise *in vivo* (Trappe et al., 2002) and *in vitro* evidence (Horsley & Pavlath, 2003; Rodemann & Goldberg, 1982) has demonstrated the integral role that PG formation and subsequent myogenic signaling exerts on muscle protein synthetic rates. Data also exists demonstrating that physically active humans and animals contain less intramuscular AA stores within skeletal muscle (Andersson et al., 2000; Helge et al., 2001). Hence, the primary rationale for AA supplementation is to replenish intramuscular AA stores available for PG formation. Furthermore, the subsequent enhancement in intramuscular protein synthesis that may accompany PG signaling in response to exercise training is the theoretical basis for AA supplementation due to the potential increases that may arise in strength and fat-free mass. Over the 50-day trial both groups experienced significant increases in fat-free mass ($p = 0.003$) most likely due to a training effect and an increase in dietary protein intake. However, there were no significant differences between groups in fat-free mass changes ($p = 0.72$).

Furthermore, there were only modest increases in bench press strength ($p = 0.20$) and no significant gains in leg press strength ($p = 0.83$); although, effect-size calculations confirmed that a small to moderate effect-size existed with AA supplementation relative to bench press strength ($d = 0.47$). In concert with significant elevations in Wingate relative peak power, it may be advantageous to further research the effect that AA exerts on bench press strength. Assuming that AA does indeed exerts its purported anabolic effects, the following paragraphs will use pertinent research in an attempt to explain why AA supplementation might not have optimized gains in fat-free mass and strength as well as to defend the rationale for further research concerning AA supplementation in the arena of sports nutrition.

The first explanation for these findings could be the lack of AA uptake into the muscle and/or the lack of its conversion into PGs. It is known that endogenous AA synthesis does occur from the enzymatic elongation of *cis*-linoleic acid. Over a 50-day period, dietary supplementation with 1.5 g/d of AA has been shown to reduce *de novo* AA production from 20 g/d of *cis*-linoleic acid and 0.2 g/d of AA from 677 mg/d to approximately 326 mg/d (Emken, Adlof, Duval, & Nelson, 1998). Another investigation (Whelan, Broughton, Surette, & Kinsella, 1992) discovered that modest increases of AA ethyl ester fed to rats increased tissue phospholipid AA levels by 39% and 57% in the liver and in peritoneal exudate cells, respectively. It should be noted that the latter cell lineage is unable to elongate *cis*-linoleic acid to AA. These increases were further enhanced to 57% and 68% in liver and peritoneal exudate cells, respectively, when dietary *cis*-linoleic acid and AA intakes were equivalent. With the aforementioned cases taken into account it is plausible that subjects in the AA group most likely experienced

increases in systemic AA implementation due to a similar dosing pattern that was used in the aforementioned experiments. However, none of the aforementioned studies investigated implementation into skeletal muscle. It should, therefore, be noted that the amount of AA present within skeletal muscle cells and/or circulating leukocytes that could contribute to the formation of PGs was not examined in the current study. Thus, at this juncture we cannot definitively conclude that the provided AA dosage significantly altered intramuscular AA stores or if the AA that was ingested was used for PG synthesis following resistance training.

A second reason for the aforementioned findings could be the lack of AA uptake into the muscle and/or the lack of its conversion into PGs. A landmark study conducted by Brash and Ingram (1986) used gas chromatographic-mass spectroscopy (GC-MS) with $H_2{}^{18}O$ to analyze the molecular weight of hydrolyzed fatty acids as well as their PG and leukotriene products that were synthesized in leukocytes in lieu of exogenous AA. This model was employed to ascertain if a majority of *de novo* eicosanoid biosynthesis occurs from exogenous AA or from esterified AA embedded in membrane phospholipids. It should be noted that ^{18}OH was implemented in endogenous AA stores upon liberation from membrane phospholipids via phospholipase-mediated hydrolysis to yield $C_{19}H_{31}CO^{18}OH$ and that the quantities of $C_{19}H_{31}CO^{18}OH$ and its eicosanoid products can be detected by GC-MS and compared to exogenous AA and its eicosanoid products. The results from the aforementioned study indicated that in absence of exogenous AA, eicosanoid products contained ^{18}OH which verifies that esterified AA is used for eicosanoid biosynthesis. However, when 5 and 50 μM of deuterated AA was added to ionophore-stimulated leukocytes, the synthesized eicosanoids were formed mainly from

the deuterated, exogenous AA. Therefore, based upon these previous results and assuming the PG biosynthesis is similar in skeletal muscle, it may have been advantageous to supplement our subjects with exogenous AA prior to resistance training in order to provide exogenous substrate for immediate PG synthesis following an exercise stimulus as well as to spare intramuscular and/or leukocyte AA stores.

Albumin acts as a fatty acyl transporter that facilitates the solubility of long chain fatty acids in the aqueous environment of mammalian plasma. In fact, the physiological concentrations of albumin in our subjects more than likely reduces any given amount of free, bio-available AA in the plasma to nM concentrations (Brash, 2001; McArthur *et al.*, 1999), thus limiting the ability of exogenous sources to be metabolized and/or implemented into tissue membranes. Thus, the third possible confounding factor as well as a severe limitation to most *in vitro* evidence studying the physiological effects of AA on various cell culture models is the absence of albumin within the cell culture medium which is present at high concentrations in human plasma [an estimated 35 mg/ml by Brash et al. (2001) and 40-50 mg/ml from our subject pool according to our clinical chemistry analyzer].

Finally, a plausible issue of supplement responders (i.e. people who do not ingest foods that contain higher AA content and/or physically active individuals) versus non-responders (i.e. individuals who consume foods high in AA and/or less active individuals) needs to be further explored. Although there was a weak correlation associated with overall dietary fat intake and circulating $PGF_{2\alpha}$ levels and/or PGE_2 levels, we were not able to discern whether subjects ingesting high levels of *cis*-linoleic acid and/or AA were able to further benefit from AA supplementation. Li *et al*. (1998) found

that on average, the visible fat of raw beef, lamb, pork, chicken, duck and turkey contained 20-180 mg AA/100 g fat versus the lean variations of these meats which contained 30-99 mg AA/100 g fat. Past studies have also demonstrated that subjects consuming diets rich in higher fat meats contain more serum and/or intracellular AA. Phinney *et al*. (1990) also show that vegetarians and semi-vegetarians contain a significantly lower content of AA in serum phospholipids than omnivores. A similar study by Sinclair *et al.* (1994) confirms the previous findings, showing that healthy subjects fed 500 g lean beef daily for 4 weeks increased the AA content of serum phospholipids from 11.3% to 14.1%. Thus, it is to be expected that individuals consuming less meat products would have the physiological potential to increase his/her endogenous pool of AA available for eicosanoid production and respond more favorably to AA supplementation than a person who consumes an overabundance of meat products.

Significant Changes in Relative Peak Power

The AA group observed significant increases in relative peak power production ($p = 0.015$) and exhibited a statistical trend in the increase of total work performed ($p = 0.09$) during the 30-sec Wingate anaerobic peak power test in comparison to the P group. Effect-size calculations confirmed that a moderate relationship existed between AA supplementation and increases in total work as well ($d = 0.62$). Due to a potential learning curve that might exist with the Wingate anaerobic bike sprint, subjects from both groups were familiarized with and completed the Wingate protocol during their familiarization session prior to the first day of testing. Thus, the significant increase in relative peak power in the AA group as well as the increases in total work is not likely to be attributed to the learning involved with the exercise test.

