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The blood glucose and insulin responses to acute maximal and submaximal aerobic exercise are well characterized (Marliss & Vranic, 2002), though our understanding of the molecular mechanisms effecting improved insulin sensitivity following acute exercise remains incomplete. Apelin has been reported to enhance glucose uptake and insulin sensitivity *in vitro* and *in vivo* (Dray et al., 2010). Apelin, therefore, merits examination as a potential regulator of the acute enhancement of insulin sensitivity *in vivo*. **PURPOSE**: To investigate apelin's response to acute bouts of maximal and submaximal aerobic exercise, so as to elucidate apelin's influence on glucose homeostasis and insulin sensitivity. **METHODS**: Twelve (22.8 ± 2.9 yrs) apparently healthy male (n=7) and female (n=5) subjects completed maximal (VO_2max) and submaximal (70-75% VO_2max) aerobic treadmill tests, as well as a 54g glucose challenge (GC), each separated by at least 3 days but not more than 14 days. Blood was sampled four times (Pre-, Post-, 1 hr post- and 24 hrs post-treatment) and assessed immediately for hematocrit and blood glucose and assayed for plasma apelin and plasma insulin. Insulin resistance was determined via HOMA-IR index. **RESULTS**: No main effect existed for apelin by condition (p=0.324) or by time (p=0.633). Blood glucose and plasma insulin were significantly elevated following VO_2max and GC, but not following submaximal exercise. Insulin resistance, as measured via HOMA-IR score, was

significantly improved at one-hour post-submaximal exercise. Resting plasma apelin was significantly correlated with plasma insulin (R=0.702, p=0.011), as well as with resting HOMA-IR score (R=0.738, p=0.006). Following submaximal exercise, plasma apelin was significantly correlated with HOMA-IR score (R=0.672, p=0.017).

CONCLUSION: Though plasma apelin was not significantly altered by either acute aerobic exercise or GC, our results suggest that elevated plasma apelin is associated with increased incidence of desensitization to insulin and that lower levels may be related to an enhancement in insulin sensitivity.

THE EFFECTS OF ACUTE EXERCISE ON PLASMA APELIN AND ITS RELATION

TO GLUCOSE HOMEOSTASIS AND INSULIN SENSITIVITY

by

Justin Daniel Waller

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Approved by

_____
Committee Chair

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of The

Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

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Date of Acceptance by Committee

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Date of Final Oral Examination

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3T3-L1   Fibroblast-like 3T3 mouse adipocyte cell line

AC   Adenylate cyclase

ACTH   Adrenocorticotropic hormone

ADH   Antidiuretic hormone

Akt   Protein kinase B, serine/threonine-specific

AMPK   5' Adenosine monophosphate-activated protein kinase

APJ   Apelin receptor

APJ-apelin   Shorthand for the apelinergic system

APKO   Apelin gene knockout

APLN   Apelin gene

AS160   Akt substrate, 160 kDa; now known as TBC1D

AT_1 Angiotensin II receptor type 1

BMI   Body mass index

BV   Blood volume

cAMP   3'-5' cyclic adenosine monophosphate

$CD4^+$ Cluster of differentiation 4 T-helper cells

CORT   Corticosterone

DAG   Diacylglycerol

EDTA   Ethylenediaminetetraacetic acid

ELISA   Enzyme-linked immunosorbent assay

eNOS	Endothelial nitric oxide synthase
Epi	Epinephrine
ERK	Extracellular signal-regulated kinases
FFA	Free fatty acid
FPG	Fasting plasma glucose
FPI	Fasting plasma insulin
GC	Glucose challenge
GH	Growth hormone
G_i	Inhibitory G protein
GLUT1	Facilitated glucose transporter type 1, solute carrier family type 2
GLUT4	Facilitated glucose transporter type 4, solute carrier family type 2
GPCR	G protein-coupled receptor
G_q	Other G protein
G_s	Stimulatory G protein
HbA_{1c}	Glycated hemoglobin
HGF	Hepatocyte growth factor
HIF-1α	Hypoxic-inducible factor 1 alpha
HIV	Human immunodeficiency virus
HOMA-IR	Homeostatic Model of Assessment of Insulin Resistance
HPA	Hypothalamic-pituitary axis
HPLC	High-performance liquid chromatography
ICV	Intracerebroventricular

IGT	Impaired glucose tolerance
IR	Insulin Resistance
KLF2	Krüppel-like factor 2
KO	Knockout
KOR	Kappa opioid receptor
L-arg	L-arginine
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase, serine/threonine/tyrosine-specific
MEF2	Myocyte enhancer factor 2
mRNA	Messenger ribonucleic acid
NCX	Sodium-Calcium Exchanger
NE	Norepinephrine
NGT	Normal glucose tolerance
NHE	Sodium-Hydrogen Exchanger
NO	Nitric Oxide
NRF1	Nuclear respiratory factor 1
OGTT	Oral glucose tolerance test
P70S6K	S6 ribosomal protein serine/threonine-specific protein kinase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PI3K	Phosphatidylinositol-3 kinases
PIP$_2$	Phosphatidylinositol 4,5-bisphosphate
PIP$_3$	Phosphatidylinositol 3,4,5-triphosphate

PKA	Protein kinase A, cAMP-dependent protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
Pyr^1-apelin-13	Pyroglutamated apelin-13
RF/6A	Rhesus fetus choroid-retinal endothelial cell line
RMANOVA	Repeated measures analysis of variance
ROS	Reactive oxygen species
SNS	Sympathetic nervous system
TBC1D	TBC1 domain family member 1; current designation of AS160
TFAM	Mitochondrial transcription factor A
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
VO$_2$max	Maximal aerobic capacity or maximal oxygen consumption
VSMC	Vascular smooth muscle cell
WHR	Waist hip ratio

CHAPTER I

INTRODUCTION

Novel treatments for diabetes mellitus – particularly type II diabetes mellitus (T2D) – are readily studied, as the continual rise in T2D diagnoses elevate global healthcare costs. Total and direct healthcare costs related to diabetes mellitus in 2007 reached $174 and $118 million, respectively. To combat this debilitating disease, a variety of potential targets have been investigated over recent decades, leading to treatments focused on elevating plasma insulin levels, enhancing tissue insulin sensitivity and reducing carbohydrate absorption rates, among other interventions (Verspohl, 2012). The hallmark of diabetes is insulin resistance, resulting in chronically elevated levels of blood glucose, control of which is the ultimate goal of therapy (Cavallo et al., 2012; Ringström et al., 2010). Nearly 1000 therapies have been described, the majority of which fail to begin clinical testing, as they neglect the pathophysiological foundations of this multifaceted disease. Potential targets must be mined to reverse the worldwide economic burden of T2D and its associated complications. Continual assessment of the molecular players involved in appropriate glucose control is the vehicle by which research in this area may progress.

