ACUTE REGULATION OF IGF-1 BY DIFFERENTIAL BINDING PROTEIN

EXPRESSION, INHIBITION, AND PROTEOLYSIS

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ACUTE REGULATION OF IGF-1 BY DIFFERENTIAL BINDING PROTEIN EXPRESSION, INHIBITION, AND PROTEOLYSIS

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DISSERTATION ABSTRACT

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The purposes of this investigation were to examine the acute regulation of IGF-1 via insulin-like growth factor binding-protein (IGFBP) expression, inhibition, and proteolysis. Eight recreationally-active college-aged males completed three identical high-intensity interval training protocols followed by each of three randomly ordered post-exercise nutritional protocols: 1) non-caloric placebo; 2) carbohydrate-only (0.85 g/kg lbw); and 3) essential amino acid/carbohydrate (0.35 g/kg and 0.5 g/kg lbw, respectively). Blood samples were obtained pre-exercise, immediately post-exercise, at ten minute intervals for 80 minutes post-exercise, and at 20 minute intervals until 160 minutes post-exercise. Blood samples were analyzed for GH, free IGF-1, insulin, hematocrit, hemoglobin, plasma amino acid concentrations, and matrix metalloproteinase

(MMP) activity. Analytes were compared relative to baseline and across conditions using ANOVAs with repeated measures. Additionally, total area under the curve scores were calculated for insulin and analyzed using one-way ANOVAs with repeated measures on condition. The *a priori* significance level for all analyses was $p < 0.05$. Significant post-exercise increases in free IGF-1 concentrations were observed in the essential amino acid/ carbohydrate group only. Additionally, significant increases in post-exercise MMP activity were observed in all groups. These results indicate that post-exercise macronutrient ratio is a determinant of free concentrations of IGF-1 and that high-intensity exercise results in increased MMP activity.

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CHAPTER I

INTRODUCTION

Acute Regulation of IGF-1 by IGFBPs

It is widely accepted that skeletal muscle contraction in the form of resistance-type exercise stimulates muscle protein synthesis during post-exercise recovery. Additionally, an additive effect has been demonstrated with certain post-exercise macronutrient ratios (39, 53, 68, 77). Positive effects on muscle protein synthesis have been measured in as little as one hour post exercise (39) and may last for up to 24 hours in trained (102) and 48 hours in untrained subjects (119). These increases in muscle protein signaling are mediated, in part, by the activation and/or inhibition of various intracellular signaling pathways.

Mammalian target of rapamycin (mTOR) is an intracellular signaling pathway that has been shown to act as a sort of metabolic 'master switch', allowing protein synthesis to commence during times of nutrient and growth factor abundance while remaining subject to inhibition by other regulatory pathways within the cell. Current theory suggests that mTOR's main mechanism of action lies in the control of protein translation initiation and elongation (53, 69, 80, 101, 134). mTOR has been shown to be directly responsible for activation of regulatory proteins leading to increases in protein synthesis rates in skeletal muscle (39, 53, 77). It is thought that activation of mTOR

requires integration of signals from at least four different sources, including phosphoinositide 3-kinase (PI3K) activation, amino acid availability, adequate cellular ATP levels, and lipid availability (23, 69, 134).

One of the most important signaling pathways regulating mTOR activity is the PI3K pathway. Many growth factors, including insulin and insulin-like growth factor-1 (IGF-1), activate PI3K (69, 134). Abundant evidence has shown that, in mammals, stimulation by growth factors results in increased protein synthesis (38, 53, 69, 77, 134). Additionally, it has been demonstrated that IGF-1 activation of the PI3K pathway suppresses protein breakdown and decreases intracellular proteolytic activity (127). Current evidence seems to indicate that PI3K acts as a sort of 'growth-factor regulator' in the control of mTOR (39, 53, 69, 134).

The IGFs are growth-promoting peptides that share significant structural homology with pro-insulin (122). Although many tissues in the body have been shown to produce and secrete IGFs, the majority of IGF in the general circulation is believed to originate from hepatic production (45, 99, 122). The general consensus in the literature is that basal levels of circulating IGFs are determined largely through the actions of human growth hormone (GH) on hepatic GH receptors (15, 16, 66, 100). IGFs exert the majority of their biological actions by binding to the Type-1 IGF receptor, which has a high degree of homology to the insulin receptor and is the only IGF receptor to have known IGF-mediated signaling function (75).

Unlike insulin, IGFs circulate in plasma complexed to a family of structurally related binding proteins (IGFBPs). Currently, IGFBPs are attributed with

three main roles within the IGF system: 1) modulation of IGF action; 2) storage facilitation of IGFs in the extracellular matrix; and 3) exertion of IGF-independent effects. While a significant body of literature exists in support of all of these, this review will focus only on the action of IGFBPs on the modulation of IGF activity.

The plasma concentrations of total IGF-1 are 50-100 times higher than the concentrations of free insulin (50). However, in contrast to circulating insulin, a small percentage of circulating IGF-1 is present in the free form in the healthy state, with the majority of circulating IGF-1 bound to IGFBPs. The IGFBPs regulate the ability of free IGF-1 to leave the vasculature and associate with the type 1 IGF receptor (72). Approximately 95% of circulating IGF-1 is bound in a ternary complex consisting of IGF-1, IGFBP-3, and a hepatically-produced acid labile subunit (ALS) (4, 73). IGF-1 bound in binary complexes with IGFBP-1, -2, -4, -5, and -6 constitute an additional 4% of circulating IGF-1, with the remainder existing as 'free' IGF-1 (4). The current theory posits that this free fraction of IGF-1 is able to bind to the type 1 IGF receptor and elicit biological effects (4, 73, 87, 114).

There is an abundance of evidence supporting the premise that all of the IGFBPs have inhibitory roles in the regulation of IGF-1 within the systemic vasculature (12, 44, 67, 75, 79, 95, 101, 117, 122, 137, 142). Additionally, there is a large body of evidence supporting the role of many of the binding proteins in facilatory and IGF-1-independent roles within the extracellular matrix (16, 26, 28, 44, 60, 67, 79). However, since this investigation centers on IGFBP regulation of IGFs within the systemic vasculature, only the inhibitory roles will be considered within this review.

A significant body of literature supports the fact that IGFBP-1 is primarily regulated by insulin (51, 60, 97). The majority of the literature suggests that IGFBP-2 is subject to dual control, with GH and amino acid availability serving as the primary regulators (24, 123, 136). IGFBP-3 has been shown to be acutely regulated via proteolysis (48, 91, 93), but no literature to date has examined the role of exercise-induced matrix metalloproteinase (MMP) secretion/release as being responsible for this proteolysis. There is, however, a significant amount of evidence supporting this in the oncology literature; and high-intensity exercise has been shown to be a potent stimulus for peripheral MMP release (47, 49, 85, 86, 126, 138). IGFBP-4 has been shown to be regulated primarily by intact IGFBP-3 and IGF-1 (48, 142).

In summary, there is a large body of evidence supporting the roles of the IGFBPs in regulating IGF-1 bioavailability, as well as some information regarding their individual regulation dynamics. However, there is a paucity of information regarding the specific integrated regulatory dynamics of IGFBPs in exercising humans. Thus, the purpose of this study was to investigate the differential contributions of IGFBPs on the bioavailability of IGF-1 and to develop additional insight into the individual regulation of each of these binding proteins in exercising humans. Eight recreationally-active college-aged males completed a high-intensity exercise protocol followed by one of three post-exercise nutritional protocols: 1) no nutritional supplement; 2) protein only; and 3) protein/carbohydrate. Blood samples were obtained at pre-determined intervals throughout the exercise sessions. Blood samples were analyzed for GH, free IGF-1, insulin, hematocrit, hemoglobin, plasma protein concentrations, IGFBP-1, IGFBP-2, IGFBP-4, and MMP activity.

Hypotheses and Rationale

Research Question 1:

Is it possible to maximize the acute expression of free, dissociable IGF-1 via an acute exercise protocol combined with manipulation of post-exercise macronutrient ratio?

Hypotheses:

H_{O1}: An acute exercise protocol and manipulation of post-exercise macronutrient ratio will not affect the acute expression of free, dissociable IGF-1.

H_{A1}: An acute exercise protocol and manipulation of post-exercise macronutrient ratio will affect the acute expression of free, dissociable IGF-1.

Rationale:

A large percentage of circulating IGF-1 is bound in a ternary complex consisting of IGF-1, IGFBP-3, and a hepatically-produced acid labile subunit (ALS) (4, 73). IGF-1 bound in binary complexes with IGFBP-1, -2, -4, -5, and -6 constitute an additional 4% of circulating IGF-1, with the remainder existing as 'free' IGF-1 (4). The current theory posits that this free fraction of IGF-1 is able to bind to the type 1 IGF receptor and elicit biological effects (4, 73, 87, 114). Exercise combined with proper post-exercise nutrition has been shown to elicit metabolic signals that would serve to maximize the bioavailability of free, dissociable IGF-1 (38, 53, 77). Thus, it is hypothesized that the

combination of an acute exercise intervention with post-exercise nutrition will maximize the bioavailability of IGF-1.

Research Question 2:

Does high-intensity exercise of the type utilized within this study promote increased MMP activity?

Hypotheses:

H_{O2}: High-intensity exercise merely increases MMP concentration, and not activity.

H_{A2}: High-intensity exercise increases both MMP concentration and activity

Rationale:

In serum, most of the IGFs are found in a ternary complex, formed by IGFs, IGFBP-3, and a glycoprotein known as the acid labile subunit (ALS) (8). This ternary complex does not cross the capillary barrier, and serves to protect, and consequently prolong, the half-life of both IGFBP-3 and IGFs. This ternary complex has been found to be subject to cleavage by certain proteases within the circulation (9, 105, 110), decreasing the affinity of IGFBP-3 for IGF-1 and releasing free IGF-1 (27, 28, 91, 92). The results from several experiments demonstrate that there is a definite link between plasma levels of MMPs and IGFBP-3 proteolysis (38, 47, 49, 53, 77). As exercise has been shown to elicit significant increases in plasma MMPs (22, 86, 126, 138), it stands to reason that these MMPs could be responsible for exercise-induced increases in IGFBP-3 proteolysis.

While previous studies have shown increased post-exercise MMP concentrations, no studies to date have shown increased MMP activity.

Assumptions

1. The college-age male subjects sampled from Auburn University are representative of the general population response to acute exercise and post-exercise nutritional protocols.

2. Participants complied with the instructions to keep dietary intake constant and forego previous exercise on testing days.

3. The theory is correct that only free, dissociable IGF-1 can bind to the Type 1 IGF receptor and potentiate biological effects.

4. The theory is correct that IGFBPs, at least in the peripheral vasculature, have primarily inhibitory effects on IGF-1 bioavailability.

Limitations

1. Dietary intake and non-study physical activity were quantified using a self-report measure.

2. Only male participants were recruited.

Delimitations

1. Only males meeting specific health guidelines were recruited.

2. Participants did not have known cardiovascular, metabolic, or pulmonary diseases.

3. Participants were not taking any medications known to influence growth hormone or insulin secretion.

4. A high-intensity interval training (HIIT) exercise protocol was utilized in the study.

Significance of the Study

The information obtained within this investigation is of benefit in both applied and clinical settings. By determining the mechanics of IGF regulation through exercise and nutrition, better programs for naturally improving athletic performance can be designed. Furthermore, it is hoped that the information obtained within this investigation will shed more light upon the complex regulatory dynamics of the IGF system and the associated intracellular signaling pathways. Clinically, this information will assist physicians in treating persons with cancer- or AIDS-related cachexia and other tissue-wasting disorders. Additionally, this investigation will be the first to link the exercise-induced production/release of MMPs to increased MMP activity.

CHAPTER II

REVIEW OF LITERATURE

Introduction

This review of literature provides background information on the acute regulation of insulin-like growth factor-1 (IGF-1). A brief overview of the key intracellular signaling pathways will first be provided. Next, a brief history of the progression of the theory of IGF-1 regulation will be presented. Subsequently, evidence for the regulatory dynamics of the individual insulin-like-growth-factor binding proteins (IGFBP) will be explored in detail. Finally, the role of an exercise and nutrition protocol on the acute regulation of IGF-1 will be discussed.

Intracellular Signaling Pathways

It is widely accepted that skeletal muscle contraction in the form of resistance-type exercise stimulates muscle protein synthesis during post exercise recovery. Additionally, an additive effect has been demonstrated with proper post-exercise nutrition (39, 53, 68, 77). Positive effects on muscle protein synthesis have been measured in as little as one hour post exercise (39) and may last for up to 24 hours in trained (102) and 48 hours in untrained subjects (119). These increases in muscle protein signaling are mediated, in part, by the activation and/or inhibition of various intracellular signaling

pathways. Numerous signaling cascades exist in mammalian cells and these pathways are highly regulated at multiple levels. There is substantial cross-talk between pathways producing a highly sensitive, complex transduction network.