The Wingate test generally measures the ability to produce high power outputs using intramuscular phosphagen and glycogen stores for energy production. Regardless, no literature exists to our knowledge demonstrating that AA and/or PG signaling elicits intracellular increases in creatine and/or glycogen concentrations. Although an explanation for the increases in relative peak power in the AA group is difficult to ascertain, recent literature exists demonstrating that 10 days of soybean-derived phosphatidylserine supplementation significantly increases the time to fatigue and thus work performed in active males during an 85% VO_2 max cycling bout (Kingsley, Miller, Kilduff, McEneny, & Benton, 2006). These results are somewhat pertinent to our findings due to the fact that phosphatidylserine is a soy bean-derived cellular membrane phospholipid in which AA is present and able to re-esterify at the sn-2 position of this compound (Yamashita, Sugiura, & Waku, 1997). However, to our knowledge the enzyme-mediated esterification of AA into phosphatidylserine has only been shown to occur in rat liver microsomes (Yamashita et al., 1997). Furthermore, AA incorporation into phospholipids is known to occur at a greater rate with phosphatidylethanolamine (Kaya & Miura, 1982) and AA content is known to be higher with phosphatidylcholine and phosphatidylinositol (in which AA content is the highest) (Bos et al., 2004; Zhou & Nilsson, 2001). Kingsley *et al.* (2006) stipulated that the increases in exercise capacity due to phosphatidylserine supplementation may occur due to an up-regulation in Ca^{2+} ATPase pump in the sarcoplasmic reticulum which delays the onset of fatigue in skeletal muscle by sustaining calcium homeostasis (Kingsley et al., 2006). But, the authors have not quantitatively evaluated these intracellular events and the results from their study have not been successfully duplicated to date. Furthermore, AA is known to stimulate

intramuscular Ca^{2+} release in both skeletal and cardiac muscle cells, which is counterintuitive to the aforementioned explanation (Dettbarn & Palade, 1993). Although AA and phosphatidylserine share similar physiological roles in humans, a common ergogenic property that may exist between AA and phosphatidylserine remains largely unknown.

One possible manner in which AA supplementation could contribute to increases in peak power without significantly altering muscle mass involves an increase in motor neuron excitability via an increase in 5-lipooxygenase metabolites (i.e. leukotriene B_4, -C_4) from skeletal muscle which, in turn, enhances the release of neurotransmitters from pre-synaptic neurons at the neuromuscular junction (Coffey, Herrero, Sihra, Sanchez-Prieto, & Nicholls, 1994; Herrero, Miras-Portugal, & Sanchez-Prieto, 1992). Past research has also shown that AA increases the secretion of the neurotransmitter glutamate from nerve terminals (Herrero et al., 1992), inhibits the resequestering of glutamate into pre-synaptic terminals, and/or potentiates *N*-methyl-D-aspartate-type receptor channel currents (Breukel, Besselsen, Lopes da Silva, & Ghijsen, 1997). Related literature has shown that an elevation in glutamate secretion correlates with an increase in motor neuron activity (McCall & Aghajanian, 1979), and that an increase in glutamate secretion is associated with neural stimulation and an increase force production (deGroot, Zhou, & Carlton, 2000). On a related note, an increase in motor neuron activity has been shown to occur through plyometric resistance training and is purported to be conducive to generating a higher power output (Masamoto, Larson, Gates, & Faigenbaum, 2003).

From an exercise perspective, a possible increase in neurotransmission that may occur with AA supplementation could lead to a metabolic condition deemed post-

activation potentiation whereby there is a concomitant, prolonged elevation in intramuscular calcium levels and, thus, more myofibrillar cross-bridging that is apt to occur (Sale, 2004). However, procedures such as surface electromyography and/or twitch interpolation were not performed in this study and, thus, an increase in neuromuscular activity that could potentially occur with AA supplementation can not be concluded. Therefore, it can only be hypothesized that AA supplementation increases neuromuscular activation through the production and leaching of eicosanoids out into the neuromuscular junction which subsequently increases the secretion of neurotransmitters that are able to increase skeletal muscle contractility. It should be noted that a sustained elevation in cytosolic calcium levels may also occur independent of neurotransmission in the AA group through an increase in Ins(1,4,5)P_3 ligand binding which also leads to an elevation in cytosolic calcium levels via possible increases in FP receptor ligand binding. Regardless, this is the first study to demonstrate that AA supplementation elicits significant increases in relative peak power during an anaerobic bike sprint and the aforementioned neuromuscular mechanisms should be further explored.

Alterations in FP and EP_3 Receptor Densities

Ample *in vivo* and *in vitro* evidence exists demonstrating that exogenous hormone supplementation at supra-physiological levels can up-regulate intracellular levels of its complimentary receptor (Doumit, Cook, & Merkel, 1996; Grino, Griffin, & Wilson, 1990). These elevations in explicit complimentary receptor levels, as with elevations in intramuscular androgen receptors, potentiates increases in strength and fat-free mass through receptor-mediated signaling (Inoue, Yamasaki, Fushiki, Okada, & Sugimoto, 1994). Despite the fact that $PGF_{2\alpha}$ levels approached statistical significance ($p = 0.14$)

and demonstrated a moderate effect size with AA supplementation (d = 0.53), there were no significant differences between groups in FP receptor density (p = 0.64) in skeletal muscle tissue. Previous research has shown that the FP receptor gene is up-regulated by IL-1ß in a time- and concentration-dependent manner (Narko, Ritvos, & Ristimaki, 1997) as well as chorionic gonadotropin (a hormone produced by the placenta during pregnancy) and 8-bromo-cAMP (a related derivative of cAMP) (Ristimaki, Jaatinen, & Ritvos, 1997). However, the present results suggest that AA supplementation, at the dosage employed, is ineffective in increasing FP receptor density in resistance-trained males.

Data in regards to EP_3 receptor densities between groups demonstrate that AA supplementation is also ineffective in increasing EP_3 receptor density despite the fact that increases in PGE_2 approached statistical significance in the AA group (p = 0.06) and demonstrated a moderate effect size (d = 0.68). Although the EP_3 receptor is present at concentrations lower than other EP receptor subtypes in skeletal muscle (Tran, Gleason, & Robertson, 2002), it is still considered to be expressed at moderate levels that are certainly able to affect physiological processes (Kotani *et al.*, 1995). As previously mentioned, the EP_3 receptor is known to activate the G_i-protein which subsequently inhibits adenyl cyclase activity and diminishes cAMP production (Bos et al., 2004). Interestingly, cAMP has been shown to oppose MAPK signaling and is thought to inhibit to cell growth during cancerous states (Filardo, Quinn, Frackelton, & Bland, 2002). Due to the fact that MAPK pathways, specifically p38 and ERK1/2 are also activated during muscular concentric/eccentric actions reminiscent of resistance training (Martineau & Gardiner, 2001; Wretman *et al.*, 2001), it is conceivable that the generation of cAMP also

inhibits skeletal muscle hypertrophy by inhibiting myogenic signaling upstream from MAPK-dependent gene transcription. However, recent literature also suggests that the generation of cAMP through β-adrenergic receptor stimulation strongly inhibits intramuscular Ca^{2+}-dependent proteolysis (Navegantes, Machado, Resano, Migliorini, & Kettelhut, 2003; Navegantes, Resano, Migliorini, & Kettelhut, 2000, 2001). Therefore, an intramuscular signaling paradigm may exist whereby PGE_2-EP_2 ligand binding could either inhibit Ca^{2+} proteolysis and/or diminish MAPK signaling. Hence, it is not known whether the seemingly higher PGE_2 levels in the AA group can be viewed as advantageous or deleterious for skeletal muscle growth. Additional research correlating EP_3 receptor signaling in skeletal muscle with concomitant MAPK protein activity, calpain protease activity, alterations in MHC mRNA expression, and/or changes in translational markers following a bout of resistance training should be conducted to unveil the extent that EP_3 receptor signaling has on muscular hypertrophy.