A molecular target of recent interest is apelin, an adipokine with myriad physiological effects and secreted from a variety of tissues, including the heart, vasculature, brain and adipose tissue (Kleinz & Davenport, 2005). Chief among its

effects is apelin's purported function in glucose homeostasis and enhancement of insulin sensitivity (Dray et al., 2008; Yue et al., 2010). Recent research suggests that apelin is an inextricable component of insulin sensitivity, as reported in studies in apelin knockout (APKO) mice compared to wild type controls (Li, Ding, Hassan, Abdelkader, & Shang, 2013; Yue et al., 2010). Likewise, acute exercise is reported to confer peripheral and hepatic insulin-sensitizing effects, while simultaneously stimulating skeletal muscle glucose uptake via insulin-independent translocation of GLUT4 proteins (Devlin, Hirshman, Horton, & Horton, 1987; Henriksen, 2002). An important distinction is to be made, as contractile activity increases GLUT4 translocation and subsequent skeletal muscle glucose uptake through signaling mechanisms dissimilar from that of insulin signaling; however, it is proposed that these paths converge on some downstream molecule (Treebak et al., 2014; Vendelbo et al., 2014). Enhanced contraction-induced glucose uptake lasts for up to two hours post-exercise, though the duration of improved insulin sensitivity is much longer (Borghouts & Keizer, 2000).

The mechanism(s) of enhanced peripheral insulin sensitivity following acute exercise remains incompletely understood, though it is well known that these benefits are transient (Fontana, Klein, & Holloszy, 2010). Studies vary widely on the topic of time to insulin sensitivity reversal following the most recent bout of exercise, providing a range of estimates (Borghouts & Keizer, 2000; Heath et al., 1983; Kirwan, Bourey, & Kohrt, 1991; Magkos, Mohammed, & Mittendorfer, 2010). Generally it is accepted that these benefits are lost within 12-48 hours after the most recent exercise bout (Magkos, Tsekouras, Kavouras, Mittendorfer, & Sidossis, 2008). Exercise training naturally offers

an improvement in insulin sensitivity, though trained individuals experience a reversal of insulin sensitivity similar to untrained individuals following periods of inactivity (Heath et al., 1983; LeBlanc, Nadeau, Richard, & Tremblay, 1981; Ren et al., 1994). Research now suggests that insulin sensitivity enhancement is a benefit facilitated primarily by acute alterations to metabolism mediated by single exercise bouts – not exercise training. Whereas exercise training has important implications for both healthy and non-healthy (i.e. obese, T2D) populations in terms of chronic metabolic control – such as enhanced lipid metabolism or increased muscle mass, among other benefits – acute exercise is the principal means by which glucose homeostasis is regulated, in terms of the contribution to this phenomenon by physical activity (Goodyear & Kahn, 1998; Heath et al., 1983; Holloszy, 2005).

Congruent with our limited understanding of acute exercise's enhancement of insulin sensitivity, apelin's role in this regard has not been firmly established. Focused study of apelin may provide the insight needed to elucidate our incomplete understanding of the mechanisms by which exercise enhances the action of insulin and facilitates blood glucose control. Apelin is reported to enhance peripheral glucose uptake in both normal and insulin-resistant rodent models, as shown by intravenous-injected apelin-induced blood glucose reductions (Dray et al., 2008). This study also reported that glucose uptake in skeletal muscle was enhanced following apelin injection during a hyperinsulinemic-euglycemic clamp, demonstrating apelin-induced enhancement of glucose uptake under conditions of high blood insulin and normal blood glucose. Apelin-stimulated glucose uptake was confirmed in isolated adipocytes obtained from healthy individuals (Attané et

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al., 2011; Than et al., 2014) and T2Ds (Dray et al., 2010). These results collectively support apelin's role as an *exogenous* insulin sensitizer under conditions of high blood insulin. Whether apelin exhibits similar insulin sensitizing effects under periods of low to normal blood insulin in humans is yet to be determined. In addition, the role of apelin as an *endogenous* insulin sensitizer and blood glucose regulator in normal, healthy individuals must be defined.

Several studies have utilized exercise-training models in attempting to define apelin's proposed insulin sensitizing effects. There is much dispute across these studies, however, and their limitations are multifactorial. These limitations, detailed herein, have resulted in equivocal findings. The primary issue regarding exercise training-related apelin studies is their use of non-healthy subjects; the majority of literature involves the response of apelin to exercise training of varied duration in obese and T2D subjects only. Obese and T2D populations exhibit considerable variability in apelin concentration, an occurrence that is to be expected given the vast number of metabolic contributors known to affect blood apelin (Castan-Laurell, Dray, Knauf, Kunduzova, & Valet, 2012; Dray et al., 2010; Weir et al., 2009). Additionally, these studies neglect to report the amount of time in the interim between the final exercise bout and post-training blood collection, a consideration that must not be ignored when interested in evaluating endocrine and/or cytokine response.

A limitation present in *all* studies regarding apelin is an indefinite baseline concentration in humans. Reported mean plasma apelin concentration in humans varies considerably across literature, from 0.0018–1.25 ng/ml in healthy control subjects, with a

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collective reported standard deviation of 0.140 ng/ml (Alexiadou et al., 2012; Castan-Laurell et al., 2008; Cavallo et al., 2012; Dray et al., 2010; Krist et al., 2013; Weir et al., 2009). This variability in healthy subjects, not to mention the significant variance in non-healthy populations, clearly highlights the need for establishing appropriate baseline data so that acute and chronic responses of apelin may be appropriately assessed.

Acute exercise represents an excellent standard by which apelin's relationship with glucose control and insulin sensitivity may be evaluated, given our rich understanding of the blood glucose and insulin responses to acute aerobic exercise. These responses vary as a function of aerobic intensity and are readily reproducible. High intensity aerobic exercise, such as that exhibited during a VO_2max test, elevates blood glucose and modestly inhibits insulin secretion. In contrast, moderate intensity, steady state aerobic exercise decreases blood insulin while maintaining blood glucose (Marliss & Vranic, 2002). Immediately following bouts of aerobic exercise at high intensity (>88% VO_2max) there is a spike of blood insulin. Following both moderate and high intensities of acute aerobic exercise insulin sensitivity is transiently enhanced. This phenomenon may be at least partially attributable to apelin's purported role as an endogenous insulin sensitizer. Apelin synthesis and release is affected by a multitude of factors and care must be taken in considering its role across and within specific populations. Thorough evaluation must occur even in apparently healthy individuals, as apelin is tightly regulated by blood insulin, hypoxic conditions and adiposity, all of which acutely influence glucose flux. Insulin is considered the greatest regulator of apelin's plasma concentration, a potent signal stimulating apelin's synthesis and release from

adipose tissue, amongst other tissues (Bastard & Feve, 2013; Boucher et al., 2005; Sakurai et al., 2013; Than et al., 2014). In return, it has been shown that apelin exerts considerable influence on insulin, acting via APJ receptors located on pancreatic islet cells (Ringström et al., 2010; Sörhede Winzell, Magnusson, & Ahrén, 2005). This dynamic interplay represents the crux of this study and no doubt becomes quite complex during and following exercise.

Purpose

The primary purpose of this study was to assess the response of plasma apelin to two bouts of varied intensity of acute aerobic exercise, as well as to a 54g glucose challenge (GC), and to examine the relationship of apelin over time with observed alterations in glucose homeostasis and insulin sensitivity.