The process of mRNA translation is functionally divided into three phases: initiation, during which the 40S and 60S ribosomal subunits bind to mRNA and locate the start codon; elongation, during which the ribosome moves along the mRNA, translating stored information into a growing peptide chain; and termination, a process resulting in release of the completed protein from the ribosome (76). Because the majority of the examples of translational regulation occur at the initiation phase, this section will focus on the regulation of initiation by mammalian target of rapamycin (mTOR), 5' AMP-activated protein kinase (AMPK), and phosphoinositide 3-kinase (PI3K).

mTOR has been shown to act as a sort of metabolic 'master switch', allowing protein synthesis to commence during times of nutrient and growth factor abundance while remaining subject to inhibition by other regulatory pathways within the cell. Current theory suggests that mTOR's main mechanism of action lies in the control of protein translation initiation and elongation (53, 69, 80, 101, 134). While mTOR is undoubtedly responsible for the activation and/or inhibition of multiple targets within the cell, the proteins receiving the most attention (and generally the proteins most examined when quantifying mTOR activation) are S6 ribosomal protein kinase 1 (S6K1) and the eukaryotic initiation factor 4E (eIF4E) binding protein (4EBP1) (69, 134). mTOR has

been shown to be directly responsible for phosphorylation of both of these proteins, leading to significant increases in protein synthesis rates (39, 53, 77) in skeletal muscle.

It is thought that activation of mTOR requires integration of signals from at least four different sources including PI3K activation, amino acid availability, adequate cellular ATP levels, and lipid availability (23, 69, 134). A growing body of evidence points to the fact that these signals converge on the tuberous sclerosis complex (TSC1/2), a heterodimeric complex lying immediately upstream of mTOR (43, 69, 134). This complex has been shown to serve as an upstream signal integrator, receiving direct inputs from AMPK (14, 80, 108), PI3K (43, 69, 134), and possibly, amino acids (39, 52, 53, 68, 77). The TSC1/2 complex is a tumor suppressor complex, serving largely as an anabolic pathway inhibitor in the absence of appropriate signaling. Loss of function of this complex has been strongly implicated in the pathogenesis of unregulated growth conditions such as cancer, Proteus Syndrome, and the development of non-cancerous tumors (101, 134).

AMPK is a protein kinase activated by both high AMP/ATP ratios (in response to increased intracellular AMP levels) and contraction-stimulated calcium-calmodulin kinase (CamK). Several groups have shown that AMPK indirectly modulates mTOR activity via direct communication with the TSC1/2 complex (14, 43, 69, 80, 108, 134). Although the mTOR complex has been implicated as an intracellular energy sensor (36), AMPK is thought to be a much more sensitive energy sensor because the intracellular AMP concentration is much lower than that of ATP (14, 43, 69, 80). Thus, a small change in ATP levels could greatly increase AMP/ATP ratio, suggesting that AMPK may

function as a primary physiological sensor of the intracellular energy charge to inhibit the mTOR signaling pathway (69).

Another important signaling pathway regulating mTOR activity is the PI3K pathway. Many growth factors, including insulin, activate PI3K, which subsequently activates 3-phosphoinositide-dependent protein kinase 1 (PDK1) and protein kinase B (PKB, also known as AKT) (69, 134). Abundant evidence has shown that, in mammals, stimulation by growth factors results in the phosphorylation/activation of S6K and 4EBP1 and, in turn, increased protein synthesis (38, 53, 69, 77, 134). Additionally, it has been demonstrated that IGF-1 activation of the PI3K pathway suppresses protein breakdown and expression of atrophy-related ubiquitin ligases, thereby decreasing intracellular proteolytic activity (127). In the past, it was thought that PI3K and mTOR constituted parallel pathways leading to the initiation of protein synthesis (101, 128). Current evidence, however, seems to indicate that PI3K lies upstream of mTOR, acting as a sort of 'growth-factor regulator' in the control of mTOR (39, 53, 69, 134).

It is widely accepted mTOR is in a pathway linking intracellular amino acid levels to the regulation of protein synthesis and cellular growth. Some recent studies have revealed that the TSC1/2 complex may mediate amino acid signals to regulate mTOR function (69, 134), with several studies illustrating activation of mTOR (via TSC1/2) with amino acid supplementation *in vivo* (38, 53, 77). Additionally, recent studies have also indicated that amino acid depletion causes the TSC1/2 complex to inhibit mTOR activation and subsequently decrease protein synthesis rates (70).

The intracellular energy charge (AMP/ATP ratio) is one of the most important inputs regulating mTOR activity (36). Protein synthesis has been shown to consume approximately 20% of intracellular ATP (130). Dennis et al. (36) found that ATP depletion by D-glucose analogs potently inhibits S6K and 4EBP1 phosphorylation in cells without affecting PI3K activation and intracellular amino acid concentrations. Several lines of evidence illustrate that activation of AMPK inhibits translation in response to changes in the intracellular AMP/ATP ratio, implicating AMPK as a pivotal factor in maintaining energy homeostasis and regulating cell growth and viability under energy deprivation conditions (69, 70). Additionally, Inoki et al. (70) has shown that the AMPK complex activates TSC1/2 via direct phosphorylation, indirectly inhibiting mTOR and protein synthesis rates.

In summary, there is ample evidence that mTOR is a central regulator for cell growth, allowing signal integration from multiple inputs. Additionally, it has been shown that the TSC1/2 complex serves as an upstream signal integration point, receiving inputs from PI3K, AMPK, and possibly intracellular amino acids. Thus, the mTOR complex ensures that protein synthesis does not occur unless there is ample input from growth factors, adequate amino acid availability, and sufficient energy charge to sustain anabolic activities.

Progression of the Somatomedin Hypothesis

The original somatomedin hypothesis originated almost 50 years ago in an effort to understand how somatic growth was regulated by pituitary factors. These experiments examined the incorporation of labeled sulfate into chondroitin sulfate from cartilage; and

suggested that the effect of human growth hormone (GH) was probably indirect, utilizing an intermediary substance such as an endocrine hormone or growth factor (129). Thus, from this line of research the term 'sulfation factor' was coined to indicate a circulating substance that was increased by GH and could stimulate sulfate uptake into cartilage (129).

Further experimentation on these 'sulfation factors' led to the discovery that these substances mediated the effects of GH (also called somatotropin). Thus, they were subsequently re-termed 'somatomedins' and divided into subtypes. Somatomedin C was found to be responsive to GH, and Somatomedin A GH-independent (99). Two decades (1978) after the existence of 'sulfation factors' had first been postulated, IGF-1 and IGF-II were purified and characterized (99). IGF-1 was shown to be the somatomedin substance that was regulated by circulating GH. Both substances were termed 'insulin-like' because of their ability to stimulate glucose uptake into skeletal muscle and adipose tissue.

The theory in place in the early 1980's was that GH acted primarily on the liver, where it stimulated IGF-1 synthesis and release into the peripheral circulation. IGF-1 then circulated to target tissues in an endocrine manner. Additionally, it was postulated that circulating IGF-1 provided a feedback mechanism to the hypothalamus and pituitary, suppressing further GH release (11). At the time this model seemed to match current thinking regarding growth control via the hypothalamic-pituitary-liver axis.

The discovery that IGFs were expressed in most, if not all, tissues caused quite an expansion of the original somatomedin hypothesis. The 'altered somatomedin

hypothesis' suggested that both circulating 'endocrine' IGF-1 and locally produced IGF-1 were responsive to GH and responsible for the effects of GH. In addition, the revised hypothesis admitted the possibility that GH might have IGF-1 independent effects on tissues (99).

In 1985, Green et al. (58) proposed a new concept concerning the roles that GH and IGF-1 play in growth and differentiation known as the 'dual effector hypothesis'. This theory suggested that GH stimulated differentiation of cells, while IGF-1 stimulated cellular expansion. More recent studies have yielded data that are not consistent with the dual effector hypothesis. The suggestion that GH has direct, non IGF-1-mediated effects on tissues is supported by a large body of evidence. Additionally, a growing body of evidence has emerged regarding the effects and regulation of the IGFs.

The current theory regarding the GH-IGF axis allows for both a direct and an indirect connection between the two. Current evidence supports the premise that GH directly controls the levels of circulating IGFs and most of the IGFBPs (44, 122), with GH deficiency or surplus being manifest with corresponding IGF deficiency or surplus, respectively (45). Indirectly, some evidence suggests that GH might have an acute effect on the control of IGF-1 bioavailability (24, 79, 84). It is here where the theories linking GH and IGF must part; as the majority of the current evidence suggests that GH plays a minimal role in the actual regulation of the IGF system. Thus, the remainder of this review will focus on the role that GH plays in the acute regulation of the IGF system, and not on the chronic effects of GH on the establishment of basal levels of IGFs.

IGF System, Receptors, and Transport Properties

The IGFs are growth-promoting peptides that share significant structural homology with pro-insulin (122). Although many tissues in the body have been shown to produce and secrete IGFs, the majority of IGF in the general circulation is believed to originate from hepatic production (45, 99, 122). The general consensus in the literature is that basal levels of circulating IGF are determined largely through the actions of GH on hepatic GH receptors (15, 16, 66, 100).

There are two known receptors that specifically recognize the IGFs. The IGF-1 receptor (also known as the type 1 IGF receptor) has a high degree of homology to the insulin receptor and is the only IGF receptor to have known IGF-mediated signaling function (75). The IGF-II receptor is identical to the mannose 6-phosphate receptor, has no known IGF signaling function, and has been shown to largely serve as a means of controlling circulating levels of IGF-II (3, 115). The insulin receptor can also bind the IGFs with low affinity, and hybrid IGF/insulin receptors have been isolated that bind the IGFs and potentially transmit a cytoplasmic signal (75), The physiological significance of these hybrid receptors is not yet known.

IGF-II has been shown to have a large role in fetal development, as it is the predominant IGF in fetal life (3), and mice in which the IGF-II gene is disrupted are growth-retarded at birth (35). In adults, high levels of IGF-II are found in bone, where it may be a mediator of remodeling, and in the central nervous system (35). While there is a distinct possibility that IGF-II plays a significant role in post-fetal growth and cellular

signaling, the remainder of this review will focus on the acute regulation of IGF-1 and IGF-1-mediated signaling.

Unlike insulin, IGFs circulate in plasma complexed to a family of structurally related binding proteins (IGFBPs). Although the existence of IGFBPs in circulation was suspected more than three decades ago, it was not until the mid 1980's to early 1990's that the six known IGFBPs were cloned and sequenced (122). Currently, IGFBPs are attributed with three main roles within the IGF system: 1) modulation of IGF action; 2) storage facilitation of IGFs in the extracellular matrix; and 3) exertion of IGF-independent effects. While a significant body of literature exists in support of all of these, the remainder of this review will focus only on the action of IGFBPs on the modulation of IGF activity.

The plasma concentrations of total IGF-1 are 50-100 times higher than the concentrations of free insulin (50). Indeed, Hall et al. (60) has shown that normal IGF concentrations are 100-fold higher in serum than levels required for biological effects *in vivo*. However, in contrast to circulating insulin, only 0.5-1.0% of circulating IGF-1 is present in the free form in the healthy state, with the majority (99%) of plasma circulating IGF-1 bound to IGFBPs. The six IGFBPs regulate the ability of free IGF-1 to leave the vasculature and associate with the type 1 IGF receptor (72). The IGFBPs are important regulators of the proliferative and mitogenic effects of IGF-1 on cells, and bind IGF-1 with an affinity equal or greater than that of the type 1 IGF receptors (75).

Approximately 95% of circulating IGF-1 is bound in a 150 kDa ternary complex consisting of IGF-1, IGFBP-3, and a hepatically-produced acid labile subunit (ALS) (4,

73). IGF-1 bound in binary complexes with IGFBP-1, -2, -4, -5, and -6 constitute an additional 4% of circulating IGF-1, with the remainder existing as 'free' IGF-1 (4). The current theory posits that this free fraction of IGF-1 is able to bind to the type 1 IGF receptor and elicit biological effects (4, 73, 87, 114).

Circulating free IGF-1 has been shown to be directly transported to the extravascular space via a paracellular route (4, 13, 73). However, another possibility is that some circulating free IGF-1 can associate with one of the other low molecular weight IGFBPs, forming binary complexes. These binary complexes are so small (<50 kDa) that they function as carriers and, like free IGF-1, can be transported to the extravascular space (6, 26). Binding affinity analyses have shown that IGF-1 has a higher affinity for the type 1 IGF receptor than for any of the lower molecular weight IGFBP's, suggesting that binary-complexed IGFs in the extravascular space have a high likelihood of dissociating and activating the IGF receptor (4, 12, 13, 73, 114).

There is an abundance of evidence supporting the premise that all of the IGFBPs have inhibitory roles in the regulation of IGF-1 *in vivo* (12, 44, 67, 75, 79, 95, 101, 117, 122, 137, 142). Thus, evidence supporting these inhibitory roles will not be discussed within the context of this review. Additionally, there is a large body of evidence supporting the role of many of the binding proteins in facillatory and IGF-1-independent roles within the extracellular matrix (16, 26, 28, 44, 60, 67, 79). As the purpose of this review is the exploration of the acute regulation of IGF-1 by IGFBPs, only the inhibitory regulatory roles of the IGFBPs will be discussed.

There is ample evidence to support the hypotheses that only free IGF-1 is able to potentiate biological effects and IGFBPs are likely regulators of free IGF-1 activity. The following section will consist of more detailed analyses of the regulatory dynamics of IGFBPs one, two, three, and four. As IGFBPs five and six have been shown to have a much higher affinity for IGF-II than IGF-1 (28, 59, 67), and are not thought to play a large role in IGF-1 regulation (7, 44, 122), information concerning them will not be presented within this review.