Changes in Prostaglandins and Other Hormones

Past research has demonstrated that exogenous AA supplementation increases circulating PG levels in animals (Whelan, Li, & Birdwell, 1997) and humans (Kelley et al., 1998). However, this study demonstrated that there were no significant differences in resting serum concentrations of PGE_2 ($p = 0.06$) and $PGF_{2\alpha}$ ($p = 0.14$); although, the increases in both prostaglandins approached statistical significance in the AA group. Based upon effect-size calculations, however, there seems to be a correlative relationship between elevations in both prostaglandins and AA supplementation ($PGF_{2\alpha}$: $d = 0.53$, PGE_2: $d = 0.67$). Therefore, more research should be conducted to further evaluate the effects that differing dosages [i.e. 1.5 g/d as seen with the study by Kelly *et al*. (1998)] or

temporal patterns of AA supplementation exerts on circulating prostaglandin levels. Potential increases in $PGF_{2\alpha}$ levels in the AA group may increase FP receptor ligand binding which might contribute to the significant alterations seen in gene expression and peak power output. As mentioned previously, $PGF_{2\alpha}$ has also been shown to exert favorable increases in muscle protein synthesis following resistance training *in vivo* (Trappe et al., 2002) and is known to increase intracellular calcium levels and muscle hypertrophy *in vitro* via myocellular accretion (Horsley & Pavlath, 2003). Again, the latter effect is proposed to be caused by the calcineurin-mediated NFAT nuclear translocation which has also been shown to modulate the myogenic gene transcription patterns and mediates fiber type transition that occurs in response to resistance training (Allen & Leinwand, 2002; Dunn et al., 1999). However, this hypothesis is inconclusive at this juncture and warrants further investigation (i.e. following an acute training bout) into whether or not the calcineurin-NFAT pathway is significantly impacted with AA supplementation and concomitant resistance training.

It is also unknown if the non-significant increases in PGE_2 levels impacted EP_3 receptor ligand binding in skeletal muscle and contributed to the significant alterations seen in gene expression and peak power output. Increases in PGE_2 levels can lead to various pleiotropic events due to the presence of numerous EP receptor subtypes that exist in most tissues (Bos et al., 2004). For instance, intramuscular proteolytic activity has been associated with increases in PGE_2 levels and concomitant increases in cAMP via the ligand binding of the $EP_{2/4}$ receptors (Gibson, Poyser, Morrison, Scrimgeour, & Rennie, 1991; Rodemann et al., 1982). Other compelling data shows that PGE_2 significantly decreases MAPK signaling in aortic smooth muscle cells through the

generation of cAMP and activation of protein kinase A (Graves *et al.*, 1993). These decrements in MAPK activation are thought to exist via a PKA-dependent inhibition of Ras activation downstream from tyrosine kinase receptor signaling (which is prevalent in IGF-1 signaling and pertinent to skeletal muscle hypertrophy). However, the fact that that there were no significant group x time interactions in strength and fat-free mass changes in the AA group practically negates the contention that AA supplementation could lead to perpetual skeletal muscle atrophy via sustained increases in PGE_2 levels. Thus, the physiological influence that PGE_2 exerts on skeletal muscle remains largely unknown and should be further explored.

Interestingly, there is a statistical trend in the decrement in IL-6 levels in the AA over the course of the intervention ($p = 0.07$). Likewise, effect-size calculations revealed that a moderate relationship existed between AA supplementation and IL-6 decreases ($d = 0.67$). IL-6 is a proinflammatory cytokine that is generally produced by T-cells, macrophages, and endothelial cells (Ray et al., 1989) and is secreted in response to infection and/or cellular trauma. IL-6 also signals the hepatic formation of acute-phase proteins as well as B- and T- cell growth and differentiation (Castell *et al.*, 1989; Muraguchi *et al.*, 1988; Takeda *et al.*, 1998) and is also deemed a potentially catabolic cytokine that is largely responsible for sustaining glucose homeostasis (Steinacker, Lormes, Reissnecker, & Liu, 2004). The genetic expression of IL-6 mRNA is known be up-regulated in skeletal muscle in response to exercise training (Steensberg *et al.*, 2000) and IL-6 has been shown to instigate intramuscular proteolytic pathways by enhancing lysosomal cathepsin and ubiquitin activities (Ebisui *et al.*, 1995; Fujita *et al.*, 1996; Tsujinaka *et al.*, 1995; Tsujinaka *et al.*, 1996; Zamir *et al.*, 1992). Furthermore,

Tsujinaka *et al.* (1996) discovered that the over-expression of IL-6 in transgenic mice causes significant muscle atrophy through directly mediated effects and/or potential decrements in IGF-1 circulation. Thus, a potential decrement of IL-6 levels that could exist with AA supplementation could be advantageous due to the possible catabolic and/or proteolytic nature of this cytokine and future research needs to be conducted to definitively ascertain if the timing and/or dosage patterns of AA supplementation exacerbates IL-6 decreases as well as the diminishing effects that these decrements may exert on the lysosomal and/or Ub-proteolytic pathways in skeletal muscle.

MHC mRNA and Protein Expression

Although there were no significant changes between groups in MHC I, IIa, and IIx mRNA expression, statistical trends and moderate effect-sizes exist for the down-regulation in all of these MHC isoform genes. In general, the concomitant down-regulation MHC IIx protein expression and up-regulation in MHC IIa protein expression is purported to occur as an adaptation to chronic resistance exercise due to the hormonal alterations and energy requirements required to lift heavy loads repetitiously (Fry, 2004). Furthermore, acute changes in MHC mRNA expression following a bout of resistance training provide the genetic template that is transcribed into MHC proteins. Unfortunately, MHC mRNA expression was not determined immediately following resistance training but rather 48 hrs following exercise. As a result, it is conceivable that by the time point following exercise, there could have been a perpetual down-regulation in the mRNA of all three MHC isoforms.

Interestingly, a research group has identified a CArG-like element containing a consensus binding site for serum response factor (SRF) that inhibits MHC IIx promoter

activity both *in vitro* and *in vivo* (Allen et al., 2001; Allen, Weber, Sycuro, & Leinwand, 2005). In order for SRF to bind to DNA binding elements it must first be phosphorylated by upstream kinases (Prywes, Dutta, Cromlish, & Roeder, 1988) and then form a ternary complex with other activated transcription factors, including Elk-1, that are most likely phosphorylated by MAPK (ERK1/2 and FRK) substrates. However, Allen and colleagues (Allen et al., 2001; Allen *et al.*, 2005) concluded that the transcription factor that acts as a repressor to the MHC IIx promoter is not SRF and has not yet been identified. Regardless, an increase in FP/EP_3 receptor ligand binding may exist with AA supplementation that leads to the increase in downstream ERK phosphorylation. This potential up-regulatory effect in ERK signaling may also activate the unidentified repressor protein that is known to decrease MHC IIx mRNA expression.