Objectives and Hypotheses

Although regular exercise is justifiably recommended for T2Ds, obese and other non-healthy populations in order to mitigate metabolic dysfunction, the mechanisms by which acute exercise improves insulin sensitivity and glucose homeostasis needs to be more clearly defined. Apelin's contribution to these metabolic alterations must also be appropriately assessed. In order to do so, appropriate baseline apelin data must be established in healthy populations before characterizing the response of apelin to acute and chronic exercise in clinical populations. In manipulating blood glucose and insulin via two acute aerobic exercise conditions and a positive control 54g GC, and by performing select assays at four time points (pre-, immediately post-, one hour post- and 24 hours post-condition), we achieved the following threefold objectives:

1. Determined the biological response of plasma apelin to two distinct acute aerobic exercise conditions and a 54g glucose load while defining baseline plasma apelin concentration.

2. Evaluated plasma apelin's relationship with insulin sensitivity, via a validated measure of the inverse of sensitivity – insulin resistance, per HOMA-IR – under these three conditions.

3. Assessed the contribution of plasma apelin in the reduction of insulin resistance following acute aerobic exercise.

Following acute aerobic exercise of high-intensity (VO_2max), it was hypothesized that plasma apelin concentration would be significantly increased from baseline immediately post- and 60 minutes post-exercise, resulting from significant increases in blood insulin following high-intensity exercise. In contrast, it was hypothesized that acute aerobic exercise of sustained (30 minute) moderate (~70-75% VO_2max) intensity would not significantly increase apelin concentration immediately post-exercise or 60 minutes post-exercise, as blood insulin is not shown to increase significantly during these periods. It was also hypothesized that the 54g GC would elicit significant increases in apelin concentration immediately post- and 60 minutes post-challenge, resulting also from significant increases in blood insulin and blood glucose.

In addition, it was hypothesized that neither exercise condition would elicit significant differences in apelin 24 hours post-exercise from baseline, consistent with the transient improvement and subsequent reversal of insulin sensitivity following acute bouts of exercise. It was, however, hypothesized that insulin resistance, as measured by

the HOMA-IR index, would be mitigated 60 minutes post-exercise in both conditions, with sensitivity improvements reversing thereafter and not significantly different from baseline at 24 hours post-exercise; it was hypothesized that insulin sensitivity would not be affected by GC one hour and 24 hours post-challenge. The observed responses of plasma apelin and insulin resistance to two varied acute bouts of exercise and 54g GC allowed us to assess the endogenous activity of apelin as a circulating insulin sensitizer and contributor to whole body glucose homeostasis.

Assumptions and Limitations

1. It was assumed that acute maximal, graded aerobic exercise (VO_2max) and submaximal exercise would elicit well-defined alterations in blood glucose, blood insulin, catecholamines, growth hormone and glucagon.

2. It was assumed that no gender differences existed regarding baseline apelin levels, apelin responses to acute exercise or apelin's influence on glucose homeostasis and/or insulin sensitivity.

3. Blood was drawn solely from an antecubital arm vein and may not have reflected whole body or cell-level apelin response, activity or influence on glucose homeostasis.

4. Results may not be wholly generalizable, as they are representative of young, healthy human subjects, aged 18-35.

Delimitations

1. Apparently healthy subjects, aged 18-35, were recruited to limit between-subject variability.

2. Subjects were required to keep dietary records prior to each session to assess and control for dietary influences on findings.

3. Subjects were required to discontinue supplementation (>2 weeks) that may influence markers of oxidative stress or alter glucose metabolism.

4. Subjects could not currently be on any medications that might influence catecholamines and/or glucose, insulin or fat metabolism.

Dependent Variables

1. Plasma Apelin Concentration: ng/ml

2. Blood Glucose Concentration: mg/dl

3. Plasma Insulin Concentration: mIU/L

4. Insulin Resistance: per HOMA-IR index score computation (see Methods)

5. Resting and Exercise Variables

   a. Heart Rate (HR): in bpm

   b. Oxygen Consumption (VO_2) and Maximal Oxygen Consumption (VO_2max): in L O_2/min and ml $O_2 \cdot kg^{-1} \cdot min^{-1}$

   c. Plasma Volume, as hematocrit (%)

   d. Blood Pressure and Mean Arterial Pressure (MAP): in mmHg

   e. Total Work Completed: in Watts (W)

   f. VO_2max Test Duration: in mins

6. Other Descriptive Variables – Resting Values

   a. Body Fat Percentage (BF%) – Siri and Brozek computations

   b. Body Mass Index (BMI): kg/m^2

Definition of Terms

1. Adipokine: A cytokine, or cell signaling molecule, released predominantly from adipose tissue

2. Apelin: A classic adipokine, first discovered in 1998 by Tatemoto et al., and the endogenous ligand of its receptor, APJ

3. G Protein-Coupled Receptor (GPCR): A cell surface receptor that initiates intracellular signaling via G protein-coupled second messaging

4. Glucose Challenge: An oral bolus consumption of glucose of a given volume

5. Glucose Transporter, Type 1 (GLUT1): The predominant glucose transporter isoform found in erythrocytes and endothelium of blood-brain barrier

6. Glucose Transporter, Type 4 (GLUT4): The predominant glucose transporter isoform found in skeletal and cardiac muscle and adipose tissue

7. Homeostatic Model of Assessment of Insulin Resistance (HOMA-IR): An indirect method of quantifying insulin resistance and β-cell function, developed in 1985 by Matthews et al. and correlating strongly with glucose clamp methodology

8. Insulin Sensitivity: The inverse of insulin resistance, signifies the degree to which a cell or tissue responds to the physiological action of insulin

9. Maximal Aerobic Capacity (VO_2max): Or maximal oxygen consumption, refers to the maximal volume of oxygen consumption and utilization, typically expressed as a volume of oxygen consumed per body weight over a given time

10. Pyr^1-apelin-13: The most biologically potent isoform of apelin, following post-translational modification of pyroglutamation.