IGFBP-1 Regulation

IGFBP-1 is a 25 kDa protein that is produced, like all IGFBPs, in the liver, decidua, and kidneys (26). Serum IGFBP-1 levels are regulated mainly by metabolic factors- primarily insulin. Post prandially, IGFBP-1 levels fall to less than 10 ng/mL, whereas during fasting IGFBP-1 levels rise to more than 100ng/dL (78). Insulin and corticosteroids are the main regulators of serum IGFBP-1 levels, through the transcriptional control of the hepatic production of IGFBP-1 (26). Additionally, there is some evidence that IGFBP-1 clearance is increased in conjunction with high insulin levels (5).

Nyomba et al. (114) examined the effects of acute changes in serum insulin on free IGF-1, total IGF-1, IGFBP-1, and IGFBP-3 in 11 healthy subjects. Glucose and insulin were infused at zero and twenty minutes, and blood samples were drawn at defined intervals for three hours. Although the total IGF-1 level remained constant, free IGF-1 decreased significantly along with IGFBP-1. IGFBP-3 increased linearly to 20% above basal levels by the end of the experiment, mirroring the decrease in free IGF-1.

These data suggest that IGFBP-1 and free IGF-1 is acutely regulated by insulin, suggesting that it may play a role in glucose homeostasis. As GH and insulin are known to stimulate hepatic IGFBP-3 production (7, 44, 67, 122), and this study included no exercise intervention to induce IGFBP-3 proteolysis (26, 48, 49, 87), it is not surprising that the decrease in IGFBP-1 was accompanied by a decrease in free IGF-1.

To examine the relative roles of GH, insulin, and glucose in IGFBP-1 regulation, Conover et al. (29) conducted a study in which seven healthy individuals were administered standard meals during three separate conditions: saline infusion, GH infusion, and glucose infusion. The glucose infusions were administered to match glucose excursions during the GH infusions, and insulin levels were allowed to fluctuate normally during all three protocols. IGFBP-1 levels increased during fasting and decreased rapidly after meals, and were correlated directly with insulin levels. No independent effects of GH were demonstrated during any of the three protocols, even while using supraphysiological doses. Additionally, this study proposed a 'critical' suppressive plasma insulin concentration of approximately 90 pmol/L, above which IGFBP-1 levels decreased.

Since insulin levels were not controlled in the preceding experiment, the Conover group performed a series of follow-up protocols (98). After an overnight fast, seven subjects were subjected to a continuous euglycemic hyperinsulinemic clamp, causing a rapid fall in plasma IGFBP-1. Subsequently, during a euglycemic pancreatic clamp (somatostatin + GH infusion) after an overnight fast, IGFBP-1 levels rose greater than four-fold over three hours (98). Next, with a hypoinsulinemic pancreatic clamp, a

sequential stepped increase in glucose concentrations from five to nine mmol/L (three hours at each concentration) did not have a suppressive effect on the rate of IGFBP-1 increase (98). Taken together, this series of experiments provides strong evidence that IGFBP-1 regulation is largely insulin-mediated, and any indirect effects of GH and glucose concentrations are directly attributable to insulin regulation.

There is some data that glucagon, somatostatin, and glucocorticoids increase IGFBP-1 production *in vitro* (97). Conover et al. (30) studied the effects of hypercortisolemia during a hypoinsulinemic euglycemic clamp. During the control saline infusion, IGFBP-1 displayed the expected rise during hypoinsulinemia. When infused with cortisol, the same subjects showed a greater than three-fold increase in IGFBP-1 levels over the six hour study period as compared to saline controls. It should be mentioned here that there were no significant increases within the first two hours after cortisol infusion. Studies examining glucagon infusion on IGFBP-1 levels have found no significant effect in healthy persons (74) and a small, but significant, increase in GH-deficient adults (64). These date provide evidence that cortisol and glucagon do seem to play a role in IGFBP-1 regulation, although probably not a large role in its acute regulation.

Filho et al. (42) examined the effect of protein intake on serum IGF-1 and IGFBP-1 in rats and found significant increases in IGFBP-1 with reduced protein intakes. Their interpretation of the data was that IGFBP-1 was correlated with protein/amino acid availability, and indeed, it was. However, a much stronger correlation could be drawn

between insulin levels, which they measured, and IGFBP-1 levels. In line with other similar studies, as insulin levels fell, IGFBP-1 levels increased concurrently.

Brismar and colleagues (17) examined the effects of insulin on hepatic production of IGFBP-1 in insulin-dependent diabetics. Seven insulin-dependent diabetics in whom insulin was withheld for twelve hours were studied in the overnight fasted state. Basal levels of IGFBP-1 were elevated eight-fold compared to healthy subjects. Fasting IGFBP-1 levels were inversely correlated to insulin levels. Insulin infusion significantly inhibited splanchnic IGFBP-1 production at 60 minutes post-infusion, with complete inhibition occurring at 120 minutes. It should be noted that IGFBP-1 levels continued to decrease for 60 minutes following the hepatic inhibition at 120 minutes, suggesting an increased clearance or tissue uptake of the protein.

There is limited evidence for an insulin-induced increase in IGFBP-1 tissue uptake. Bar et al. (5) examined the transcapillary movement of IGFBP-1 and IGFBP-2 in the rat heart. Tissue uptake was measured in a beating heart preparation using ^{125}I-labeled binding proteins. The insulin concentrations utilized in the study were 1, 10, and 100 ng/mL, corresponding to 172, 1720, and 17200 pmol/L, respectively. The lowest insulin infusion concentration displayed a trend for increased IGFBP-1 tissue uptake, but only the two higher concentrations showed a significant increase. While 172 pmol/L is within the physiological realm, the two higher concentrations are not likely, even within the hepatic portal circulation (135). This is an important point in the examination of this study, as it is oft-cited as evidence for the increased tissue uptake of IGFBP-1 *in vivo*. It should be noted that with all of the insulin concentrations tested, there was an increase in

tissue uptake within 30 minutes. Unfortunately, this line of research has not been repeated within the literature. Thus, the evidence remains inconclusive as to the acute effects of insulin on the transcapillary movement of IGFBP-1.

IGFBP-2 Regulation

IGFBP-2 is a 31 kDa protein produced in the liver, kidneys and decidua. The levels of IGFBP-2 have been shown to be somewhat age-dependent, with high levels in infancy and older age and low levels in young adults (131). Unlike IGFBP-1 and IGFBP-3, there has not been a great deal of research on the acute regulation of IGFBP-2. Concentrations of IGFBP-3, the principal carrier of IGFs in the circulation, are relatively constant during the day and are not thought to change acutely with nutrient restriction. However, IGFBP-2 has been shown to rise with prolonged fasting or nutrient restriction (24, 31).

Clemmons et al. (24) investigated IGFBP-2 regulation in a population consisting of 44 normal weight subjects. The subjects were fed a normal diet for two days, followed by glucose-only infusions on the third day, and finally, triglyceride-only infusions on the fourth day. As opposed to IGFBP-1, there were no post-prandial insulin-induced changes in IGFBP-2 in any of the three conditions. Indirectly, it was observed during the study that plasma IGFBP-2 levels decreased in a diurnal rhythm, following daily GH pulses. As another part of the same study, Clemmons et al. (24) repeated the above experiments on obese patients who were receiving GH infusions (0.10 mg/kg LBW every other day). There were no significant differences in IGFBP-2 levels between any of the trials, both with and without GH infusion.

Smith and colleagues (136) compared IGFBP responses of eight healthy children and eight healthy adults to caloric and protein restriction. The groups underwent energy restriction (50% reduction intake) for six days and were refed a normal diet for an additional 6 days. A second group underwent protein restriction (decreased from 1.0 to 0.66 g/kg) for six days and was refed a normal diet for 6 days. Serum concentrations of IGFBP-2 did not change in either group in response to energy restriction, while IGFBP-1 levels increased significantly in both. IGFBP-2 levels rose significantly in both groups in response to protein restriction with no significant change in IGFBP-1. The findings from this study lend evidence to protein restriction playing a significant role in the regulation of IGFBP-2. However, GH elevations which would normally accompany both energy and/or protein restriction could have played a role in these responses (61, 66), but were not measured in this study.

In a similar study on hypopituitary subjects, Smith et al. (136) found significant elevations in IGFBP-2 concentrations. Both Ooi et al. (116) and Zapf et al. (144) showed that hypophysectomized rats have increased IGFBP-2 mRNA abundance compared to controls. Hardouin et al. (62) found significantly increased IGFBP-2 in human hypopituitary plasma. These results suggest that in normal animals GH acts to suppress IGFBP-2 synthesis. However, IGFBP-2 regulation seems to be more complex. Both Ooi et al (116) and Margot et al. (104) showed that administration of GH alone to hypophysectomized rats did not result in suppression of IGFBP-2 mRNA.

Cohick et al. (25) found that in pituitary-intact, continuously fed cows, GH is a potent suppressor of IGFBP-2 plasma concentrations. This differs markedly from the

results of Clemmons et al. (24), who showed that obese, calorically restricted adults have no change in serum IGFBP-2 after GH administration. Taken together, these findings suggest that a normal caloric intake may be required to achieve suppression by GH. However, when combined with the results of Smith et al. (136), it seems likely that IGFBP-2 is under dual control- with both protein/amino acid availability and GH playing distinct roles in its regulation. Unfortunately, there have been no studies to date specifically investigating this theory.

IGFBP-3 Regulation via Proteolysis

The molecular weight of IGFBP-3 in its nonglycosylated form is 29 kDa (26). IGFBP-3 has three glycosylation sites and is present in the circulation in the glycosylated state, with a molecular weight between 40 and 44 kDa (78). Like the other IGFBPs, IGFBP-3 is produced mainly in the liver, kidneys, and decidua. Nutritional status has an important role in controlling serum levels of IGFBP-3, with GH being the primary direct modulator of IGFBP-3 levels (26, 136).

The mechanisms by which GH stimulates IGFBP-3 synthesis are not entirely known. The two proposed mechanisms are: 1) a direct effect of GH on Kupffer cells and 2) an indirect effect mediated by IGFs (26). It is believed that IGFs affect the levels of IGFBP-3 not by stimulating production, but instead protect IGFBP-3 from proteolysis (27). The literature suggests that the basal levels of IGFBP-3 are relatively static, with abnormal levels normally accompanying chronic malnutrition (31, 136, 140), GH abnormalities (125, 141), or other diseases affecting pituitary hormones (140).

In serum, most of the IGFs are found in a ternary complex, formed by IGFs, IGFBP-3, and a glycoprotein known as the acid labile subunit (ALS) (8). This ternary complex of approximately 150 kDa does not cross the capillary barrier, and ALS is found only in the intravascular space (8). The formation of the ternary complex protects, and consequently prolongs, the half-life of both IGFBP-3 and IGFs. The half-life of unbound IGFBP-3 is between 30 and 90 minutes, the half-life of free IGF-1 is less than 10 minutes, and the half-life of the 150 kDa complex is approximately 12 hours (63). The binding of IGFs to IGFBP-3 and ALS maintains IGFs in the intravascular space for steady delivery of IGF-I in contrast with the pulsatile levels of GH (8).

Most of the IGFs in the circulation (95%) are bound in the ternary complex. This ternary complex has been found to be subject to cleavage by certain proteases within the circulation (9, 105, 110), allegedly decreasing the affinity of IGFBP-3 for IGF-1 and releasing free IGF-1 (27, 28, 91, 92). There is a significant body of literature supporting the notion that IGFBP-3 proteolysis is a major regulator of IGF-1 bioavailability (7, 8, 16, 26, 44, 67, 75, 99, 122). However, there is little information regarding the identity of the protein responsible for this proteolysis within the exercise physiology literature. There is, however, a wealth of information regarding serum IGFBP protease activity within the oncology literature, with matrix metalloproteinase (MMP) consistently implicated as a key proteinase involved in IGFBP proteolysis (9, 48, 49, 103, 105, 106, 109, 110). Thus, the information presented within this section will first focus on evidence for IGFBP-3 proteolysis with exercise, followed by evidence for IGFBP-3 proteolysis via MMPs, and finally evidence for the link between exercise and MMP production.

Lamson and colleagues (93) were one of the first teams to speculate that IGFBP-3 was subject to regulation via proteolysis. They initially suspected this after some earlier studies revealed increased levels of IGFBP-3 proteolytic fragments in serum from pregnant females (56, 65). Additionally, Lamson et al. (94) were also the first to develop a specific assay designed to quantify the level of IGFBP-3 proteolysis in serum. Specifically, they designed a Western Ligand Blot (WLB) to detect the two proteolytic fragments that occurred when serum binding proteins cleaved IGFBP-3.

Lalou et al. (92) investigated IGFBP-3 proteolysis in normal human volunteers. They found evidence for IGFBP-3 proteolysis in both serum and lymph. This was one of the first studies to show that IGFBP-3, which cannot egress the vascular endothelium, is produced in peripheral tissues in addition to the liver. Additionally, they showed that the protease was most likely a serine protease, as its activity was inhibited by the addition of aprotinin (serine protease inhibitor) to serum samples.

Brismar et al. (17) investigated the effects of insulin infusion on IGFBP-1 and -3 production in insulin-dependent diabetics and found significant changes in levels of IGFBP-1, with no corresponding changes in IGFBP-3. This is in line with most other research, showing that basal levels of IGFBP-3 are relatively static and GH-dependent, while IGFBP-1 levels are acutely regulated by insulin. Bereket et al. (10) also investigated the effects of insulin therapy on insulin-dependent diabetics, with the intention of quantifying levels of IGFBP-3 proteolysis both before and after insulin therapy on previously untreated children. They found significant decreases in IGFBP-3 proteolysis at one week and one month after beginning insulin therapy, perhaps

suggesting that without insulin therapy the main means of IGF regulation was through IGFBP-3 proteolysis (as opposed to some insulin-induced regulation via IGFBP-1 in non-diabetic persons).