Based upon the concurrent trends seen in MHC I and MHC IIa mRNA decrements, it also remains possible that signaling downstream from the EP_2 receptor may oppose these effects via cAMP production/PKA activation and lead to the decrements seen in overall myogenic gene expression. As mentioned previously, an increase in cAMP production and PKA activation has been shown to disrupt Ras activation which ultimately decreases ERK1/2 phosphorylation. Hence, due to the fact that hormones such as IGF-1Ea and mechano-growth factor (IGF-1Eb) are known be locally expressed following mechanical loading and elicit hypertrophic effects (i.e. increases in myogenic gene transcription) via MEK-ERK signaling (Adams, 2002), an increase in skeletal muscle EP_2 receptor ligand binding that could occur with AA supplementation may oppose the hormonal signal transduction mechanisms of these anabolic hormones, and thus down-regulate all MHC mRNA expression patterns. Again,

since muscle biopsies were taken at 48 hrs following resistance training in this study, it is difficult to ascertain the degree in which AA supplementation affects post-exercise MHC mRNA expression.

There was a significant increase in MHC IIa protein content in both groups over the course of the study and no significant changes in MHC I and MHC IIx protein isoform levels. It is very likely that these effects can likely be attributed to the body-building nature of the training regimen (i.e. 3 sets of 8-10 repetitions using moderate lifting intensities) in concert with the addition of a protein supplement that was provided to each subject over the course of the study. Numerous studies have shown that routinely performed resistance training hypertrophies type I and IIA muscle fibers and elicits a fiber-type transformation from IIB/X fibers to IIA fibers (Fry, 2004). It should also be known that each subject in the current investigation was administered a whey protein/carbohydrate supplement and was told to ingest the supplement immediately following each training session. Previous research has shown that protein/carbohydrate feeding increases muscle protein synthesis after resistance training. One investigation (Koopman *et al.*, 2005) demonstrated that systemic protein degradation was lower while whole body protein synthetic rates were higher in subjects who consumed drinks containing carbohydrate and protein, or carbohydrate, protein, and leucine (which is prevalent in whey protein) in comparison to carbohydrate ingestion immediately following 45-min of resistance training. Experimentally investigating the aforementioned findings over more chronic time periods has demonstrated that post-exercise protein supplementation significantly increases fat-free mass and type I and IIA fiber hypertrophy in individuals engaging in regular resistance training (Andersen *et al.*, 2005).

Nonetheless, there were no significant differences in MHC I, IIa, or IIx protein levels between groups. Previous research has also shown that AA and $PGF_{2\alpha}$ up-regulate PI-3K-$p70^{S6k}$ signaling in vascular smooth muscle cells (Kunapuli et al., 1998; Neeli et al., 2003; Rao et al., 1999). The PI-3K-$p70^{S6k}$ cascade is known to initiate mRNA translation in skeletal muscle and an increase in $p70^{S6k}$ activation is highly correlated with skeletal muscle hypertrophy (Baar & Esser, 1999). However, the trends seen in the decreases in MHC mRNA expression in the present investigation may provide insight into the ineffectiveness that AA supplementation has on increasing the MHC I and IIa mRNA templates necessary for protein synthesis. Follow-up investigations applying a more mechanistic *in vivo* approach should be followed in order to further delineate if AA supplementation increases myogenic signal transduction (i.e. MAPK signaling), MHC gene expression, and the subsequent transcriptional patterns that may occur.

Changes in Whole Blood Variables and Serum Chemistry Markers

Consistent with the recent literature (Kelley et al., 1998), our study demonstrates that AA supplementation affects PG levels without altering blood leukocyte levels or immune function. The neutrophil:lymphocyte ratio is a physiological marker of stress on the immune system and has been shown to increase in response to rigorous exercise (Nieman, 1997). Therefore, the observed increases in the neutrophil:lymphocyte ratio in both groups in the present study can most likely be attributed to the strenuous prescribed resistance training regimen in which each subject was required to participate. Furthermore, although these subjects were previously trained, the volume of the prescribed regimen did typically alter from the subjects' prior training program, thus imposing a chronically differing stressor on the immune system. Regardless, neutrophil

and lymphocyte levels in both groups remained within normal clinical ranges throughout the duration of the study.

There were no significant differences between groups for serum cholesterol, liver enzyme or kidney function markers as well. However, there was a non-significant elevation in serum triglycerides in the AA group ($p = 0.09$). Related research has demonstrated that AA does significantly elevate serum triglycerides in rodents (Whelan, Surette, Li-Stiles, & Bailey, 1995), although the rationale behind this elevation remains unknown. Regardless, the elevations in the AA group did not exceed the upper range for acceptable physiological triglyceride values. Therefore, these results suggest that AA supplementation during an extended period of resistance training is physiologically well-tolerated. Furthermore, it should be advisable that individuals with dyslipidemia monitor serum triglyceride levels while supplementing with AA.

Conclusions

In conclusion, 50 days of AA supplementation in healthy, resistance-trained males significantly increases relative peak power in resistance-trained males. There is also evidence suggesting that AA supplementation appears to diminish circulating levels of IL-6, which may be advantageous for building muscle due to the possible proteolytic activity of IL-6 while leading to non-significant elevations in PGE_2 and $PGF_{2\alpha}$ levels. AA supplementation may also decrease the basal expression of MHC I, IIa, and IIx mRNA. However, it should be noted that the aforementioned findings in regards to hormonal and transcriptional alterations were non-significant trends with moderate effect-sizes. Regardless, this is the first investigation showing that 50 days of AA supplementation may elicit favorable alterations in training adaptations and the hormonal

status in healthy, resistance-trained males. This study warrants a need for future research in regards to the ergogenic potential that may exist with AA supplementation. Furthermore, additional research should also elucidate acute *in vivo* intramuscular responses that occur after a bout of resistance training as well as the phenotypic and chronic molecular training adaptations that occur with differing temporal and dosage quantities of AA supplementation.