CHAPTER II

REVIEW OF LITERATURE

General Apelin Information

<u>Apelin</u>

Apelin, a cytokine primarily synthesized in and secreted by adipose tissue, is the endogenous ligand of the G protein-coupled receptor (GPCR) APJ (Tatemoto et al., 1998). The family of apelin peptides is derived from a 77-amino acid preproprotein (preproapelin) encoded by a single gene, APLN, which, after transcription and translation, produces the 8.5 kDa preproapelin. Angiotensin-converting enzyme 2 (ACE2) was reported to enzymatically cleave both apelin-36 and apelin-13 peptides, though the enzymatic mechanism by which preproapelin is cleaved to its proprotein and subsequent other isoforms is unknown (Kalea & Batlle, 2010; Vickers et al., 2002). Various isoforms of apelin are biologically active, the most potent and structurally stable of which is pyroglutamated apelin-13 (Pyr[1]-apelin-13) (Kleinz & Davenport, 2005; Zhen, Higgs, & Gutierrez, 2013). Moreover, it has been demonstrated that, as with many peptides, the post-translational addition of pyroglutamate to the N-terminus of apelin-13 (i.e. Pyr[1]-apelin-13) prevents enzymatic degradation while enhancing biological potency (Kleinz & Davenport, 2005). All identified bioactive isoforms in humans (apelin-12, -13, Pyr[1]-apelin-13, -17, and -36) are known to activate the APJ receptor, ostensibly a

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requisite for biological activity (Dennis K Lee, George, & O'Dowd, 2006; Zhen et al., 2013). The predominant isoforms in humans are apelin-36, apelin-13 and Pyr[1]-apelin-13 (O'Carroll, Lolait, Harris, & Pope, 2013) and it has been posited that the length of the apelin isoform defines its tissue-specific function, though no studies comparing the disparity of isoforms' efficacies have been carried out (Kleinz, Skepper, & Davenport, 2005). The C-terminus region of the apelin peptides seems to dictate its biological function, as it has been shown that N-terminal deletions do not appreciably diminish function (O'Carroll et al., 2013). From this point we will collectively refer to the family of bioactive isoforms simply as apelin. The results and discussion sections refer to the aggregate of these isoforms, as the methodology used to measure apelin is not sensitive enough to distinguish the various isoforms. Future HPLC assays may help define the relative contribution of the various isoforms to their observed function at the tissue level.

Apelin and APJ exhibit remarkable distribution amongst tissues, with significant APJ expression in the thalamus, hypothalamus, frontal cortex and lower brain stem. Endothelial and vascular smooth muscle cells (VSMCs) and other peripheral tissues such as cardiac myocytes, pancreatic islet cells, skeletal muscle and adipose tissue also exhibit considerable APJ concentration. Substantial APJ and apelin mRNA was reported in humans in the spleen and placenta, with considerable apelin content noted in human placental tissue (Kleinz & Davenport, 2005; Medhurst et al., 2003). It is increasingly evident that apelin is necessary for cardiovascular development and that this is highly conserved across all species. Apelin functions in various other roles, exerting endocrine activity as a circulating peptide, as well as paracrine and autocrine activity, seemingly

requisite for embryonic and tumor angiogenesis (Kälin et al., 2007; Kleinz & Davenport, 2005; Ronti, Lupattelli, & Mannarino, 2006).

APJ

Apelin exerts its action via binding to APJ, a 380-amino acid GPCR which functions putatively through a variety of G proteins to effect downstream signaling (Habata et al., 1999; Masri, Lahlou, Mazarguil, Knibiehler, & Audigier, 2002; O'Carroll et al., 2013). Interestingly, it was noted that continued agonist binding of APJ elicits receptor internalization and/or altered downstream phosphorylation, leading to desensitization and impaired target cell responsiveness (Reaux, Gallatz, Palkovits, & Llorens-Cortes, 2002). Another study showed that duration of receptor internalization was correlated with both ligand concentration and isoform type, with longer isoforms resulting in longer durations of receptor internalization (Zhou et al., 2003). Recently, Chun et al. (2008) reported that APJ may heterodimerize with other GPCRs, such as angiotensin-II type 1 (AT_1) in atherosclerotic mouse models, to act constitutively – independent of apelin binding. Li et al. (2012) corroborated these findings, providing evidence of APJ heterodimerization with the kappa opioid receptor (KOR). In the presence of either apelin-13 *or* the endogenous KOR ligand, dynorphin A, in cells stably transfected with both receptors, greater extracellular signal-regulated kinase 1/2 (ERK 1/2) phosphorylation was observed compared with cells transfected with either receptor alone. The variety with which APJ is distributed in concert with its ever-expanding list of biological activity implies great tissue- and cell-type-specific functionality.

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APJ shares remarkable amino acid homology with AT_1 (31% total sequence

homology; 84% transmembrane residue homology), leading to its initially being termed

AT_1-like receptor; yet, angiotensin is not an endogenous ligand of APJ (Ishida et al.,

2004). GPCRs are abundantly expressed in the cardiovascular system, including

VSMCs, and APJ is no exception, indicating apelinergic (APJ-apelin) importance in

regulating blood pressure, angiogenesis and inotropy (Cox, D'Agostino, Miller, Heimark,

& Krieg, 2006; Kasai et al., 2004; Newson, Pope, Roberts, Lolait, & O'Carroll, 2012).

Studies of APJ knockout (KO) mice compared with wild-type controls have confirmed

APJ-apelin influence on cardiovascular homeostasis – both from its action at the

peripheral tissues and in the forebrain and brainstem – as well as helped define its role in

regulating hypothalamic-pituitary-adrenal (HPA) activity and the stress response and

fluid homeostasis. This perhaps explains the great homology of APJ with AT_1 and their

complementary functionality of fluid and blood pressure regulation (Chandrasekaran,

Dar, & McDonagh, 2008; Ishida et al., 2004).

APJ-Apelin on Glucose Homeostasis and Insulin Sensitivity

Recently APJ-apelin has been identified as a contributor of glucose homeostasis

and insulin sensitivity (Attané et al., 2011; O'Carroll et al., 2013; Schenk, Harber,

Shrivastava, Burant, & Horowitz, 2009; Yue et al., 2010). Numerous studies utilizing

animal models have reported apelin injection-induced glucose uptake (Attane et al., 2012;

Dray et al., 2008; Zhu et al., 2011), while similar findings have been confirmed in human

subjects *in vitro* (Attané et al., 2011; Dray et al., 2010). Indeed, it has been suggested

that apelin is a critical mediator of insulin sensitivity, as Yue et al. (2009) demonstrated

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by administering apelin to apelin knockout (APKO) mice. This study also examined indices of insulin sensitivity in APKO and wild-type mice and found that APKO mice had significantly impaired glucose regulation and insulin resistance. Another study (Zhu et al., 2011) observed apelin-stimulated glucose uptake following TNF-α-induced reductions in glucose uptake in 3T3-L1 mice adipocytes, further highlighting the significance of apelin's mitigation of insulin resistance.

Currently it is incompletely understood how apelin binding to APJ regulates blood glucose and purportedly enhances insulin sensitivity. Specifically, this problem relates to whether meaningful apelin action that may lead to enhanced glucose regulation occurs centrally or peripherally (i.e. APJ activity at the hypothalamus or via signal transduction alteration in insulin-sensitive tissues, such as adipose tissue or skeletal muscle), or in concert (Schwartz et al., 2013). It is well known that alterations of hypothalamic neuronal activity may result in altered peripheral glucose metabolism (Castan-Laurell et al., 2012). Nevertheless, the preponderance of research to this point has shown that the majority of glucose uptake is mediated by apelin action at skeletal muscle, utilizing *in vitro* animal models and isolated human adipocytes (Boucher et al., 2005; Castan-Laurell et al., 2012; Dray et al., 2008). Still, the determination as to whether APJ-apelin mediates glucose homeostasis through insulin-dependent mechanisms or independent of insulin (or some combination) has yet to be clarified. Purported mechanisms of action on glucose regulation and other known physiological effects of APJ-apelin are discussed in greater detail below.