Borst et al. (15) studied the effects of resistance training on IGF-1 and IGFBP-3 on 31 adult men and women during a 25 week program. The participants were separated into control, single-set, and multiple-set groups. They found significant increases in total IGF-1 during the first 13 weeks in the two exercising groups, with no increases thereafter. There were significant decreases in serum IGFBP-3 in the multiple-set group, which were accompanied by significantly higher strength increases than the control group or the single-set group. While free IGF-1 and IGFBP-3 proteolysis were not directly measured in this study, there were significant correlations between the decreases in IGFBP-3 and overall strength gains. This group interpreted this to mean that the decrease in IGFBP-3 corresponded to an increase in free IGF-1, which could directly mediate the increased strength gains with the multiple-set group.

Schwarz et al. (132) measured circulating GH, IGF-1, IGFBP-3, and IGFBP-3 proteolysis in the same group of ten male subjects under three different conditions: 1) control, 2) low-intensity exercise for ten minutes, and 3) high-intensity exercise for ten minutes. They found significant increases in GH in both of the exercise conditions, but significant increases in IGFBP-3 proteolysis (44%) only with the high-intensity protocol. As the increased proteolysis of IGFBP-3 would result in an increase of free IGF-1, they postulated that it would result in increased bioavailability of IGF-1.

Doll and colleagues (34) investigated post-exercise IGFBP-3 proteolysis in a group of sixteen elite rowers. The participants performed an incremental rowing test, with blood samples withdrawn at specific intervals both during and up to 120 minutes post-exercise. In contrast to Schwarz et al. (132), they found no changes in IGFBP-3 or IGFBP-3 proteolysis after exercise. However, the exercise protocol utilized within the study was of a relatively low intensity and duration, as evidenced by the low magnitude of the post-exercise GH pulse. Additionally, they did not adjust their measured values for plasma volume shifts that occur with exercise. As some exercise protocols have been shown to elicit plasma volume shifts of more than 20% (133), this adjustment would have likely affected the results of the investigation.

Berg et al. (13) explored changes in IGF-1 and IGFBPs over an exercising leg utilizing both microdialysis and arterio-venous differences during exercise and for 60 minutes post-exercise. Eighteen healthy young men performed leg extensions for 45 minutes while blood samples were taken from the artery and vein servicing one of the exercising muscles. They found no significant arterio-venous changes in IGF-1, IGFBPs, or IGFBP-3 proteolysis across the exercising leg. There was a significant increase in IGFBP-3 proteolysis in plasma, suggesting that proteinases in the general circulation were responsible for the proteolysis. Additionally, microdialysis revealed a significant increase in free IGF-1 in the exercising muscle beginning during exercise and persisting for the 60 minute post-exercise period.

Thus, there appears to be ample evidence that with sufficient exercise intensity and duration there is a significant increase in the levels of IGFBP-3 proteolysis. As a

large percentage of circulating IGF-1 exists in the vasculature bound in the ternary complex, this proteolysis would serve to allow dissociation of bound IGF-1. Unfortunately, there seems to be a gap in the exercise physiology literature regarding the identity of the proteinase responsible for IGFBP proteolysis. The following section will present evidence supporting the role of MMPs as key players in IGFBP proteolysis.

The Role of MMPs as IGFBP Proteinases

Matrix metalloproteinases (MMPs), also called matrixins, function in the turnover of extracellular matrix components such as collagens, proteoglycans, elastin, laminin, fibronectin, and other glycoproteins (109). Currently, sixteen members are found in vertebrates, including humans. MMPs are secreted from cells or expressed as plasma-membrane-bound forms and have been found to be regulated by pro-inflammatory cytokines (40), hormones and growth factors (109), and plasmin (21).

Crown and colleagues (33) compared a group of thirty cancer patients to a control group, examining relationships between aspects of the IGF system. Specifically, the group measured the relationship between free IGF-1, IGFBP-3 proteolysis, and the inflammatory cytokine interleukin-6 (IL-6). They found significant correlations between IGFBP-3 proteolytic activity, free IGF-1, and IL-6 levels. Additionally, they found a significant inverse correlation between weight loss (independent of nutritional intake) and levels of IGFBP-3 proteolysis. Together, these results suggested that IGFBP-3 proteolysis might improve IGF-1 bioavailability to tissues and that this proteolysis might be enacted or stimulated via inflammatory cytokines such as IL-6. Several subsequent *in vitro* analyses (41, 118), however, demonstrated that IL-6 had no effect on IGFBP-3

proteolysis in serum-free media, suggesting that some other serum factor was responsible for IGFBP-3 proteolysis *in vivo*.

Due to recent epidemiological studies, it has been discovered that there is an established correlation between high levels of circulating IGFs and low levels of IGFBP-3; and relative risk of developing colon, breast, lung, and prostate cancer, all of which are known to produce high levels of MMPs. Therefore, Miyamoto and colleagues (105) investigated the *in vitro* effects of MMPs on IGFBP-3. They found that MMPs, specifically, MMP-7 had an extremely high affinity for IGFBP-3 and that the proteolytic fragments found *in vitro* were an exact match for fragments found *in vivo*. Subsequently, this same group examined the *in vitro* effects of MMPs on all IGFBPs (110). They found that MMPs degrade all IGFBPs, but seemed to have an unusually high affinity for IGFBP3 in vitro. These studies formed the bases for several follow up *in vitro* and *in vivo* studies on the role of MMPs in IGFBP proteolysis.

Kirman et al. (81) performed a series of experiments investigating the link between MMPs, IGFBP-3 proteolysis, and IGFs. In the first two experiments, the group compared plasma samples from a large group of patients which had undergone major abdominal surgery to plasma from patients that had undergone laparoscopic surgery. They incubated these plasma samples with HT29 human colon cancer cells *in vitro* and found significantly increased mitogenic activity in the plasma from the abdominal surgery group, with significant correlations to the total length of incision. Additionally, the group found significantly decreased levels of IGFBP-3 and increased levels of IGFBP-3 proteolytic fragments in the surgery group (82).

Subsequently, the Kirman group (83) examined another two groups of similar patients. However, in this experiment they included analyses of MMPs as well as IGFBP-3 proteolysis. The findings from this investigation demonstrated significant post-surgery plasma activity of MMP-9 in the surgery patients, with a strong correlation to levels of IGFBP-3 proteolysis. This plasma activity was not significantly elevated in the patients who had undergone laparoscopic surgery. Correspondingly, this group displayed no significant increases in IGFBP-3 proteolysis.

Belizon et al. (9), including many members of the Kirman group, continued this line of research utilizing an animal model. They investigated the links between MMPs and IGFBP-3 proteolysis in thirty mice. The mice were divided into three groups: sham laparotomy, carbon dioxide pneumoperitoneum, and anesthesia control. The group found significant increases in MMP activity and IGFBP-3 proteolysis only in the sham laparotomy group. Additionally, pre- to post-surgery analyses of peripheral blood mononuclear cells in the laparotomy group revealed significantly decreased levels of MMP proteins. Taken together, the results from this series of experiments demonstrate that there is a definite link between plasma levels of MMPs and IGFBP-3 proteolysis. Additionally, there appears to be ample evidence to suggest that these plasma MMPs originate from peripheral blood mononuclear cells.

Evidence for Exercise-Induced Increases in Plasma MMPs

Koskinen et al. (86) examined the plasma release of MMPs after downhill running in a group of fourteen healthy males. The exercise protocol consisted of forty-five minutes of ten-degree downhill running at 60% VO_{2max}. They found significant increases

in MMP and serum creatine kinase (CK) after exercise. However, they found no correlation between CK and MMP levels, suggesting that muscle damage may not be a pre-requisite for MMP release with exercise.

Carmeli and colleagues (22) tested the hypothesis that exercise-induced expression of MMPs is dose-dependent such that high-intensity endurance exercise would increase skeletal muscle and plasma MMP expression whereas low-intensity exercise would not. The group compared three groups of rats: high-intensity, low-intensity, and control. They found significantly elevated levels of skeletal muscle and plasma MMP only with high-intensity exercise. Additionally, they found that the influence of exercise on MMP expression is dominant in muscles containing a high percentage of fast fibers.

Rullman et al. (126) investigated the effects of a single bout of exercise on tissue and plasma expression of MMPs. Ten healthy males performed 65 minutes of incremental cycle exercise (20 minutes at 50% VO_{2max}, 40 minutes at 65% VO_{2max}, five minutes all-out). They found significant increases in skeletal muscle MMP mRNA, protein expression, and plasma MMP levels post-exercise. Unfortunately, the group only performed biopsies pre-exercise, immediately post-exercise, and at 120 minutes, so a time-course of plasma MMP expression could not be elucidated.

The Suhr group (138) examined the effect of high-intensity cycling on circulating MMPs. Twelve male cyclists completed 90 minutes of cycling (10 minutes warm up at 50% VO_{2max} followed by 10 intervals of 3-minute high load at 80-85% VO_{2max} and 5 minute recovery at 55-60% VO_{2max}). They found significant increases in post-exercise

plasma MMPs under all exercise conditions, with a time course beginning immediately post-exercise and persisting for at least four hours. It should be noted that the experiment included four exercise conditions: 1) normoxia with vibration, 2) normoxia without vibration, 3) hypoxia with vibration, and 4) hypoxia without vibration. However, there were no significant main effects between any of the four exercise conditions.

There appears to be ample evidence to suggest that exercise of a sufficient intensity and/or duration elicits a significant increase in plasma levels of MMPs. There have been no studies to date, however, showing increased post-exercise MMP activity. MMPs have been shown to play a substantial role in the proteolysis of IGFBPs in general, specifically IGFBP-3. Thus, if intense exercise results in significant increases in MMP activity, the increased proteolysis of IGFBPs could then permit dissociation of IGF-1 from the ternary complex, allowing the potentiation of its biological effects by binding to the Type 1 IGF receptor.

IGFBP-4 Regulation

Of all of the IGFBPs, IGFBP-4 is undoubtedly the least researched. The small amount of literature available regarding IGFBP-4 regulation all suggests that its primary mode of regulation is via proteolysis (48, 120, 142). Fowlkes et al. (48) performed *in vitro* studies examining IGFBP-4 regulation. They found that intact, unbound IGFBP-3 inhibits IGFBP-4 proteolysis. However, when bound to IGFs, IGFBP-3 loses its capability to inhibit IGFBP-4 proteolysis, thus enhancing IGF activity by facilitating the degradation of IGFBP-4. This protection was also lost when IGFBP-3 was

proteolytically cleaved by proteinases. Additionally, the group found that the addition of IGFs induced IGFBP-4 proteolysis via an unknown proteinase.

Van Doorn and colleagues (142) investigated plasma concentrations of IGFBP-4 under normal and pathological conditions. They sampled various hospital populations and determined that IGFBP-4 did not change with fasting, thyroid status, GH deficiency, or parathyroid hormone status. They did find increased levels with chronic renal failure, but all binding proteins were elevated in this condition. It should be noted that they found relatively high concentrations of IGFBP-4 in amniotic and follicular fluid. Both body fluids also contained significant IGFBP-4 proteolytic activity. These findings would suggest an important role of IGFBP-4 in the regulation of IGF effects in the female reproductive system.

Price et al. (120) performed in *vitro studies* investigating IGF-1 regulation utilizing rat lung fibroblasts. They found that lung fibroblasts secreted an IGF-dependent IGFBP-4 protease and that these cells express RNA for pregnancy-associated polypeptide-A (PAPP-A), a known IGFBP protease. Secretion of this IGFBP-4 protease likely has physiological significance because native IGFBP-4 is cleaved in the presence of IGF-1 if no protection is available in the form of free, intact IGFBP-3. Together, the results from these studies seem to indicate that IGFBP-4 is regulated directly by IGFBP-3 and IGF-1. Thus, indirectly IGFBP-4 would be regulated by many of the same factors that regulate IGF-1 bioavailability (e.g. IGFBP-1, 2, and 3 regulators).

Conclusions and Purpose of Study

In conclusion, there is a large body of evidence supporting the roles of the IGFBPs in regulating IGF-1 bioavailability, as well as some information regarding their individual regulation dynamics. Thus, the purpose of this study is to investigate the regulatory dynamics of IGF-1 and to gain more insight into the individual regulation of IGFBPs *in vitro*.

To accomplish this goal, we utilized a group of healthy, recreationally-active college-aged males. The participants performed a high-intensity exercise protocol on three separate occasions, with each session followed by one of three nutritional interventions: one session was followed by no nutritional intervention; one was followed by a carbohydrate-only (CHO) nutritional intervention; and the last was followed by a mixed essential amino acid (EAA)/CHO supplement. The purposes and reasoning behind each of these sessions will be outlined in the following paragraphs.

High-intensity exercise has consistently been shown to elicit significant elevations in post-exercise plasma concentrations of GH (32, 96) and MMP (22, 86, 126, 138). Thus, the purposes of the exercise session were two-fold: 1) to examine the effect of an increase in plasma GH on IGFBP-2, and 2) to examine the effect of an increase in plasma MMPs on MMP activity. As IGFBP-2 has been shown to possibly be under dual control of GH and protein/amino acid availability, the purpose of the exercise session without post-exercise nutrition was to specifically examine the role of GH regulation (in the absence of exogenous amino acid supplementation) on this binding protein. Additionally, the link between exercise-induced MMP expression and MMP activity was

examined in all of the exercise sessions. The exercise session followed by CHO-only supplementation served to examine the role of insulin on IGFBP-1 regulation.