APPENDICES

APPENDIX A

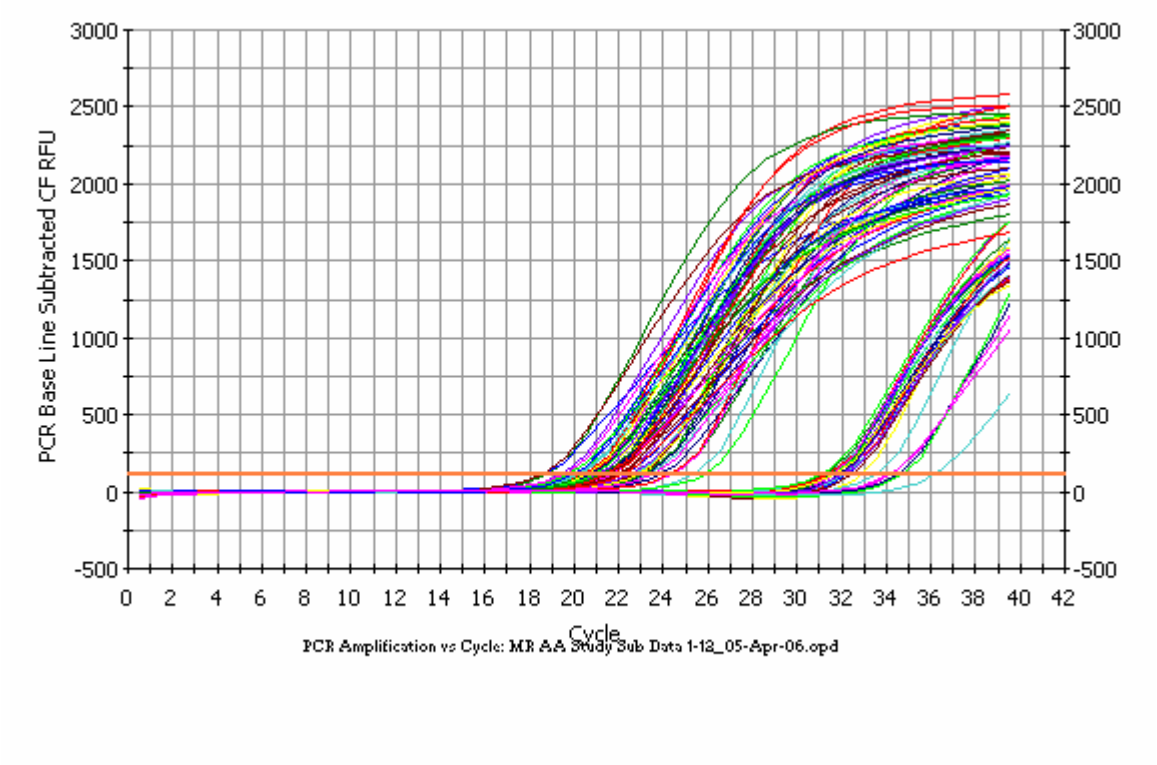

Fig. A.1. This graph represents MHC I, -IIa, and –IIx amplification by allocating using real-time PCR using the iCycler® (Bio-Rad, Hercules, CA). The complimentary DNA for MHC I, -IIa, and –IIx mRNA was amplified over 40 cycles. β-actin cDNA was also amplified and used as an internal standard in order to express relative gene expression.

APPENDIX B

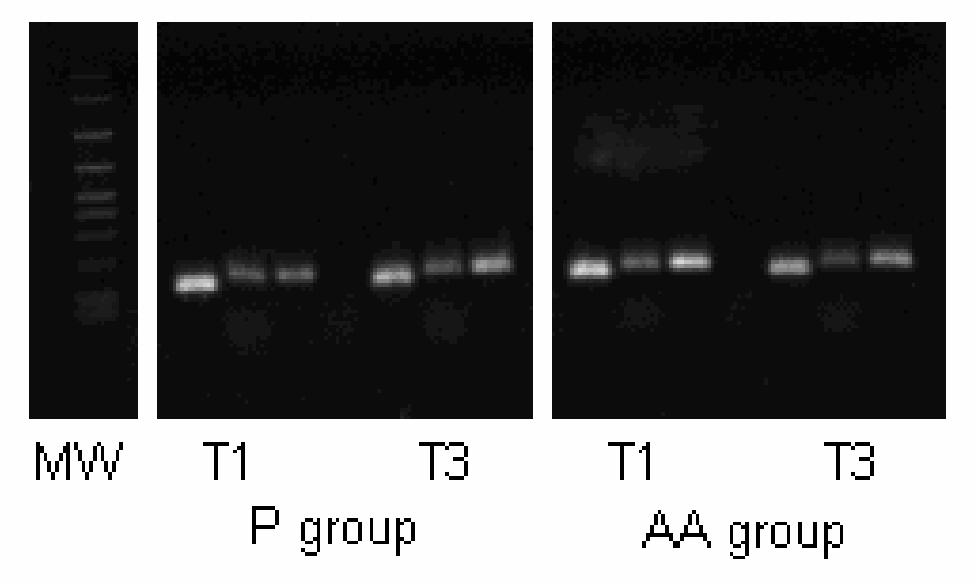

Fig. B.1. This is an agarose gel confirming the positive amplification of MHC I, -IIa and -IIx mRNA.

APPENDIX C

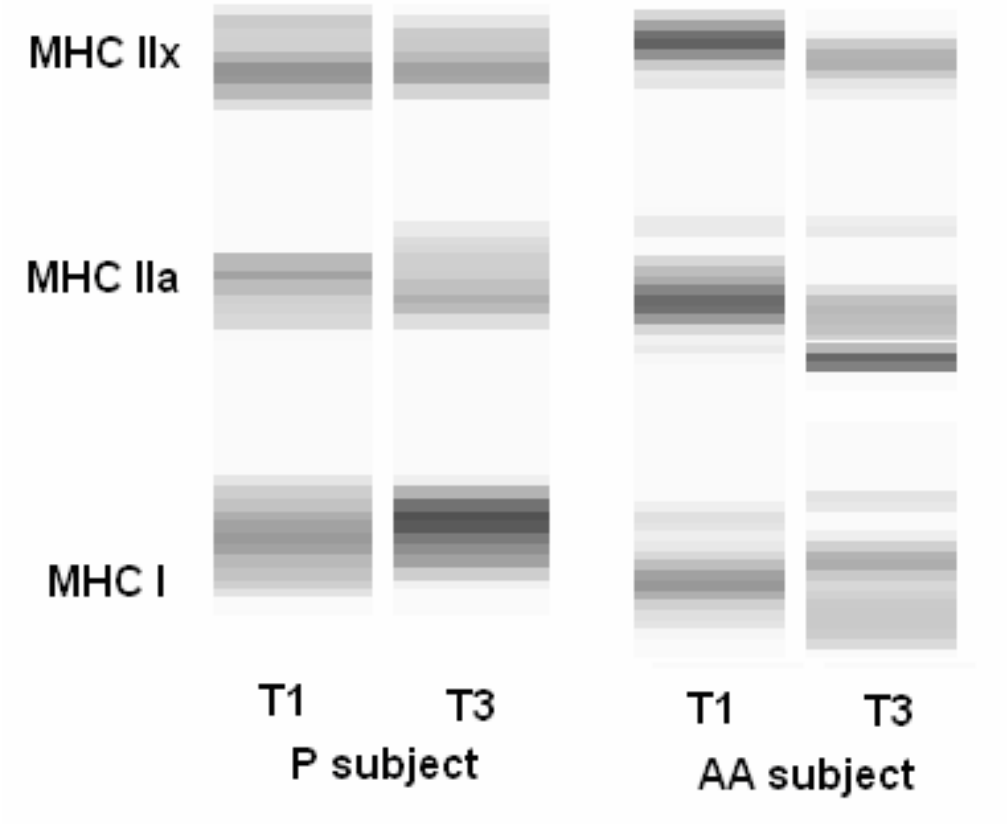

Fig. C.1. This figure represents protein staining using the Experion Pro260 Automated Chip Protein Electrophoresis System.
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APPENDIX D