Proposed Mechanism(s) of APJ-Apelin Signaling Action and Physiological Function

General Signaling and Function of APJ-Apelin

Apelin does not act independently but through a variety of G proteins coupled to APJ, which acutely alter cell signaling cascades and participate in cross-talk (Kleinz et al., 2005; Langelaan et al., 2013; Masri et al., 2002). Fundamentally, however, it seems that the bioactive isoforms of apelin are, to an extent, distinctly tied to tissue-specific responsiveness. For example, short apelin isoforms, such as apelin-12 and -13, are linked to a greater extent in regulating cardiovascular homeostasis and lowering blood pressure in rats than their longer peptide counterparts (Hosoya et al., 2000; Zhen et al., 2013); short forms of apelin, particularly Pyr[1]-apelin-13, also predominate in the rodent hypothalamus (O'Carroll et al., 2013; A Reaux et al., 2002). In essence, although short and long isoforms of apelin certainly cross-react to activate APJ and display similar functionality, they differ by way of tissue distribution, receptor affinity and potency (Kleinz et al., 2005). Research endeavors have adopted a systems approach to identifying both the tissue-specific function of APJ-apelin and its participation in downstream signaling pathways.

As aforementioned APJ-apelin acts via G protein coupling, the multifaceted effects of which are dependent on three factors: tissue-isoform specificity, intact tissue function and the type of G protein stimulated (Falcão-Pires, Ladeiras-Lopes, & Leite-Moreira, 2010; Zhen et al., 2013). APJ-apelin activation has also been shown to induce either immediate cellular effect or delayed transcription-level function (Castan-Laurell et al., 2012). To appreciate the salience of intact tissue function, it is helpful to consider

APJ-apelin at the endothelium and smooth muscle cells of the peripheral vasculature. Herein, apelin acts in an autocrine and/or paracrine fashion, as it is synthesized locally in the endothelium, among other tissues. In the functional endothelium, APJ-apelin acts first by stimulating nitric oxide (NO) production through a phosphoinositide 3-kinase (PI3K)/Akt mechanism. APJ-apelin works subsequently on adjacent VSMCs via APJ-G-inhibitory (G_i) protein coupling with simultaneous NO diffusion into VSMCs, inhibiting adenylate cyclase (AC) activity and cyclic adenosine monophosphate (cAMP) production and reducing intracellular Ca^{2+}. This cascade manifests in relaxed vasomotor tone and induction of vasodilation. When considering a non-functional endothelium, however, APJ binds apelin directly at the VSMCs, resulting in vasoconstriction from reduced NO synthesis coupled with increased Ca^{2+} production via a phospholipase C (PLC)/PIP_2/PIP_3 mechanism – ostensibly through another G protein (G_q) (Falcão-Pires et al., 2010).

APJ-apelin may work through G_i, G_q or G stimulatory (G_s) proteins, though it is widely accepted that APJ-apelin acts, with rare exception, predominantly via G_i protein coupling (Falcão-Pires et al., 2010; Masri et al., 2002; O'Carroll et al., 2013). It has been previously shown that APJ activation induces ERK1/2 phosphorylation via direct stimulation of Raf by a DAG/PKC mechanism, independent of Ras (Masri et al., 2002). The signaling mechanisms, downstream effector molecules and associated physiological effects are highlighted in a system-by-system fashion in the following paragraphs.

APJ-Apelin and Cardiovascular Physiology

APJ-apelin plays a critical role in cardiovascular physiology, in terms of development, improved cardiac performance and mitigation of dysfunction. Indeed,

apelin's relation with cardiovascular physiology is the most widely studied. In 2006, Cox et al. reported apelin-dependent vascular development in frog embryos, with apelin exhibiting considerable angiogenic activity and induction of endothelial cell proliferation; strikingly, this stimulation proceeded in a VEGF-independent manner. Apelin was found to be a potent chemotactic agent in inducing endothelial cell migration. These findings have been corroborated across species in multiple subsequent studies (Cox et al., 2006; Kälin et al., 2007; Karpinich & Caron, 2014). Kasai et al., in 2004, also presented evidence of apelin-induced capillary tubule formation *in vitro* in human RF/6A retinal endothelial cells, with neovascularization stimulated equally by Pyr[1]-apelin-13 and apelin-36 in a dose-dependent manner and independent of vascular trophic factors such as VEGF and HGF. In addition it has been established that APJ KO mice suffer lethal consequences *in utero* due to cardiovascular development defects, such as underdeveloped hearts and poorly established embryo vasculature.

Investigators have identified APJ-apelin downstream signaling involvement originating from $G\alpha_{13}$ protein activation of myocyte enhancer factor 2 (MEF2), a transcription factor requisite for embryonic development (Kang et al., 2013). From the perspective of apelin's importance in angiogenesis, it has also been reported that apelin-deficient mice exhibit decreased eNOS and lower NO production resulting from reductions in Krüppel-like factor 2 (KLF2), a protein of the zinc finger transcription factor family necessary for vascular and pulmonary endothelial development and upregulated by MEF2 and laminar shear stress (Kang et al., 2013; Takeda et al., 2012). The introduction of exogenous apelin has been shown to abrogate these deleterious

effects via targeting of AMPK, up-regulating both eNOS and KLF2 (Dray et al., 2008; Young et al., 2009). APJ-apelin initiates a twofold signaling cascade: 1) activation of shear stress-responsive channels, which activate MAPK/ERK and MEF2, and 2) activation of PI3K, increasing the stability of KLF2 mRNA and the proliferation of VSMCs (Young et al., 2009).

Apelin has also been identified as a potent cardiac inotrope, mediated through APJ-apelin activation of PLC, PKC and the Na^+/H^+ and Na /Ca^{2+} exchangers (NHE and NCX, respectively) (Földes et al., 2003; Szokodi et al., 2002). Ultimately, activity of these mediating proteins increases intracellular Ca^{2+} levels. It has been suggested that apelin may be an endogenous Ca^{2+} sensitizer (Chandrasekaran et al., 2008), though it is more likely that this positive inotropic function is related wholly to increased calcium levels (Falcão-Pires et al., 2010). Normal and pathological *in vivo* models have consistently shown that the introduction of apelin increases myocardial contractility and that APJ presence is obligatory. In two other studies, it was noted that exogenous delivery of apelin did not alter Ca^{2+} transients, but increased myocardial contractile elements' sensitivity to calcium, indicating potential apelin autocrine activity and upregulation during periods of heightened cardiac stress (Charo et al., 2009; Farkasfalvi et al., 2007). Hypoxic conditions initiate the pathways mentioned via hypoxic inducible factor alpha-1 (HIF-1α) (Glassford et al., 2007; Ronkainen et al., 2007).