The final exercise session was followed by mixed EAA/CHO supplementation. The purpose of this protocol was to examine the insulin-induced regulation of IGFBP-1 in combination with exercise and EAA availability. It should be noted that free, dissociable IGF-1 was measured in all of the exercise protocols. Additionally, insulin, GH, blood glucose, plasma amino acid concentrations, and plasma MMP activity were measured at pre-determined intervals throughout all sessions.

CHAPTER III

METHODS

Study Overview

Eight males completed three identical exercise protocols followed by one of three different post-exercise nutritional supplements: 1) no supplement; 2) CHO-only supplement; and 3) EAA/CHO supplement. The purpose of the investigation was to elucidate the effects of an exercise and nutritional intervention on the acute regulation of IGF-1. The participants performed the exercise protocol and then immediately ingested the nutritional supplement. Blood samples were obtained for analysis at pre-determined intervals throughout the protocol. Exercise sessions were separated by a minimum of 48 hours.

Participants

Volunteer Recruitment

Volunteers were recruited from Auburn University by posted flyers (Appendix A) and word of mouth. Male volunteers aged 19 to 35 meeting the following inclusion criteria were invited to enroll in the study: 1) equal to or above the 50^{th} percentile for age-dependent maximal oxygen uptake (1); 2) healthy, as determined by Medical History Questionnaire (Appendix B); and 3) currently engaging in at least three to four days per week of moderate- to high-intensity strength and/or endurance training for 30 to 60

minutes duration (self-reported, Appendix C). Volunteers taking prescription medications with known effects on GH or insulin secretion, or those with known cardiovascular, pulmonary, or metabolic diseases were excluded from the study. The study was approved by the Auburn University Institutional Review Board, and all subjects granted informed consent (Appendix D).

Preliminary Procedures and Assessments

Preliminary Screening

Preliminary screening was done in person. Qualifying volunteers were scheduled for an initial visit to the Thermal Lab for further screening. Upon arrival, volunteers were verbally informed about the study, provided with an opportunity to ask questions and signed an institutionally-approved informed consent document prior to any screening processes (Appendix C). Volunteers meeting all of the inclusion criteria who decided to participate then continued with anthropometric measurements and preliminary physiological assessments.

Physiological Assessment

Anthropometric measurements including height and weight were obtained. Height was determined to the nearest 0.25 inch with a stadiometer and weight was measured to the nearest 0.25 pound using a calibrated balance scale. Three-site skinfold assessment was obtained using skinfold calipers (Lange Skinfold Caliper, Beta Technology Inc., Cambridge, MD), and body density was determined via the manner of Jackson et al (71). Participants performed a 30-second Wingate maximal anaerobic power test with resistance determined by bodyweight (.75g/kg). Participants also performed a graded exercise test on an electrically-braked cycle ergometer (Quinton

Excalibur, Quinton Instrument Company, Bothell, WA). Each participant pedaled against 200 watts for five minutes, 250 watts for 3 minutes, 300 watts for 3 minutes, and then wattage increases of 25 watts per minute until volitional exhaustion. Oxygen uptake was measured with an automated metabolic testing system (True Max 2400 Metabolic Testing System, Parvo Medics, Salt Lake City, UT). The highest observed oxygen uptake over three consecutive 15-second averages was considered the peak oxygen consumption (VO_{2peak}).

Experimental Procedures

Participants completed three identical exercise protocols, followed by each of three post-exercise nutritional supplements in a randomized order. Participants were instructed to continue normal outside physical activity and dietary practices. On exercise testing days, participants were instructed to refrain from any exercise preceding the testing session and to arrive at the lab after a two hour fast. Subjects were tested at the same time of day, separated by no less than 48 and no more than 72 hours.

Upon arrival at the lab, participants provided a urine sample for determination of hydration status. Urine samples were testing utilizing a refractometer (American Optical Corp., Keene, NH), and the participants were cleared to exercise if urine specific gravity was less than 1.020 g/mL. A venous catheter was placed in a dorsal wrist vein and the participant remained seated for 15 minutes before the pre-trial blood draw. The participant then performed the exercise session and a post-exercise blood draw was taken. The post-exercise nutritional supplement was consumed within five minutes of the completion of the exercise protocol. Blood draws were then taken, with the participant

seated, at ten minute intervals for 80 minutes post-exercise and at 20 minute intervals until 160 minutes post exercise.

Nutritional Supplements

Three different nutritional supplements were utilized in the study: 1) non-caloric placebo; 2) CHO-only supplement; and 3) EAA/CHO supplement. The non-caloric placebo was prepared utilizing a commercially available non-caloric flavoring, and administered in a manner consistent with the other supplements. The carbohydrate-only supplement was prepared using sucrose mixed with water and non-caloric flavoring. It was administered at 0.85 g/kg lean bodyweight (LBW). The EAA/CHO supplement was prepared using an EAA mixture, sucrose and non-caloric flavoring. It was administered at 0.35 g/kg LBW EAA and 0.50 g/kg LBW CHO (38, 53). The participants were blinded to which of the supplements they were receiving.

Composition of the EAA + CHO Solutions

The leucine enriched EAA + CHO solution consisted of essential amino acids in the following proportions: histidine, 8%; isoleucine 8%; leucine, 35%; lysine, 12%; methionine, 3%; phenylalanine, 14%; threonine, 10%; and valine, 10%; and has been used in previous studies (38, 53). LBW as determined by skinfold analysis was used to calculate the proportion of each EAA (0.35 g/kg LBW) added to the nutrient solution. Similarly, carbohydrate (sucrose) was added at 0.85 g/kg LBW (CHO only) or 0.5 g/kg LBW (EAA/CHO). All ingredients were dissolved in a noncaloric, caffeine-free, flavored beverage to increase palatability.

Exercise Conditions

The participants performed a high-intensity interval training (HIIT) protocol on an electrically-braked cycle ergometer (Quinton Excalibur, Quinton Instrument Company, Bothell, WA). The participants performed a 20-minute protocol, which consisted of four minutes of cycling at 15% of maximum anaerobic power (MAP) followed by 30 seconds at 90% of MAP. These percentages were based upon pre-trial Wingate tests. This cycle was repeated four times within each protocol, ending with two minutes at 15% MAP. It should be noted here that the cycle ergometer did not transition wattages in a square-wave fashion. Wattage increased/decreased at each power transition at a rate of 150 watts per second.

Blood Sampling Procedures

Participants were asked to report to the lab on testing days after a two hour fast. A venous butterfly catheter (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, 23G x 3/4) was inserted into a dorsal wrist vein and then capped with an intermittent injection port (Kawasumi Laboratories, Inc., Tampa, FL). Blood samples were drawn into 5.0 mL syringes (Becton Dickinson & Co., Franklin Lakes, NJ) with no additives. Catheter patency was maintained by a 2mL injection of sodium heparin lock flush (Abbott Laboratories, North Chicago, IL, 10 USP U/mL) following collection of each blood sampling time point and as needed. Whole blood samples were analyzed immediately for hematocrit, hemoglobin, and blood glucose concentrations. The remainder of the sample was allowed to clot at room temperature prior to centrifugation at 1500 X g for 10 minutes for isolation of serum. Serum aliquots were isolated in 2.0 mL ultracentrifuge tubes and stored at -70 ˚C for subsequent analysis.

Blood samples were taken pre-exercise, immediately post-exercise, at 10 minute intervals for 80 minutes post-exercise, and subsequently at 20 minute intervals until 160 minutes post-exercise. Each blood draw consisted of approximately 5 mL, with 3mL discarded as waste. The total amount of blood volume for this experiment was approximately 210 mL (5 mL/draw, 14 draws/session, 3 sessions).

Hematocrit and Hemoglobin

Hemoglobin and hematocrit were determined immediately following each blood sample using a small portion of the whole blood sample. Hematocrit was determined in duplicate utilizing the microhematocrit method. Hemoglobin was determined utilizing an automated CO-oximeter (Instrumentation Laboratories 682, Lexington, MA). Hemoglobin and hematocrit concentrations from whole blood samples were used to estimate plasma volume shifts resulting from the exercise sessions via the method of Dill and Costill (37).

Blood Glucose and Amino Acid Concentrations

Blood glucose concentrations were determined in duplicate via the glucose oxidase method using an automated handheld analyzer (True Track Smart System, Home Diagnostics, Inc., Fort Lauderdale, FL). Leucine was used as a representative marker for plasma amino acid presence and concentration determination. Leucine concentrations were determined in duplicate utilizing high-performance liquid chromatography apparatus (Waters HPLC Analyzer, Waters Inc., Milford, MA) in the method of Wu et al. (143).

Growth Hormone

Serum GH concentrations were determined in duplicate utilizing a coated tube immunoradiometric assay (IRMA) kit (DSL-1900, Diagnostic Systems Laboratories, Webster, TX). Intraassay coefficient of variation was 4.5%, and recovery of added mass was 102.9%. Assay sensitivity was 0.01 ng/mL.

Insulin

Insulin was determined utilizing a human insulin-specific radioimmunoassay (RIA) kit (HI-14K, Linco Research, St. Charles, MO). Interassay coefficient of variation was 4.9%, intraassay coefficient of variation was 3.3%, and recovery of added mass was 97.8%. Assay sensitivity was 2µU/mL.

Free IGF-1

Free IGF-1 concentrations were determined using an active free IGF-1 enzyme-linked immunosorbent (ELISA) kit (DSL-10-9400, Diagnostic Systems Laboratories, Webster, TX). Interassay coefficient of variation was 10.3%, intraassay coefficient of variation was 4.1%, and recovery of added mass was 102%. Assay sensitivity was 0.015 ng/mL.

MMP-9 Activity

MMP-9 activity was determined using a human active MMP-9 fluorescent assay kit (F9M00, R&D Systems, Minneapolis, MN). Interassay coefficient of variation was 7.4%, intraassay coefficient of variation was 3.3%, and recovery of added mass was 95.7%. Assay sensitivity was 0.005 ng/mL.

Statistical Analysis

This experiment was a within-subjects design with each participant serving as his own control. Group characteristics were reported as means ± sd. Multiple three (condition) x ten (time) analysis of variance (ANOVAs) with repeated measures on both condition and time were used to compare GH, blood glucose, amino acid concentrations, insulin, MMP activity, and free IGF-1 responses to the different conditions.

The independent variables for this experiment were as follows: condition (control, CHO, and EAA/CHO) and time (all of the pre- and post-exercise serum measurements at each of the blood sampling time points). The dependent variables for this study were serum free IGF-1 concentrations and MMP activity. Significant between-group differences were further explored utilizing a Bonferroni confidence interval adjustment. The *a priori* significance level for this study was $p < 0.05$. Data analysis was completed with the Statistical Package for the Social Sciences (SPSS, version 16.0, SPSS, Inc., Chicago, IL).

CHAPTER IV

RESULTS

Participant Selection

Ten individuals inquired about the study in response to recruiting methods. Two of the individuals were excluded based upon results of preliminary testing. In total, eight volunteers met the study inclusion criteria and agreed to participate in the study. These eight participants began and completed the entire study protocol.

Baseline Physiological Characteristics

Participant baseline physiological characteristics are presented in Table 1.

Table 1. Baseline physiological characteristics

Variable	Mean ± SD	Minimum	Maximum
Age (yrs)	22 ± 2	19	27
Height (cm)	180.5 ± 5.5	173.1	191.2
Weight (kg)	86.0 ± 11.5	77.4	114.5
Body fat %	10 ± 3	6	16
VO_{2peak} (mL/kg/min)	41.3 ± 3.1	36.0	45.2
Wingate (Watts)	906 ± 162	726	1217

All values are presented as mean ± sd and minimum and maximum values.

Exercise Intervention

All participants were able to complete each of the three exercise conditions. Each of the exercise sessions lasted exactly 20 minutes, and was identical in power settings and cycle ergometer settings for each participant. Participants completed the exercise protocol following at least a two hour fast and ingested the post-exercise supplement within five minutes of completing the exercise protocol.

The participants completed the exercise protocols within no less than 48 and no more than 72 hours. Each of the three exercise protocols was identical within each of the eight participants. Percentage of VO_{2peak} was based upon each participant's power output at VO_{2peak}. Exercise intervention data are presented in Table 2.

Table 2. Exercise Session Data

Variable	**15% MAP**	**90% MAP**
Average power (Watts)	138.4 ± 8.8	832.0 ± 53.5
% VO_{2peak}	46.5 ± 1.8	279.8 ± 11.6
HR (bpm)	145.9 ± 4.7	180.6 ± 4.4
% HR_{peak}	73.6 ± 2.2	91.2 ± 2.2
Total time (min)	18.0	2.0*

All values are presented as mean ± sd. MAP = maximum anaerobic power as measured by Wingate; % VO_{2peak} = percentage of peak oxygen uptake at each intensity based on power output at VO_{2peak}; % HR_{peak} = percentage of peak heart rate; Total time = total exercise time at each intensity for each exercise session. * = Wattage increased/decreased to set value at 150 watts per second at each power transition.