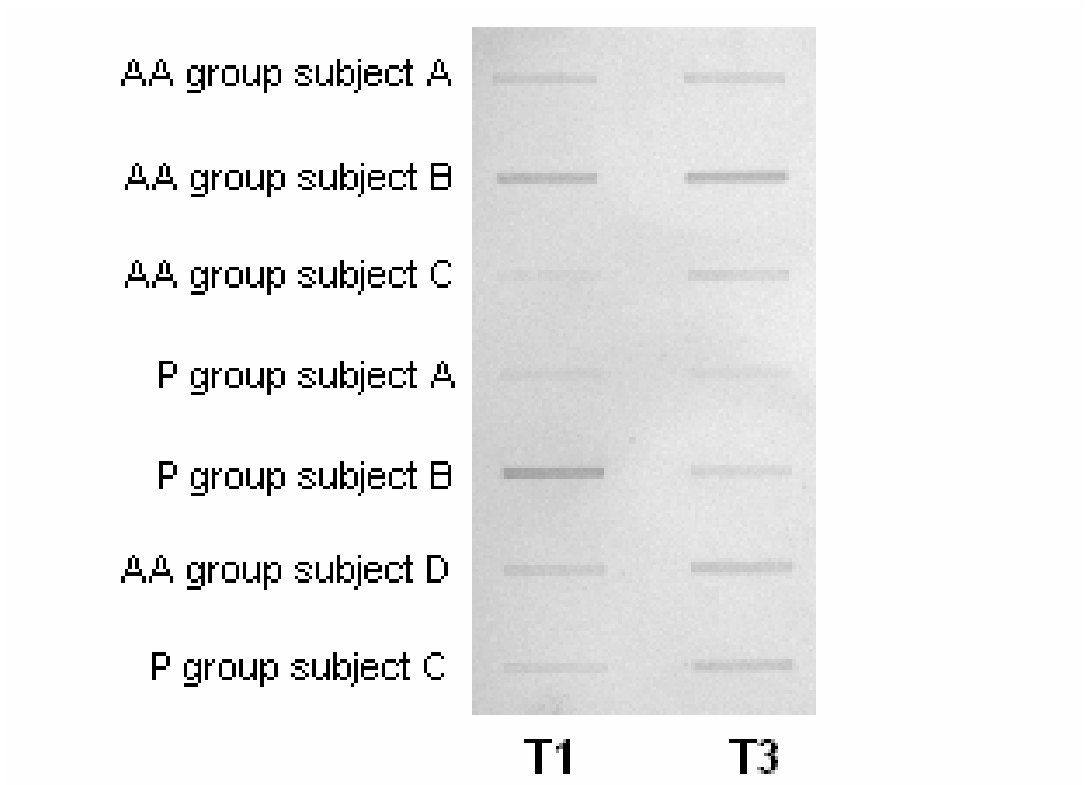

Fig. D.1. Band density was determined using the Chemi-Doc Imaging System (Bio-Rad, Hercules, CA) and relative changes in band density were used to assess changes in receptor levels from baseline (Day 1) values.

APPENDIX E

BAYLOR UNIVERSITY
Department of Health, Human Performance, & Recreation
Informed Consent Form

Title of Investigation:	**Effects of Arachidonic Acid Supplementation on Body Composition and Training Adaptations**
Principal Investigator:	Richard B. Kreider, PhD, FACSM, MX Chair, Department of HHPR, Baylor University
Coinvestigators:	Chris Rasmussen, MS, MX, CSCS, EPC Coordinator, Exercise and Sport Nutrition Lab, Baylor University
	Darryn Willoughby, PhD Director, Exercise Biochemical and Nutrition Lab, Baylor University
	Mike Greenwood, PhD Graduate Program Director, Department of HHPR, Baylor University
	Mike Iosia, PhD, Exercise & Sport Nutrition Lab, Department of HHPR, Baylor University
	Ron Wilson, MD Department of HHPR, Baylor University
Sponsor:	Molecular Nutrition, LLC (Jupiter, FL)

Rationale:

Arachidonic acid (AA) is a polyunsaturated essential fatty acid (PUFA) which is primarily found in fatty parts of red meat and fish and is normally consumed in small amounts in the regular diet [1]. Research has indicated a number of potential health benefits from AA supplementation. Of particular interest in this study, research has indicated that AA stimulates localized hormones (prostaglandins) that influence protein synthesis. Consequently, dietary supplementation of AA has been theorized to stimulate muscle growth during resistance training. The purpose of this study will be to examine the effects of AA supplementation (1 gram/day for 8-weeks) on body composition and training adaptations in experienced resistance-trained males.

Description of the Study:

I will be one of approximately 30 apparently healthy trained (at least 1 year of training 3 times per week) male weight lifters between the ages 18 to 50 who will participate in this study lasting approximately 10 weeks. During an initial familiarization session, I will be informed of the requirements of the study and sign an informed consent statement in compliance with the Human Subjects Guidelines of Baylor University and the American College of Sports Medicine. A trained individual will examine me to determine if I am qualified to participate in this study. If I am cleared to participate in the study, I will be familiarized to the training and supplementation protocol and practice the exercise tests to be evaluated in this study on three occasions (30-second cycling sprint, bench press, and leg extension test). These sessions will take approximately 30 minutes to complete. Once I complete the familiarization sessions, I will be scheduled for baseline testing.

Prior to reporting to the lab for baseline testing, I will record all food that I eat on dietary record forms for four days (including one weekend day). I will not exercise for 48 hours nor eat for 8 hours prior to reporting to the lab for baseline testing. Once I report to the lab, I will be weighed and have my total body water determined using a bioelectrical impedance analyzer (BIA). The BIA analysis will involve lying down on my back on a table and having two small electrodes placed on my right hand and my right foot. The analyzer wires will be attached and a small and safe current (500 micro-amps at a frequency of 5- kHz) will pass through my body so that the amount of water can be measured. This analyzer is commercially available and has been used in the health care/fitness industry as a means to assess body composition and body water for over 20 years. The use of this device has been approved by the Food and Drug Administration (FDA) to assess total body water and the current to be used has been deemed safe.

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My body composition and bone density will then be determined by using a Hologic 4500W dual energy x-ray absorptiometer (DEXA). This will involve lying down on my back on the DEXA exam table in a pair of shorts or a gown for about 6 minutes. I understand that a low dose of radiation will scan my entire body to determine the amount of fat weight, muscle weight, and bone weight. I understand that I will be exposed to an X-ray dose that is similar to the amount of natural background radiation a person would receive in one month while living in Waco. After this test, I will have resting blood pressure determined using a standard sphygmomanometer and heart rate determined by assessing pulse rate from my wrist. This testing will take about 20 minutes to complete.

I understand that I will then donate about 20 milliliters (4 teaspoons) of venous blood from a vein in my arm. Blood samples will be obtained using standard/sterile procedures using a needle inserted into a vein in my arm. I understand that personnel who will be taking my blood are experienced in phlebotomy (procedures to take blood samples) and are qualified to do so under guidelines established by the Texas Department of Health and Human Services. This will take about 5-minutes.

I will then perform a one repetition maximum (1RM) test on the isotonic bench press and leg press. I will then rest 10 minutes and perform a 30-second Wingate anaerobic capacity test on a Lode computerized cycle ergometer. These tests will help determine if arginine-AKG supplementation affects exercise capacity and take about 45 minutes to complete.

I understand that I will also donate muscle biopsies prior to and following training in order to determine how my muscle adapts to training and the supplementation protocol. I understand that Darryn Willoughby, PhD (Director of the Exercise Biochemical and Nutrition Lab [EBNL] at Baylor University) and a research nurse will be responsible for collecting muscle biopsy samples. The muscle biopsy procedure basically involves sterilizing and anesthetizing the biopsy site on the outside middle of my thigh. Then, a small incision (about 1 centimeter or the width of a finger) is made in my skin and fascia in order to expose the underlying muscle. Approximately 80 to 160 milligrams of muscle tissue is then extracted from the thigh muscle using a sterile muscle biopsy needle according to standard clinical procedures. Once the sample is obtained, the area of incision is cleaned and dressed. I will then be given a list of post-biopsy instructions on how to clean and care for the incision in order to promote healing. I understand that I will have to return to the lab within 24 to 72 hours so that the biopsy sites can be checked for infection and improper healing.