Apelin's function as a regulator of vasomotor tone seems to be dependent on tissue function, APJ concentration and, as has been posited, the *in vivo* activity of AT_1 (Jia et al., 2007; Kasai et al., 2004; Liu et al., 2014). In rodent models, apelin induces

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acute hypotensive responses in normal and hypertensive animals, and opposes the

vasopressor activity of the receptor AT_1, seeming to suggest that APJ-apelin exerts a

counter regulatory action to angiotensin II. To a lesser extent, apelin has been shown to

be a vasoconstrictor in humans, as well, though often this is a result of pathological

conditions (Katugampola, Maguire, Matthewson, & Davenport, 2001). APJ-apelin

induction of vasodilation is NO-dependent and occurs in stepwise fashion via activation

of the L-arginine (L-arg)/NOS/NO pathway, potentiating eNOS activity and facilitating

L-arg transport. In rat aortic tissue, apelin incubation (10^{-7} mol/L) increased NO

production in a dose- and time-dependent manner (Jia et al., 2007). Despite these effects,

the literature is controversial with regards to blood pressure, as studies have cited a wide

range of responses from considerable decreases (Cheng, Cheng, & Pang, 2003; D K Lee

et al., 2000; Tatemoto et al., 2001) to slight increases in mean arterial blood pressure

(MABP) in response to apelin treatment in animal models (Katugampola et al., 2001). It

is highly likely that apelin-induced vasoconstriction occurs as a response to inactivity of

angiotensin II vasopressive action or via direct activation of APJ at VSMCs (O'Carroll et

al., 2013) and predominates in the presence of a functionally intact endothelium.

Apelin has been identified as a marker of cardioprotection and as a direct

contributor to lessening the severity of ischemic-reperfusion (IR) injury. Tao et al.

(2011) observed that IR injury cardioprotection occurs via a PI3K/p70S6K mechanism, a

finding confirmed in two other studies (Ronkainen et al., 2007; Zeng et al., 2009). Some

dissension has been created on the topic of the mechanism by which apelin induces IR

cardioprotection. Another group reported no diminution of Pyr[1]-apelin-13-induced

protection when PI3K and p70S6K inhibitors (wortmannin and rapamycin, respectively) were co-administered; no mechanism was posited and this remains a controversial point (Kleinz & Baxter, 2008). Apelin protection in this study occurred only when apelin perfusion was initiated just prior to reperfusion/hyperemic period, the period when apelin mRNA was drastically upregulated, suggesting that apelin works as an endogenous cardioprotective agent upregulated during periods of ischemia. Human and rodent models of heart failure have shown increases in cardiac performance parameters and hemodynamics (Ashley et al., 2005; A G Japp et al., 2010).

APJ-Apelin and Fluid Homeostasis

APJ-apelin is proven to play a critical role in fluid homeostasis (Kälin et al., 2007). APJ and apelin are expressed highly in the paraventricular and supraoptic nuclei within the hypothalamus, two of the major sites of hypothalamic regulation of fluid homeostasis. These two sites are major producers of antidiuretic hormone (ADH). Apelin acutely alters ADH production *in vivo* in rodent models; it was observed that intracerebroventricular (ICV) injection of apelin-13 and -17 resulted in decreases in plasma ADH by 47% and 42.6%, respectively, further suggesting a role for centrally-acting apelin in the regulation of fluid balance (Reaux et al., 2002; Reaux et al., 2001). Ostensibly, apelin exerts similar counter regulatory effects on ADH as it does on angiotensin II.

The effects of apelin injection on rodent water intake have been extensively studied, though literature regarding this topic has been equivocal. Several studies have noted greatly reduced or no significant change in water intake upon apelin injection, even

in those animals previously deprived of water (De Mota et al., 2004; Reaux et al., 2001; Taheri et al., 2002), while others have observed significant increases in water intake (Lee et al., 2000). Both outcomes suggest a critical role for apelin in fluid homeostasis. However, it is worth noting that those noting decreases in water intake in rodents administered apelin centrally, while the latter chose a peripheral injection route. The dichotomy suggests great importance of APJ-apelin site of activity. Further study in this area is needed to clarify apelin's role in the regulation of fluid homeostasis.

Other Physiological Roles

APJ-apelin is complicit in a variety of other physiological processes, including inflammation and immune function (Falcão-Pires et al., 2010; Hosoya et al., 2000; O'Carroll et al., 2013), while also exhibiting renal and gastrointestinal function (i.e. opposing angiotensin-II response and gastric cell proliferation, respectively) (Hus-Citharel et al., 2008; Kleinz & Davenport, 2005). Apelin was also reported to be a potent inhibitor of HIV viral entry into CD4^{+} cells co-expressing APJ (Zhou et al., 2003). APJ-apelin undoubtedly plays an important role as a neuroendocrine, as evidenced by its central role in fluid homeostasis and plentiful mRNA and protein expression in higher centers. Newson et al. (2013) reported APJ-apelin importance as it relates to HPA-mediated stress responses, as assessed by CORT (corticosterone) and ACTH change; in their study, they noted that apelin modulated the stress response and CORT/ACTH release from two imposed stressors: infusion of lipopolysaccharide (LPS) and insulin-induced hypoglycemia, with only ACTH altered by hypoglycemic condition. The authors herein note that an intact APJ is necessary to induce appropriate ACTH response

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to these stressors. This last point, specifically as it relates to APJ-apelin's compulsory

role in modulating ACTH, nicely highlights the movement of the literature devoted to

apelin and its inextricable effects on energy balance. Recent trends have focused on APJ-

apelin significance to glucose homeostasis and enhancement of insulin sensitivity.

APJ-Apelin on Energy Metabolism and Glucoregulation

Apelin is most well known as an adipocyte-derived peptide cytokine (adipokine)

and has become a recent target of study for its implications in energy metabolism. Apelin

is reported to inhibit lipolysis and reduce FFA release through a theorized G_q and G_i

activation of AMPK (Attané et al., 2011; Yue et al., 2011), and was shown to stimulate

mitochondrial biogenesis in rodent skeletal muscle through a PGC-1α-directed manner,

as noted by apelin-induced increases in NRF1 and TFAM (Scarpulla, 2008; Wu et al.,

1999). These findings have been confirmed in a variety of studies utilizing both animal

and human models, assessing a variety of mitochondrial markers (Attane et al., 2012;

Frier, Williams, & Wright, 2009; Yamamoto et al., 2011). Attane et al. (2012) observed

apelin-induced increases in fatty acid oxidation, while other groups have noted no

changes in lipolysis, FFA release or oxidation. Disparity in the literature may be a

function of methodology, with some studies utilizing *in vivo* rodent models and others

using 3T3-L1 cells to monitor changes *in vitro*.

The unclear effect of apelin on lipolysis and lipid oxidation notwithstanding, there

is clear evidence of apelin's efficacy as a glucoregulatory factor (Dray et al., 2008).