Effect of Exercise on Blood Variables

Plasma Volume Shifts

Plasma volume was significantly decreased from pre- to post-exercise ($p < 0.01$), with most participants displaying non-significant post-exercise plasma volume expansion from 20 to 60 minutes. Thus, all blood and serum measurements were adjusted for plasma volume shifts during this time period. The average post exercise plasma volume decrease was 12%. There were no significant differences at any time points across the three exercise conditions, so data were pooled. Post-exercise plasma volume shifts are shown in Figure 1.

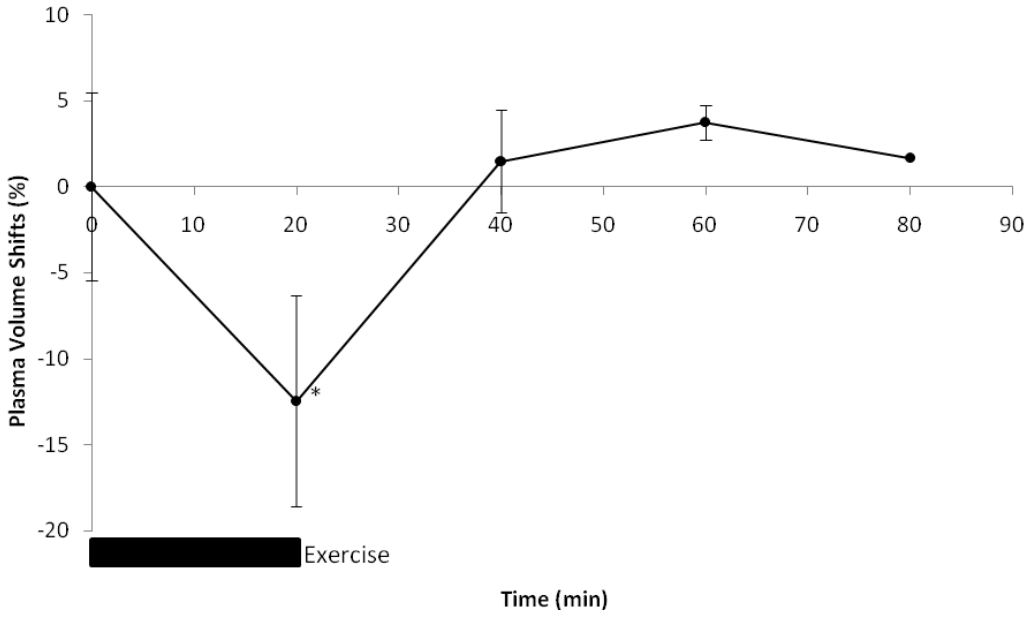

Figure 1. Post-exercise plasma volume shifts

Values are reported as mean ± sd. Means are collapsed across conditions. * = significant difference from pre-exercise value. Plasma volume expansion from 20 to 60 minutes did not reach statistical significance.
GH

Serum growth hormone concentrations increased approximately 60-fold from pre- to post-exercise ($p < 0.01$), remaining significantly elevated until 100 minutes post-exercise. There were no significant differences at any time across the three exercise conditions, so data were pooled. Growth hormone responses to exercise are presented in Figure 2.

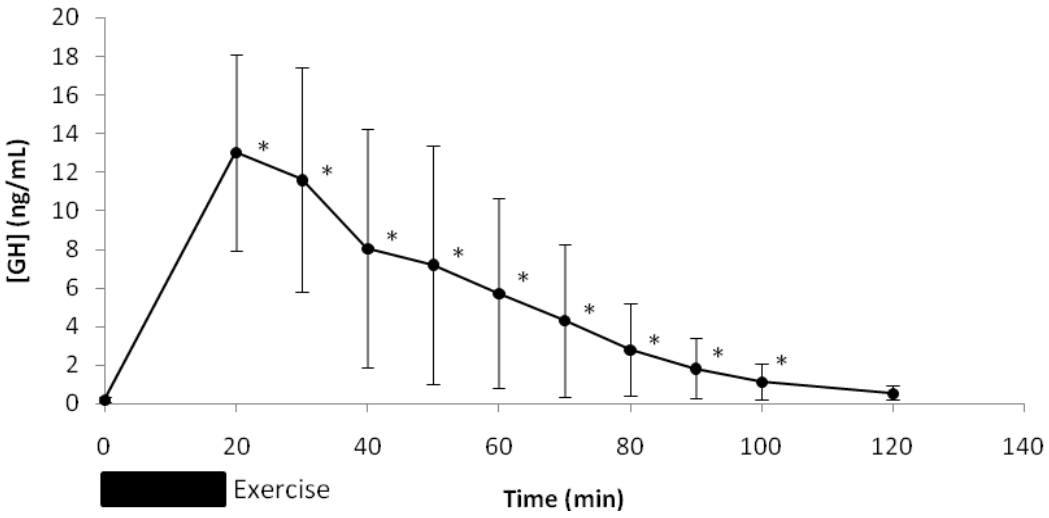

Figure 2. Growth hormone response to exercise

Values are reported as mean ± sd. Means are collapsed across conditions. * = significant difference from pre-exercise value. Serum growth hormone concentrations remained significantly elevated until 100 minutes post-exercise.

MMP-9 Activity

MMP-9 activity significantly increased by 89% from pre- to post-exercise ($p < 0.01$), remaining significantly elevated until 40 minutes post-exercise ($p < 0.01$). MMP-9 activity again increased significantly at 140 minutes post-exercise ($p = 0.05$) and

remained significantly elevated until 160 minutes post-exercise ($p = 0.02$). There were no significant differences at any time points across the three exercise conditions, so data were pooled. MMP-9 activity is presented in Figure 3.

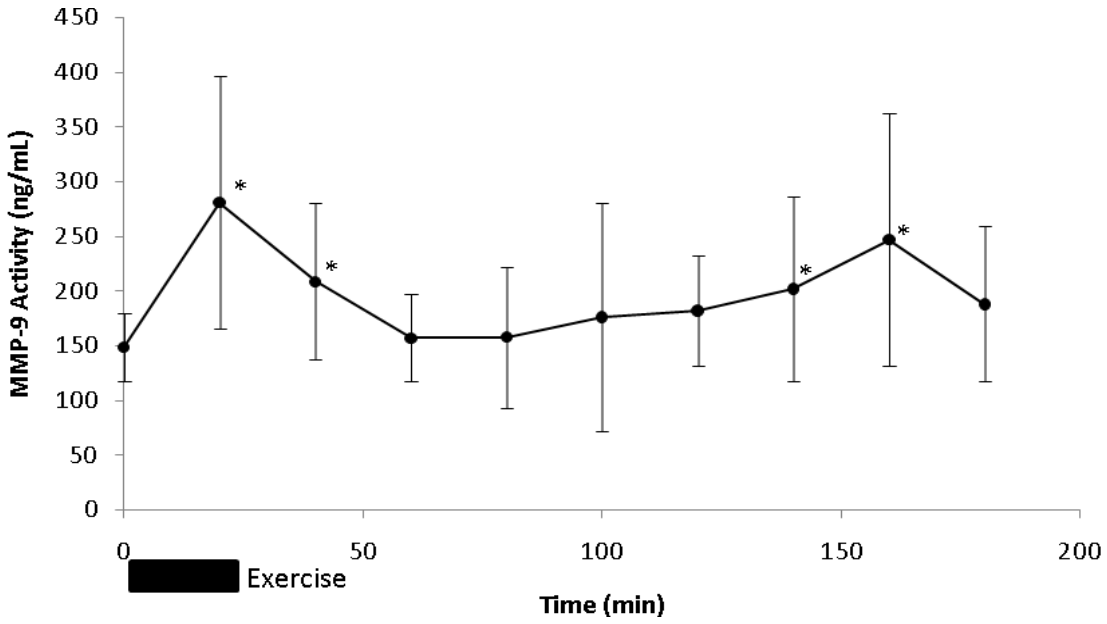

Figure 3. MMP-9 activity response to exercise

Values are reported as mean ± sd. Means are collapsed across conditions. * = significant difference from pre-exercise value. MMP-9 activity significantly increased from pre- to post-exercise, and again at 120 minutes post-exercise.

Effect of Post-Exercise Nutritional Intervention On Blood Variables

Blood Glucose

There were no signficant increases or decreases in blood glucose from pre- to post-exercise at any time. However, blood glucose concentrations in the CHO group was significantly elevated above the EAA/CHO group at 40 minutes post-exercise ($p = 0.01$), remaining significantly elevated until 60 minutes post-exercise ($p = 0.01$). Additionally,

blood glucose concentrations in the EX groups were significantly elevated above the CHO group at 100 minutes post-exercise (p = 0.01) and above the EAA/CHO group at 120 minutes post-exercise (p = 0.01). Blood glucose concentrations across the three conditions are presented in Figure 4.

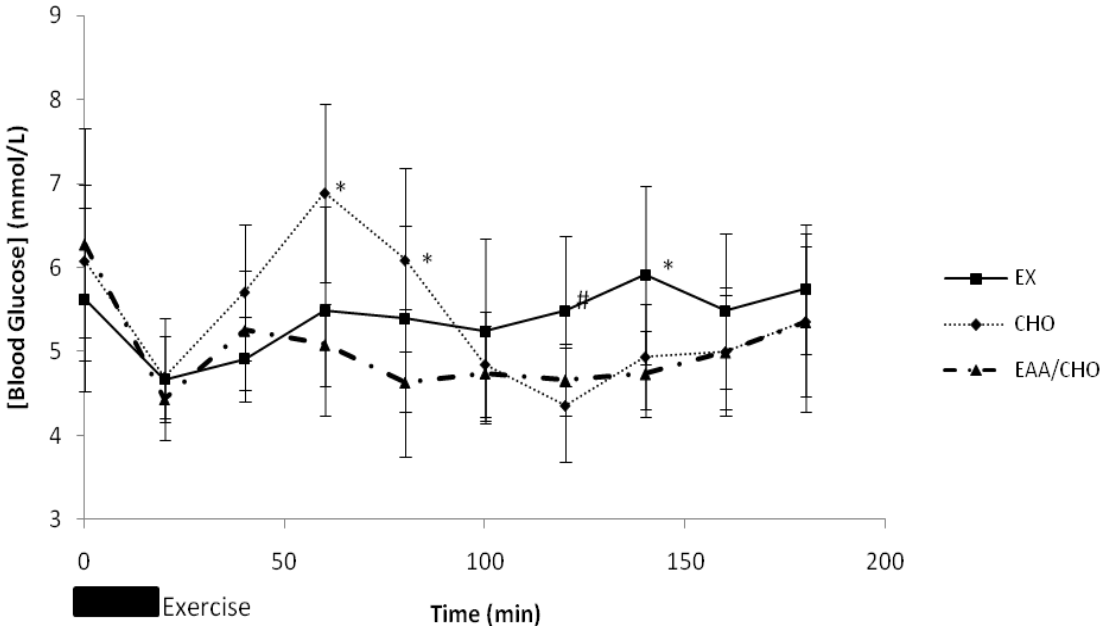

Figure 4. Blood glucose concentrations across conditions

Values are reported as mean ± sd. * = significant difference from EAA/CHO. # = significant difference from CHO.

Insulin

Insulin concentrations for both CHO and EAA/CHO conditions increased sharply beginning at 20 minutes post-exercise, reaching statistical significance at 40 minutes (p = 0.01, p = 0.03), 60 minutes (p = 0.01, p = 0.04), and 80 minutes (p = 0.03, p = 0.02) post-exercise. There were no statistical differences at any time between the CHO and

EAA/CHO conditions. Integrated total area under the curve (tAUC) analysis revealed statistically significant increases in both CHO and EAA/CHO ($p = 0.03$) compared to EX. Insulin concentrations by time and tAUC across the three conditions are presented in Figures 5 and 6.

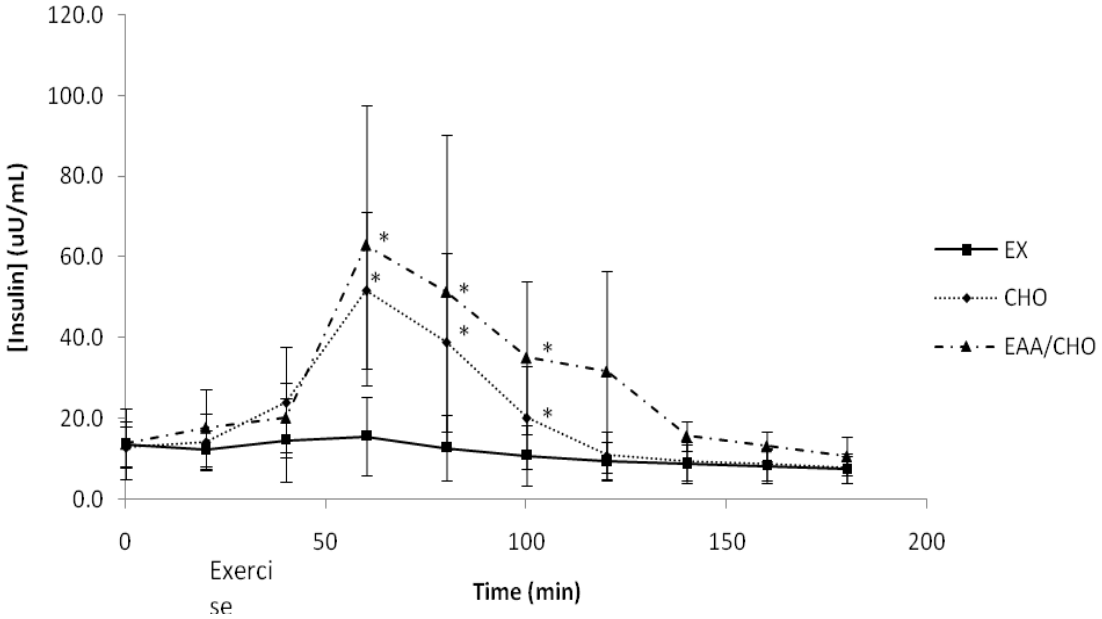

Figure 5. Insulin concentrations across conditions.