I understand that after baseline testing, I will be matched based on age, muscle mass and training history to randomly and blindly ingest (meaning I nor the investigators will know which groups until the end of the study) capsules containing a placebo or arachidonic acid (*X-Factor™, Molecular Nutrition LLC, Jupiter, FL*). I understand that I must take one capusule (250 mg) of the supplement four times per day (morning, lunch, dinner, and prior to sleep) for 8-weeks. This will provide a total daily intake of 1 gram/day. I understand that the supplements will be prepared in gel capsule form and packaged in generic bottles for double blind administration by Molecular Nutrition, LLC (Jupiter, FL). I understand that my compliance in taking the supplements will be monitored by returning empty bottles to the ESNL when I report back for testing after 4 and 8 weeks of supplementation. I understand that if I do not take my supplements I will be removed from the study. In addition, I understand that I should eat a normal but slightly hypercaloric diet (an extra 500 kcals/day) consisting of at least 2 grams per kilogram (2.2 lbs) of protein per day (e.g., 180 – 220 grams/day for individuals weighing 90 – 110 kg) in order to ensure I have enough calories and protein to promote gains in muscle during training. In order to accomplish this goal, I will be provided a protein supplement to take two times per day during the study. I also understand that I should not regularly consume foods or supplements known to be high in omega-3 fatty acids (e.g., fish oil, flax seed oil, omega-3 fatty acids, omega-6 fatty acids, cold water fish [e.g., salmon, cod], olive oil, sesame oil, peanut butter, N-Acetyl-Cysteine, conjugated linoleic acid) or take over-the-counter anti-inflammatory medications (e.g., Acetaminophen, Ibuprofen, Aspirin, Non-Steroidal Anti-Inflammatory Drugs).

I understand that during the supplementation period, I must adhere to a standardized 4-day per week resistance training program (two upper body and two lower body workouts). The upper body resistance-training program will consist of nine exercises (bench press, lat pull, shoulder press, seated rows, shoulder shrugs, chest flies, biceps curl, triceps press down, and abdominal curls) and lower extremity program will consist of seven exercises (leg press or squat, back extension, step ups, leg curls, leg extension, heel raises, and abdominal crunches) performed twice per week. I understand that I should perform 3 sets of 10 repetitions with as much weight as I can lift (typically 60 – 80% of 1RM) for each lift. Rest periods between exercises should last no longer than 3 minutes and rest between sets should last no longer than 2 minutes. I understand that I should conduct training at the Student Life Center (SLC) at Baylor University or at my local gym and that I must document my training on exercise cards and have them signed off by a floor supervisor to verify compliance and monitor progress. I understand that the workouts will take about 60-75 minutes to complete.

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I understand that I will be required to call a research assistant on a weekly basis to describe my progress in the study as well as whether I have experienced any unexpected problems or adverse events from participating in this study. If I don't call in, I understand that the research assistant will call me. I understand that if clinically significant side effects are reported, I will be referred to discuss the problem with a research nurse. Upon their discretion, I may be referred to discuss the matter with Dr. Ronald Wilson to determine whether any medical treatment is needed and/or whether I can continue in the study. I understand that if I fail to report my progress and health status to the research assistant I may be removed from the study.

I understand that following 4 & 8 weeks of supplementation, I will be scheduled to return to the lab to repeat the battery of tests described above. I agree to do my best to: 1) follow the instructions outline by the investigators; 2) show up to all scheduled testing times; and 3) take the supplements as instructed. I agree not to take any other nutritional supplements or performance enhancing aids during this study (i.e., vitamins/minerals, creatine, HMB, androstenedione, DHEA, etc). I agree not to take any non-medically prescribed medications and to report any medication that is prescribed for me to take during this study. I understand that if I take any other nutritional supplements or medications during the course of the study that may affect body composition or strength that I may be removed from the study.

Exclusionary Criteria

I understand that in order to participate in the study, a trained individual will examine me to determine whether I qualify to participate. I understand that I will not be allowed to participate in this study if: 1.) I have any metabolic disorder including known electrolyte abnormalities; heart disease, arrhythmias, diabetes, thyroid disease, or hypogonadism; 2.) I have a history of hypertension, hepatorenal, musculoskeletal, autoimmune, or neurologic disease; 3.) I am taking thyroid, hyperlipidemic, hypoglycemic, anti-hypertensive, or androgenic medications; 4.) I have taken nutritional supplements that may affect muscle mass (e.g., creatine, HMB), anabolic/catabolic hormone levels (androstenedione, DHEA, etc), or weight loss (e.g., ephedra, thermogenics, etc) within three months prior to the start of the study; and/or, 5.) I report any unusual adverse events associated with this study that in consultation with the supervising physician recommends removal from the study. I understand that the only exception will be if my personal physician does not feel that any condition that I have or medication that I am currently taking would prohibit me from participating in this study. In this case, I must obtain a letter signed by my physician approving my participation in this study.

I have reported all nutritional supplements, medically prescribed drugs, and non-medically prescribed drugs that I am presently taking. I have completed medical history questionnaires and am not aware of any additional medical problems that would prevent me from participating in this study. I agree to report all changes in medical status, nutritional and/or pharmacological agents (drugs) that I take during the course of the investigation to Richard Kreider, PhD (254/710-4003). I understand that if I experience any unexpected problems or adverse events from participating in this study I may be referred to discuss the problem with a research nurse (Melyn Galbreath, RN or Sherri Williams, RN). Upon their discretion, I may be referred to discuss the matter with Dr. Ron Wilson to determine whether any medical treatment is needed and/or whether I can continue in the study.

Risks and Benefits

I understand that the supplements to be investigated in this study have been studied for various medical uses in humans and that research has demonstrated that oral administration of these compounds is not associated with any significant medical side effects. These nutrients are currently available in over the counter nutritional supplements sold in United States. However, as with the vast majority of nutritional supplements, I understand that the FDA may not have evaluated the safety or marketing claims of arachidonic acid.

I understand that I will be exposed to a low level of radiation during the DEXA body composition tests, which is similar to the amount of natural background radiation a person would receive in one month while living in Waco, TX. In addition, a very low level of electrical current will be passed through my body using a bioelectrical impedance analyzer. This analyzer is commercially available and has been used in the health care/fitness industry as a means to assess body composition and body water for over 20 years. The use of the BIA and DEXA analyzers has been shown to be safe methods of assessing body composition and total body water and is approved by the FDA. I also understand that I will have about 4 teaspoons (20 milliliters) of blood drawn from a vein in my forearm using a sterile needle and blood tubes by an experienced phlebotomist three (3) times during this study. This procedure may cause a small amount of pain when the needle is inserted into my vein as well as some bleeding and bruising. I may also experience some dizziness, nausea, and/or faint if I am unaccustomed to having blood drawn. I will also