Apelin has been demonstrated to induce glucose uptake in humans and animal models *in

vivo* and *in vitro* (Attané et al., 2011; Friedrichsen, Mortensen, Pehmøller, Birk, &

Wojtaszewski, 2013; Yue et al., 2010) and this has been proposed to occur through an AMPK-dependent mechanism, as AMPK is a well-conserved acute sensor of energy regulation and metabolism (O'Neill, 2013); additionally, AMPK is highly activated during exercise (Friedrichsen et al., 2013). It has also been confirmed that apelin-induced glucose uptake is NO-dependent *in vivo*, suggesting an autocrine/paracrine mechanism and, perhaps, an irrevocable role for apelin in this mechanism (Duparc et al., 2011). Interestingly, apelin also significantly improved glucose uptake and restored insulin sensitivity in TNF-α-induced insulin resistant 3T3-L1 cells via a PI3K/Akt, insulin-mimicking path (Zhu et al., 2011). In essence, it seems that apelin exerts its glucoregulatory effects in an insulin-independent manner, via AMPK, and participates in cross-talk in such a way as to induce translocation of glucose transporters, GLUT4 and GLUT1, although this has not been confirmed (Higaki, Hirshman, Fujii, & Goodyear, 2001). Apelin may function to directly or indirectly alter the insulin signaling pathway and further work on this topic is necessary.

This improved glucose uptake in adipose tissue and skeletal muscle by apelin does not reach a magnitude of that induced by insulin (Attané et al., 2011), but may be a necessary adjunct to insulin, as it is posited that apelin is necessary to maintain insulin sensitivity and beneficial for the reversal of insulin resistance in pathological states. Yue et al. (2010) demonstrated this in APKO mice, which displayed markedly reduced adiponectin, significantly diminished insulin sensitivity and hyperinsulinemia; apelin injection abrogated these effects, returned insulin sensitivity and induced glucose uptake. It is well documented that both apelin and insulin regulate each other, with insulin

upregulating apelin and apelin inhibiting insulin secretion at the islet level (Sörhede Winzell et al., 2005). There exists a complex relationship between these key regulators of glucose balance and apelin acts as an obligatory metabolic contributor, upregulated in periods of energy imbalance and during pathological states, such as T2D and obesity, perhaps in a compensatory manner. A following section, 'Factors Affecting Blood Glucose and Exercise Considerations', will discuss factors that independently alter blood glucose and their relevance to apelin.

Factors Affecting Apelin Synthesis and Release

This section will consider those factors that affect apelin synthesis and release *with regards to adipose tissue*. A multitude of factors affect apelin's synthesis and circulating levels, the most important regulator of which is blood insulin. Table 1 lists the previously observed resting, post-absorptive apelin concentrations in healthy control subjects. In both *in vivo* and *in vitro* studies there is a high correlation between apelin and insulin (Boucher et al., 2005). Hyperinsulinemic mouse models exhibit significantly greater apelinemia. In turn, it has been demonstrated that apelin inhibits insulin secretion (Ringström et al., 2010). Apelin levels are increased in T2Ds and obese individuals, with apelin being correlated positively with adiposity, as well. Having said this, the literature is controversial regarding apelin's relationship with obesity, with many studies showing decreased plasma apelin concentration in obese individuals without T2D or with impaired glucose tolerance (IGT) (Reinehr, Woelfle, & Roth, 2011). In general, obesity should not be considered the main determinant of apelin increases, but merely contributory (Soriguer et al., 2009). Regional fat distribution and its relation to apelin concentration has not

been assessed, nor has visceral or subcutaneous adipose tissues' relative contribution (Blüher, 2014; Habchi et al., 2014), save for one study, which found that viscerally located adipose tissue resulted in significantly greater apelin concentration than subcutaneous (Krist et al., 2013). Further exploration into this topic is warranted.

Table 1. Summary of Apelin Concentration in Healthy Controls. Only three studies of those known to the authors to include healthy controls were omitted from this list, as they either: 1) utilized serum samples or 2) did not appropriately control for protease activity and drug action in methodology.

Study	Subjects	Apelin Concentration (ng/ml) ± SD	Procedure Utilized	Tissue
Zhen et al. (2013)	Healthy Controls	0.1292 ± 0.02	Phoenix Pharmaceuticals: Apelin-12 HRMB	Plasma
Mesmin et al. (2010)	Healthy Controls	0.369 ± 0.08	Phoenix Pharmaceuticals: Apelin-12 HRMB	Plasma
Castan-Laurell et al. (2008)	Healthy Female Controls	0.272 ± 0.069	Phoenix Pharmaceuticals: Apelin-12 HRMB	Plasma
Foldes et al. (2003)	Healthy Controls	0.0898 ± 0.013	Phoenix Pharmaceuticals: Apelin-36 Human	Plasma
Alexiadou et al. (2012)	Healthy Controls	1.25 ± 0.65	Phoenix Pharmaceuticals: Apelin-12 HRMB	Plasma
Papadopoulos et al. (2013)	Healthy Controls	0.315 ± 0.147	Phoenix Pharmaceuticals: Apelin-12 HRMB	Plasma

As expected, blockade of the renin-angiotensin-aldosterone system (RAAS) upregulated apelin in adipocytes, along with hypoxic conditions and inflammatory mediators, namely TNF-α and LPS (Boucher et al., 2005; Dray et al., 2010). Hypoxia has been shown to increase apelin expression in both human and rodent adipocytes *in vitro* (Glassford et al., 2007). Interestingly, PGC-1α is a potent upregulator of apelin in adipose tissue in both human and mouse adipocytes (Mazzucotelli et al., 2008).

Obviously a plurality of factors induces overexpression of apelin. Apelin expression may be downregulated by several factors, as well, including vitamin C, weight

loss and/or hypocaloric diets and a fasted state (García-Díaz, Campión, Milagro, & Martínez, 2007). An interesting factor that acutely downregulates apelin expression is an increased concentration of circulating glucocorticoids (i.e. cortisol), though it should be noted in the literature that it has been shown only to decrease apelin mRNA (Wei, Hou, & Tatemoto, 2005); however, in so doing, it was reported that glucocorticoid-stimulated downregulation of apelin gene expression was accompanied by a rise in angiotensin-II. This finding has implications in glucose metabolism and obesity-related hypertension (among other issues) and, thus, indirect activity on apelin expression (Castan-Laurell et al., 2012). Considering all of this information, the most appropriate assessment of apelin regulation would include discussion of insulin, glucose, TNF-α, plasma triglycerides, the Homeostasis Assessment Model of Insulin Resistance (HOMA-IR) and HbA$_{1c}$ (Castan-Laurell et al., 2008; Dray et al., 2010; Heinonen et al., 2009; Soriguer et al., 2009), as it has been found that apelin correlates significantly with triglycerides and HbA$_{1c}$.