Values are reported as mean ± sd. * = significant difference from EX and pre-exercise value..

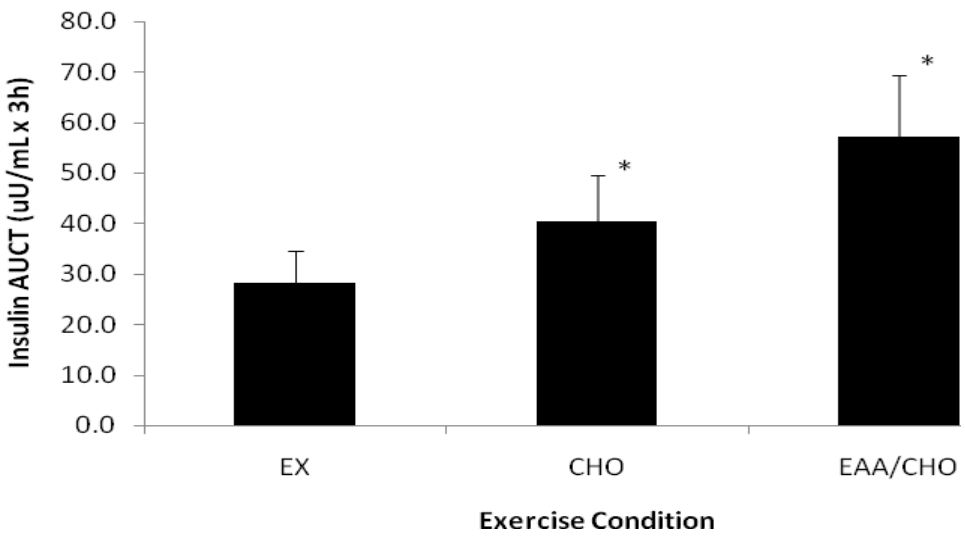

Figure 6. Total insulin integrated area under the curve by condition

Values are reported as mean ± sd. * = significant difference from EX. Both CHO and EAA/CHO were significantly greater than EX over the three hour period.

Plasma Amino Acid Concentrations

Plasma leucine concentrations were measured at four times: pre-exercise, 20 minutes post-exercise, 60 minutes post-exercise, and 120 minutes post-exercise. Leucine concentration was used as a representative marker for amino acid delivery. Plasma amino acid concentrations significantly increased in the EAA/CHO group only, beginning 60 minutes post-exercise ($p = 0.00$) and remained significantly elevated at 120 minutes post-exercise ($p = 0.00$). Plasma amino acid concentrations in the EX and CHO groups did not differ significantly from pre-exercise values at any time. Plasma amino acid concentrations across the three conditions are presented in Figure 7.

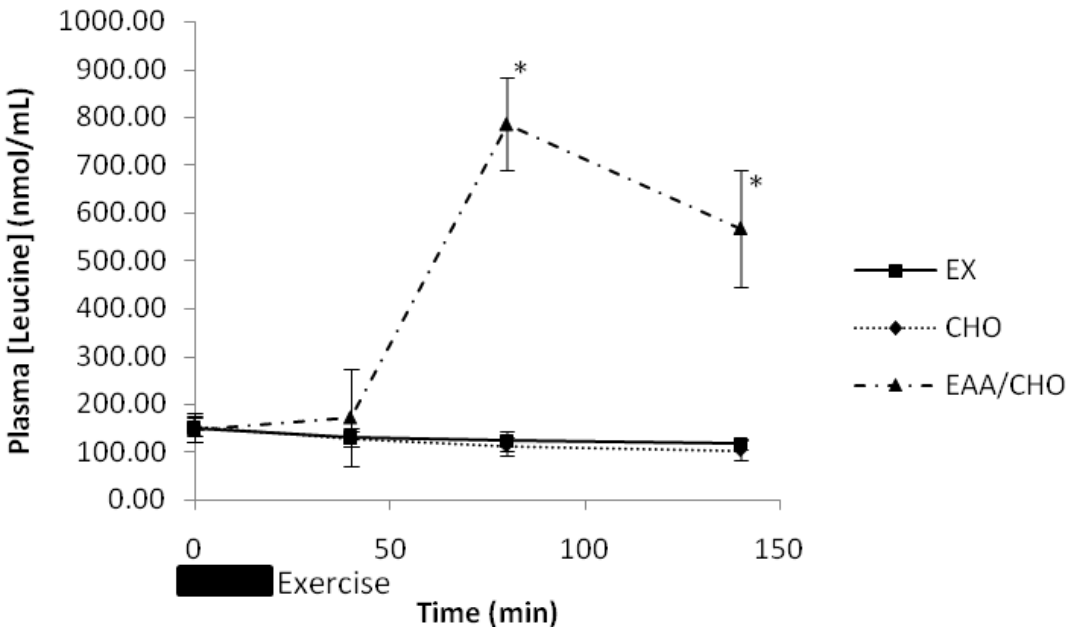

Figure 7. Plasma amino acid concentrations across conditions

Values are reported as mean ± sd. * = significant difference from EX and CHO, and significantly elevated from baseline.

Serum Free IGF-1 Concentrations

Serum free IGF-1 concentrations in the EAA/CHO condition displayed an increasing trend from pre- to post-exercise values, reaching statistical significance at 160 minutes post-exercise ($p = 0.02$). Free IGF-1 concentrations in the EAA/CHO condition were significantly elevated relative to EX at 120, 140, and 160 minutes post exercise ($p = 0.01$, $p = 0.00$, $p = 0.00$); and were significantly elevated relative to CHO at 140 and 160 minutes post-exercise ($p = 0.01$, 0.00). Serum free IGF-1 concentrations across the three conditions are presented in Figure 8.

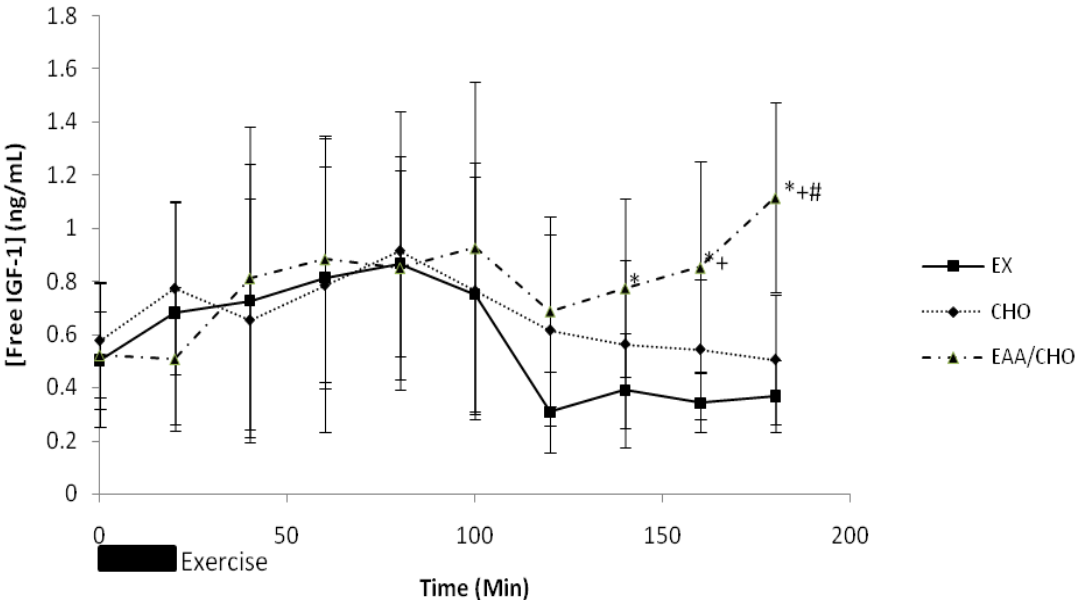

Figure 8. Serum IGF-1 concentrations across conditions

Values are reported as mean ± sd. # = significant difference from pre-exercise value. * = significant difference from EX. + = significant difference from EX and CHO.

CHAPTER V

DISCUSSION

The purpose of this study was to determine the effects of different macronutrient ratios following high-intensity exercise on the 'free' fraction of serum IGF-1 concentrations. Additionally, this investigation sought to gain evidence concerning the identity of the protease responsible for exercise-induced IGFBP-3 proteolysis. This is one of the first studies to demonstrate several important factors regarding IGF-1 regulation: 1) post-exercise macronutrient composition directly influences free IGF-1 concentrations within the peripheral vasculature; 2) high-intensity exercise promotes an immediate increase in plasma MMP proteolytic activity; and 3) by inference, MMPs are a likely candidate for exercise-induced IGFBP-3 proteolysis within the peripheral vasculature. Our data demonstrate that the inclusion of carbohydrate and amino acid supplementation following high-intensity exercise promotes significant increases in the free fraction of IGF-1 in the peripheral vasculature. Additionally, our data support the possibility that the exercise-induced production and activation of plasma MMPs may be partially responsible for this increased free IGF-1 bioavailability through increased proteolysis of IGFBP-3.

Effect of Exercise on IGFBP Regulation

High intensity exercise of various modes and/or intensities has consistently been shown to elicit significant GH release (57, 88-90, 96, 121) and recent evidence has shown

that many types of exercise increase plasma MMP concentrations (86, 124, 126, 138). This is significant because both of these factors may have implications in the acute regulation of IGF-1 by their respective roles in IGFBP regulation and/or proteolysis.

Exercise Protocol

The exercise intervention utilized in this investigation was a HIIT protocol very similar to protocols utilized in several recent studies (2, 18-20, 54, 55). HIIT was chosen because preliminary testing revealed that it elicited substantial GH release, in combination with significant post-exercise plasma MMP activity. In recent years, this type of training has been shown to be extremely effective in inducing drastic short-term increases in both aerobic and anaerobic conditioning (2, 18-20, 54, 55).

GH Response

While the GH response to high-intensity exercise has been shown to be extremely robust, the GH response to HIIT is higher than most values seen in the literature for any other type of exercise (2, 18-20, 54, 55). In the current investigation, participants displayed 60-fold serum GH concentration increases from pre- to post-exercise, remaining significantly elevated until 80 minutes post-exercise.

MMP Activity

A significant body of literature has provided evidence for increased post-exercise and/or post-trauma plasma MMP concentrations (83, 85, 124, 139). A growing body of evidence examining MMP activity from these perturbations has emerged, illustrating the importance of not only the quantification of MMP presence, but of MMP activation dynamics and quantification of proteolytic activity (9, 106, 109, 126). Thus, the assay utilized within this investigation served to quantify endogenous MMP activity induced

from the exercise intervention. The participants in this study displayed significant increases in post-exercise MMP activity, remaining significantly elevated until 20 minutes post-exercise. These results are not surprising, as they have been seen in similar studies (105, 110, 126). However, the significant increases in MMP activity seen in this study at 120 and 140 minutes post-exercise were unexpected and belie explanation from nutritional factors, as these increases were mirrored across all conditions.

MMP proteolysis of IGFBP-3

There is a significant body of literature supporting proteolysis of IGFBPs, specifically IGFBP-3, by MMPs (9, 47, 49, 105, 106, 110). While IGFBP-3 proteolysis was not measured within this study, several other lines of research have shown MMPs to be directly responsible for IGFBP-3 proteolysis (9, 46, 91, 93). Thus, while this investigation does not demonstrate concrete evidence that MMPs are responsible for IGFBP proteolysis, it does provide compelling circumstantial evidence for the case.

Macronutrient Effect on IGFBP Regulation

There is a wealth of information regarding individual IGFBP regulation by nutritional (17, 26, 42, 60, 78, 123) and stress-related factors (47, 82, 87, 93, 107, 112). However, no studies to date have examined the combination of exercise-induced factors combined with nutritional intervention on the acute regulation of IGF-1. Thus, this investigation attempted to integrate several bodies of known information into a dynamic setting by combining a high-intensity exercise protocol with different variations of post-exercise nutrition.

Insulin regulation of IGFBP-1

It is well understood that insulin is the primary hormone regulating IGFBP-1, with a large body of evidence supporting this theory (17, 79, 97, 113, 117). Current theory posits that elevated insulin concentrations in the portal vasculature directly inhibit hepatic production of IGFBP-1, with a time-course of roughly 90 minutes (60, 97). Additionally, there is some evidence that elevated insulin concentrations may increase transcapillary movement of IGFBP-1 from the peripheral vasculature (5). Within this investigation, post-exercise insulin spikes were achieved through delivery of CHO and EAA/CHO supplementation, achieving significant increases in insulin concentrations for both conditions.

Amino acid regulation of IGFBP-2

In contrast to the abundance of literature regarding IGFBP-1, there is a paucity of definitive information regarding IGFBP-2 regulation. The information that does exist suggests that IGFBP-2 is subject to the dual control of GH and protein/amino acid presence, with IGFBP-2 regulation enacted via hepatic inhibition similar to IGFBP-1 (24, 25, 104, 116, 136). The results of this study do seem to support this theory, as free IGF-1 concentrations only increased in the EAA/CHO group. However, as the individual IGFBPs were not actually measured, this is purely speculative.