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donate a muscle biopsy prior to and following the study to assess the effect of training and dietary supplementation on muscle growth. I understand that I may experience some anxiety before this procedure regarding a perception of pain or discomfort. The biopsy procedure may cause a small amount of bleeding and/or pain as the incision is made and the sample is extracted from the muscle. However, once the anesthesia takes affect, there is usually only mild pressure and a small amount of bleeding as the needle is inserted and extracted. During the biopsy procedure, I may experience a slight localized cramping followed by brief and minor aching but these symptoms usually go away when the needle is withdrawn. Frequently, subjects feel little or no sensation at all. I understand that although the muscle selected for biopsy (vastus lateralis) has no major blood vessels or nerves in the areas where the biopsy needle will be inserted, there is the rare occurrence of compressing or cutting small nerve branches, which can sometimes cause temporary tingling and numbness in the skin. These responses, when they have occurred, have dissipated in a few days or weeks. After the needle is withdrawn, pressure is applied to the site of the incision to prevent any unwarranted bleeding (there is usually very little bleeding). After the biopsy, the muscle is likely to be moderately sore for about 24 hours. This soreness is similar to muscle soreness following unusually vigorous exercise or a muscle injury especially if muscle is compressed against a bone (e.g., "charley horse"). Complications accompanying this procedure are rare and no complications have been observed in subjects who have donated biopsies in the EBNL in previous studies. The primary risks, however, include bleeding, bruising, infection, and slight scarring of the skin. Some individuals may develop keloid scarring at the site of incision. Also, some individuals may have an allergic reaction to the anesthetic, such as a local rash, or difficulty breathing. I understand that the exercise tests that will be performed may cause symptoms of fatigue, shortness of breath, and/or muscular fatigue/discomfort. The exercise tests may also cause short-term muscle soreness and moderate fatigue for several days following the tests. I understand that I may also experience muscle strains/pulls during the exercise testing and/or training program. However, these risks will be similar to the risk of participating in my normal training program. I also understand that trained, non-physician exercise specialists certified in CPR will supervise exercise assessments. I understand that a telephone and an automated electronic defibrillator is in the laboratory in case of any emergencies and that there will be no less than two researchers working with me during each testing session. I understand that emergency procedures are posted in the lab in the unlikely event that any emergency may arise.

I understand that the main benefit that I may obtain from this study is that if this nutritional supplement is effective, there is a possibility that I may experience greater training adaptations. I may also gain insight about my health and fitness status from the assessments to be performed. However, even if I do not experience individual benefit, I understand that participating in this study will help to determine whether arachidonic acid supplementation may enhance training adaptations and/or performance.

Alternative Treatments

This is not a medical treatment. Therefore, if medical treatment is needed, I must continue to obtain treatment for any medical problem I might have from my personal physician.

Costs and Payments

If I am a Baylor University student, I will not receive any academic credit for participating in this study. Eligible subjects will be paid $150 for completing all familiarization and experimental testing sessions as well as turning in all required materials (i.e., food logs). I also understand that I will be given free assessments during the course of the study as described above and may receive information regarding results of these tests if I desire.

New Information

Any new information obtained during the course of this research that may affect my willingness to continue participation in this study will be provided to me. In addition, I will be informed of any unusual/abnormal clinical findings in which medical referral to my personal physician may be warranted. If I desire, I may request that this information be provided to my physician.

Confidentiality

I understand that any information obtained about me in this research, including questionnaires, medical history, laboratory findings, or physical examination will be kept confidential to the extent permitted by law. However, I understand in order to ensure that FDA regulations are being followed, it may be necessary for a representative of the FDA to review my records from this study which may include questionnaires, medical history, laboratory findings/reports, statistical data, and/or notes taken about my participation in this study. In addition, I understand

that my records of this research may be subpoenaed by court order or may be inspected by federal regulatory authorities. I understand that data derived may be used in reports, presentations, and publications. However, I will not be individually identified unless my consent is granted in writing. Additionally, that confidentiality will be maintained by assigning code numbers to my files, limiting access to data to research assistants, locking cabinets that store data, and providing passwords to limit access to computer files to authorized personnel only. I understand that once blood samples are analyzed that they will be discarded.

Right to Withdrawal

I understand that I am not required to participate in this study and I am free to refuse to participate or to withdraw from the study at any time. Further, that my decision to withdraw from the study will not affect my care at this institution or cause a loss of benefits to which I might be otherwise entitled. If there is concern about my medical safety, I may be referred to seek medical attention.

Compensation for Illness or Injury

I understand that if I am injured as a direct result of taking part in this study, I should consult my personal physician to obtain treatment. I understand that the cost associated with the care and treatment of such injury will be the responsibility of me or my insurance carrier. In some cases, insurers may not reimburse claims submitted for a research-related injury resulting from medical procedures or treatments performed as part of a research study. I understand that Baylor University, the investigator's institutions, and the grant sponsor have not budgeted funds to compensate me for injury or illness that may result from my participation in this study and thus will not be accountable for illness or injury acquired during the course of this study. However, I may be referred to my personal physician if any clinically significant medical/psychological findings are observed during the course of this study.

I AGREE TO INDEMNIFY AND HOLD HARMLESS BAYLOR UNIVERSITY, ITS OFFICERS, DIRECTORS, FACULTY, EMPLOYEES, AND STUDENTS FOR ANY AND ALL CLAIMS FOR ANY INJURY, DAMAGE OR LOSS I SUFFER AS A RESULT OF MY PARTICIPATION IN THIS STUDY REGARDLESS OF THE CAUSE OF MY INJURY, DAMAGE OR LOSS.

Statement on Conflict of Interest

I understand that funding for this study will be obtained from Molecular Nutrition LLC (Jupiter, FL) through a grant awarded to Baylor University. I understand that researchers involved in collecting data in this study have no financial or personal interest in the outcome of results or sponsors.

Voluntary Consent

I certify that I have read this consent form or it has been read to me and that I understand the contents and that any questions that I have pertaining to the research have been answered by Richard Kreider, PhD (Professor & Chair, Department of Health, Human Performance & Recreation, 106 Marrs McLean Gymnasium, Baylor University, phone: 254/710-4003) or one of the research associates in the ESNL (254/710-7199 or 254/710-7277). I also understand that I have the right to have any questions about the research which arise in the future answered by Dr. Kreider or a research associate before proceeding with the research. My signature below means that I am at least 18 years of age and that I freely agree to participate in this investigation. I understand that I will be given a copy of this consent form for my records. If I have any questions regarding my rights as a research subject in this study, I may contact Baylor's University Committee for Protection of Human Subjects in Research. The chairman is Dr. Matt Stanford, Professor of Psychology and Neurosciences, BSB A320, P.O. Box 97334, Waco, TX 76798-7334, phone number 254-710-2236.

Date _____ Subject's Signature _____

I certify that I have explained to the above individual the nature and purpose of the potential benefits and possible risks associated with participation in this study. I have answered any questions that have been raised and have witnessed the above signature. I have explained the above to the volunteer on the date stated on this consent form.

Date _____ Investigator's Signature _____

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Table 1. Overview of Training Study Research Design

Familiarization and Entry	Week 0 (T1)	Week 4 (T2)	Week 8 (T3)
Phone interview Familiarization session General exam to determine qualifications to participate in study.	Dietary History Quality of Life & Health Questionnaires Body Mass Body Water DEXA Body Composition Resting HR & BP Fasting Blood Sample Isotonic Bench Press 1 RM Isotonic Leg Press 1 RM Anaerobic Power Test Muscle Biopsy *Subjects matched according to FFM and age for random and double blind administration of capsules containing a placebo or arachidonic acid.* Standardized training & supplementation begins.	Dietary History Quality of Life & Health Questionnaires Body Mass Body Water Resting HR & BP Fasting Blood Sample Isotonic Bench Press 1 RM Isotonic Leg Press 1 RM Anaerobic Power Test	Dietary History Quality of Life & Health Questionnaires Body Mass Body Water DEXA Body Composition Resting HR & BP Fasting Blood Sample Isotonic Bench Press 1 RM Isotonic Leg Press 1 RM Anaerobic Power Test Muscle Biopsy

* *Dietary records will be collected every 2 weeks during the study.*

Initials ____

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