Theoretical Model for Increased Free IGF-1

Several studies have examined the effects of exercise on serum total IGF-1 concentrations, with most studies finding no effect (12, 89, 111, 132). However, these studies assayed total, bound IGF-1 instead of free IGF-1. In the current study, a free IGF-1 assay kit was utilized, yielding information regarding the complex regulatory

dynamics of the individual IGFBPs. In this context, a theoretical progression of IGF-1 regulation resulting from this study becomes more apparent: 1) exercise induces GH release and stimulates MMP production/activation, cleaving IGFBP-3; 2) post-exercise CHO delivery induces insulin release, inhibiting hepatic IGFBP-1 production and possibly increasing clearance; and 3) post-exercise EAA delivery, in combination with exercise-induced GH release, inhibits hepatic IGFBP-2 production. In concert, we see significantly increased serum concentrations of free IGF-1.

Outside Variability

Participants in this study maintained normal outside activities throughout the entire protocol. To limit variation in study outcomes due to extraneous elements, participants were given instructions at the onset of the study and were reminded prior to the start of each condition pertaining to dietary and physical activity regimens. Compliance was documented verbally prior to each condition. Given that there were no significant differences in pre-trial variables between subjects and that this study examined only acute responses to the exercise and nutritional intervention, it is unlikely that outside variation influenced the present findings.

Conclusions

This is the first study to demonstrate that free IGF-1 concentrations can be altered through exercise and dietary manipulation. While the importance of exercise and post-exercise nutrition has long been known, this investigation adds valuable insight into the mechanisms of growth factor control and regulation. Additionally, this is the first study to demonstrate increased MMP activity post-exercise and provides compelling evidence identifying MMPs as the protease responsible for exercise-induced IGFBP-3 proteolysis.

While both of these findings have practical implications in general exercise prescription and nutritional counseling, their main significance lies in the fact that they bring us closer to unraveling the complex regulatory dynamics of the IGF system.

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APPENDICES

APPENDIX A

Research Study

Purpose: You are invited to participate in a research study examining the expression and regulation of Insulin-Like Growth Factor-1 (IGF-1). To complete this investigation we are recruiting participants to complete four identical exercise protocols with four different post-exercise re-feeding strategies while allowing pre- and post-exercise blood draws for analysis.

Participant Qualifications: Male, age 19-35 years old, moderate to high fitness (according to age-dependent VO2max Guidelines), healthy (as determined by a medical screening questionnaire), normally active (self-reported, at least 3-4 days/week of moderate to high intensity strength and/or endurance training for 30 to 60 minutes duration), some cycling experience, some resistance weight training experience, and available during the months of March and April.

Requirements: If you decide to participate, you will be asked to complete the medical screening questionnaire and read and sign the informed consent form. The performance trials will consist of four identical High-Intensity Interval Training (HIIT) protocols, with the intensity based upon the performance of a pre-trial 30-second Wingate maximum anaerobic power test. Each exercise trial will take approximately 20 minutes and total participation time to complete the research protocol will be about 3.5 hours for each visit. Blood draws will be taken preceding the performance trial, immediately following, and at 20 minute intervals for 160 minutes post-exercise. The study will require a total of five visits to the lab for a total time commitment of approximately 15 hours.

Contact Information: Please contact Byron Foster via email at fosteer@auburn.edu or you can come by the Thermal Lab (Memorial Coliseum 2118).

APPENDIX B

Medical Screening Questionnaire*

Please read each question carefully and answer honestly. If you do not understand the question, please ask the investigator for clarification. Check the appropriate answer.

No	Yes	
_____	_____	1. Are you under 19 or over the age of 35?
_____	_____	2. Do you presently smoke or have been a regular smoker?
_____	_____	3. Has your doctor ever said you have heart trouble?
_____	_____	4. Do you have family history of early cardiovascular death before the age of 50?
_____	_____	5. Have you ever had a heart murmur, rheumatic fever or respiratory problems?
_____	_____	6. Have you been told that you have a kidney disorder?
_____	_____	7. Have you been told that you have diabetes or that your blood sugar is too high?
_____	_____	8. Have you been hospitalized in the past year for any reason that might interfere with you being able to perform this trial or do you have any recent training injuries that would prohibit you from cycling?
_____	_____	9. Are you taking prescription medicine? (If yes, please discuss this with the person taking this information.)
_____	_____	10. Do you have any reason to believe that your participation in this investigative effort may put your health or well being at risk? (If so, please discuss this with the person taking this information.)

Signature of subject _____ Date _____

This person has been cleared to participate in this study.

Signature of investigator_____ Date _____

APPENDIX C

RECENT TRAINING HISTORY QUESTIONNAIRE

Subject code number:_____ Date:_____

Please answer these questions regarding your recent training level. Answer the questions by giving explanations provided, where indicated.

1. On average, how many days per week do you perform resistance exercise?

_____

2. On average, how many days per week do you perform endurance exercise?

_____

3. How long have you been exercising on a regular basis?

_____

4. Did you do any interval training during this time? If yes, please describe.

_____

_____

_____

_____

APPENDIX D

Auburn University
Auburn University, Alabama 36849-5323

Department of Kinesiology
2050 Memorial Coliseum
Thermal Lab (Room 2118)

Telephone: (334) 844-4483
Fax: (334) 844-1467
Thermal Lab: (334) 844-1479

Informed Consent for a Research Study Entitled
"Acute regulation of IGF-1 by differential growth-factor-binding-protein expression, inhibition, and proteolysis"

Project Overview: You are invited to participate in a research study examining the effect of different post-exercise re-feeding strategies on the expression of insulin-like growth factor (IGF-1). Participants will perform four identical high-intensity interval training (HIIT) protocols and then ingest one of four different post exercise supplements. Blood draws will be taken both before and after completion of the protocols

Purpose: The purpose of this investigation is to examine the effect of different post-exercise re-feeding macronutrient ratios on the regulation of IGF-1, a hormone shown to be extremely potent in activating anabolic (muscle building) pathways in humans.

Participation Requirements: To be eligible, you must be:

1. a male participant of 19 to 35 years of age
2. *at or greater than the 50^{th} percentile for age-related cardiorespiratory fitness guidelines (*'equal to or above the 50th percentile' means that a person is above average according to guidelines for maximal oxygen uptake with exercise. This means that they have above average aerobic fitness.
3. at low risk for medical complications (as determined from a series of questions we will ask you regarding your health).
4. currently strength and/or endurance training 3-4 days/week, moderate to high intensity, 30-60 minutes duration

If you decide to participate, we will ask you a series of questions regarding your health and fitness, and then have you read and sign this informed consent. You must meet all requirements for participation.

Participant's initials _____

"Acute regulation of IGF-1 by differential growth-factor-binding-protein expression, inhibition, and proteolysis"

Testing Procedures:

Visit 1: On the first visit to the lab, you will complete the Medical Screening Questionnaire, complete the training log, and read and sign the University-approved informed consent form. Either Ernest Byron Foster or Gordon Fisher will be present for all informed consent briefings. If you meet all requirements for the study and choose to continue, you will be included in the study.

Descriptive data will be obtained (age, height, weight, 3-site skin fold body composition assessment). You will be familiarized with both the Monark and Quinton cycle ergometers. After familiarization, you will perform a 30-second Wingate anaerobic power test. This is a 30-second all-out cycling exercise against a resistance which is determined by your lean bodyweight. As this is a high intensity test, many people feel dizzy or light-headed after the test. Because of this, we will monitor you closely and ensure that you complete a thorough cool-down by cycling for several minutes against a very low resistance after the test is complete.

The total time for the exercise testing will be approximately ten minutes (including a warm-up and cool down) and the total time commitment for the entire visit will be approximately one hour.

Visits 2-4: On four non-consecutive days, separated by at least 48 hours, you will perform High Intensity Interval Training (HIIT) on a stationary cycle ergometer. The trials will be performed at the same time of day to assure similar circadian rhythms.

You will need to consume at least 500 mL of water before arriving at the lab. During each trial, you may drink as much water as you like. Upon arrival at the lab, you will provide a small urine sample which we will test to determine your hydration status. If the test indicates that you are dehydrated, you will be given a chance to re-hydrate by consuming water before beginning the trial-or the trial will be rescheduled.

A Polar heart rate monitor will be placed on your chest for heart rate analysis. We will place a small needle into a vein on the back of your hand, and have you sit quietly for 15 minutes before the pre-trial blood sample. This venous catheter will remain in the back of your hand for the remainder of the daily trial.

You will then perform the 15-minute HIIT protocol. This is a type of interval training performed on a stationary cycle in which the intensity will vary from high to low within the protocol. Following completion of each protocol, you will ingest a commercially available post-exercise supplement with one of four different macronutrient ratios (protein only, carbohydrate only, protein/carbohydrate mix, or non-caloric placebo). Blood samples will be taken immediately post trial and at 20-minute increments for 160 minutes post-exercise. Each blood sample will consist of about 1.5 mL. There will be a total of ten blood samples. Thus, the total blood taken will be approximately 15mL, or the equivalent of about 5 teaspoons. The entire time commitment for each of these visits will be approximately 3.5 hours.

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***Summary*:** The total time commitment for all five visits will be approximately 15 hours (1 hour for the initial visit plus the 4 performance trials at 3.5 hours each). The total blood volume required for the entire study is approximately 60 mL (about 15 teaspoons).

Potential Risks:

1. While performing any exercise there is a chance of muscle strains, sprains, and even death. The American College of Sports Medicine estimates the risk at 0.5 per 10,000 individuals.
2. Due to the high intensity nature of the Wingate cycle ergometer test, many participants feel naceous and/or light-headed after completing the test.
3. With any blood draw procedure there is a risk of infection, bruising, irritation at injection site, fainting, and/or contact with blood-borne pathogens.
4. There is a chance that you could have an allergic reaction to the ingredients in the nutrition supplement. You must not participate if you are allergic to any ingredients contained in the supplements.

Note: **It is important for you to realize that you are responsible for any costs incurred in the event of an injury.**

Precautions:

1. Although the training for this trial is of a higher intensity, it is of short duration and at a comfortable environmental temperature and humidity level. All individuals recruited for this trial will exceed the 50th percentile for age-dependent cardiorespiratory fitness guidelines and are engaging in at least 3-4 days per week of moderate- to high-intensity strength and/or endurance training. Therefore, the risk of death or serious medical issues is reduced in this population.

We have additionally employed the use of a Medical Screening Questionnaire to assist in eliminating participants that have potential medical or orthopedic identified risks. During the trials you will always be accompanied by either Ernest Byron Foster or Gordon Fisher, both of which maintain current CPR Certifications. You will not be allowed to begin trials if our pre-trial testing indicates that you are dehydrated. Your heart rate will be recorded and monitored throughout the trial.

Upon termination of a trial, you will be provided with additional fluids and monitored until your heart rate reaches <100bpm and your blood pressure monitored each 15 minutes until it is <150 systolic and <90 diastolic. If you are unresponsive or need further medical care the EMS will be notified. Emergency medical numbers are listed within easy access of all locations in the Thermal Lab and there is a current emergency action plan in place in the lab.

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2. Many participants feel nauseated and/or light-headed after the completion of Wingate test. Therefore, a waste bucket will be provided in the event you feel the need to vomit. After the completion of the test you will be closely monitored by at least two investigators and will be instructed to continue pedaling against very light resistance for as long as needed (typically 4-5 minutes).
3. Both investigators participating in data collection (Ernest Byron Foster and Gordon Fisher) are certified in phlebotomy and have completed blood-borne pathogen training. Only new, sterile blood-gathering equipment and aseptic techniques will be utilized throughout all data collection and analysis processes. All sharps and contaminated paraphernalia will be disposed of in approved containers. Additionally, all lab personnel involved in data collection have completed Lab Safety and Blood-Borne Pathogen training.
4. You will be provided a comprehensive ingredient list of all the supplements utilized within the study. Additionally, you will be closely monitored for adverse reactions after ingesting any of the supplements.

Benefits: You will receive a Wingate assessment of your maximum anaerobic power and a body composition assessment. You will receive a report indicating your individual GH response to the exercise protocols as well as a comparison report to other group participants.

Your participation is completely voluntary. If you change your mind about participating, you can withdraw at any time during the study. If you choose to withdraw, your data can be withdrawn as long as it is identifiable. Your decision about whether or not to participate or to stop participating will not jeopardize your future relations with Auburn University, the Department of Kinesiology, or the Thermal Lab.

Your privacy will be protected. Any information obtained in connection with this study will remain anonymous. Information obtained through your participation may be used in scholarly publications and presentations. If you have any questions, we invite you to ask us now. If you have questions later, you can contact Ernest Byron Foster (fosteer@auburn.edu). You will be provided with a copy of this document to keep. For more information regarding your rights as a research participant, you may contact the Auburn University Office of Human Subjects Research or the Institutional Review Board phone (334) 844-5966 or email at hsubjec@auburn.edu or IRBChair@auburn.edu.

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HAVING READ THE INFORMTION PROVIDED, YOU MUST DECIDE WHETHER OR NOT YOU WISH TO PARTICIPATE IN THIS RESEARCH STDUY. YOUR SIGNATURE INDCATED YOUR WILLINGNESS TO PARTICIPATE.

_____	_____	_____
Participant's Signature	Date	Print Name
_____	_____	_____
Investigator Obtaining Consent	Date	Print Name
_____	_____	_____
Co-Investigator	Date	Print Name