Factors Influencing Blood Glucose and Insulin and Exercise Considerations

Factors Affecting Blood Glucose Concentration

Blood glucose is regulated in a complex manner and may be altered by a variety of inputs, both humoral and neural (Kumar, 1999). Maintenance of blood glucose within a tight physiological range (~80-100 mg/dl fasting; ~80-120 mg/dl at random) is essential for whole body glucose homeostasis and is of paramount importance during exercise, particularly in diabetics (Vranic, Kawamori, Pek, Kovacevic, & Wrenshall, 1976). Traditionally, glucose control has been ascribed to glandular hormone signal integration, yet it is evident that the hypothalamus plays a central role and that neural efferents to

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endocrine regulators of blood glucose, namely the pancreas, adrenal cortex and medulla and liver, are obligatory (Cherrington, 1999). APJ and apelin are found in abundance in the hypothalamic centers implicated in glucose homeostasis, particularly the paraventricular and arcuate nuclei (Knauf, Drougard, Fournel, Duparc, & Valet, 2013; McEwen & Reagan, 2004). As such, it may be that apelin partially mediates central glucose homeostasis, as evidenced by centrally-administered apelin-induced glucose uptake (Duparc et al., 2011; Knauf et al., 2013). This section, however, will not expound on the purported central role of apelin and will discuss only the hormonal inputs regulating glucose homeostasis. In addition, the key components regulating blood glucose during and after acute exercise will be considered.

The pancreas and liver play the most critical peripheral roles governing blood glucose concentration. Indeed, the liver exerts tight physiological control on glucose production *in vivo*, responding to efferent signals to stimulate hepatic glucose output during periods of low blood glucose (Cherrington, 1999) and during exercise, particularly high intensity exercise (Douglas & Rubin, 1963; Marliss & Vranic, 2002). The pancreas, as well responding to neural and humoral efferents, may stimulate either peripheral glucose uptake, via insulin, or hepatic glucose production (through glycogenolysis or gluconeogenesis), via glucagon; pancreatic α- and β-cells respond quite sensitively to blood glucose levels by modifying their secretion of insulin and glucagon with remarkable accord. So sensitive are the α- and β-cells in this process that it has been shown in canine models that a decrease in plasma glucose of <0.6 mmol/l results in a decrease of insulin output from 78 to 24 pmol/l (Cherrington, 1999). Somatostatin, also

produced by the pancreas (δ cells), suppresses insulin and glucagon secretion, an effect predicated on local secretion (i.e. paracrine activity) of somatostatin from the δ cells and evidenced via utilization of somatostatin infusion-induced endogenous α- and β-cell suppression (Galassetti, Coker, Lacy, Cherrington, & Wasserman, 1999).

An array of other hormonal inputs influences the blood glucose response and, thus, has profound implications on whole body glucose homeostasis. Thyroxine (Ellenberger et al., 1989; Harrison et al., 1988), β-endorphin (Paolisso et al., 1987; Radosevich, Lacy, Brown, Williams, & Abumrad, 1989; Schleicher, Chawla, Coan, Martino-Saltzman, & Collins, 1987), growth hormone (GH) (Pritzlaff et al., 2000; Rizza, Mandarino, & Gerich, 1982), ACTH and cortisol (Tabata, Ogita, Miyachi, & Shibayama, 1991; Vila et al., 2010) and the catecholamines, norepinephrine (NE) and epinephrine (Epi) (Marliss & Vranic, 2002; Yale, Leiter, & Marliss, 1989), have all been shown to influence blood glucose concentration. Each of these has considerable importance as it relates to their secretion during acute exercise bouts, at both moderate and high intensities, though the focus of glucoregulation during acute exercise rests decidedly on the side of catecholamine-, insulin- and glucagon-driven glucose alterations.

Marliss and Vranic (2002) eloquently depicted the various responses of glucose, insulin and NE and Epi to two acute bouts of moderate and high intensity aerobic exercise, performed, respectively, at 50% and 88% VO$_2$max; these responses are readily reproducible at intensities up to ~75% VO$_2$max (moderate intensity) and during VO$_2$max testing (high intensity) and have been corroborated by numerous studies (Marliss et al.,

1992; Marliss et al., 1991; Sigal, Fisher, Halter, Vranic, & Marliss, 1996; Yale et al., 1989). Three components of an acute exercise bout – assuming exercise is performed in a fasted state – must be observed and comprehended in order to appreciate their discrete impacts on blood glucose: 1) the exercise period; 2) immediately acute, post-exercise period and; 3) the recovery period. The responses of the catecholamines, insulin and glucagon and their effects on blood glucose at moderate and high intensities of aerobic exercise, are discussed accordingly.

During acute aerobic exercise of moderate intensity, it has been observed that plasma concentrations of NE and Epi do not significantly increase from baseline and that, in concert with a slightly increased glucagon-to-insulin ratio (G:I), plasma glucose levels are maintained, or perhaps slightly elevated. Plasma insulin levels are significantly depressed during moderate intensity exercise, being inhibited by circulating catecholamines and sympathetic (SNS) innervation of pancreatic islet cells (Marliss & Vranic, 2002; Suh, Paik, & Jacobs, 2007). At this intensity of exercise, the insulin response is vital, as it sensitizes the liver to glucagon and increases hepatic glucose output, which, since it is observed that plasma glucose levels remain relatively constant, is tightly matched to glucose uptake. Thus, at moderate intensity the G:I drives the plasma glucose response, as skeletal muscle demand for glucose corresponds nicely with hepatic production and output. Though carbohydrate utilization fuels muscle demand at the beginning of moderate intensity exercise, fat oxidation dominates as exercise continues, resulting in a decrease in observed RER with less reliance on carbohydrate for energy. Catecholamines play a minor role here and do not increase with any appreciable

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effect until exercise persists for approximately 1.5 hours or more (Chenevière, Borrani, Ebenegger, Gojanovic, & Malatesta, 2009). Immediately following moderate intensity aerobic exercise insulin increases to resting levels or slightly above, in accordance with the euglycemic response to moderate intensity, after which time it normalizes to resting levels.

Intense aerobic exercise (>80% VO_2max) provides a stark contrast of substrate utilization and glucose response and is driven almost exclusively during exercise by catecholamine effects, though it has been shown that GH contributes significantly to the glucose response herein (Pritzlaff et al., 2000). Glucagon may also stimulate hepatic glycogenolysis at high intensities, as well (Yale et al., 1989). During intense aerobic exercise there is a demonstrable inequality in glucose output and glucose uptake, such that output supersedes uptake and plasma concentration of glucose is elevated – a disparity driven by catecholamine-induced muscle glycogenolysis. Additionally, despite elevated glucose concentration, plasma insulin remains depressed below resting levels, owing to the glucose-stimulated insulin secretion inhibitory activity (Chiasson, Shikama, & Chu, 1981) of the catecholamines, the plasma concentrations of which rise 14- to 18-fold, or higher (Marliss & Vranic, 2002; Sigal et al., 1996). Upon cessation of exercise, catecholamine concentration drops precipitously, replaced by significant increases in plasma insulin that may last for up to 60 minutes post-exercise in humans (Marliss et al., 1992; Marliss et al., 1991; Sigal et al., 1996).

Acute Exercise and Insulin Sensitivity

At either intensity of aerobic exercise, acute bouts are known to enhance peripheral insulin sensitivity, specifically being relegated to the skeletal muscles exercised, but with overall improved whole body insulin sensitivity (Bordenave et al., 2008; Fontana et al., 2010; Heath et al., 1983; Hecksteden, Grütters, & Meyer, 2013; Holloszy, 2005). This is unsurprising given that skeletal muscle, comprising ~45% of total human body mass, assumes the vast majority of glucose disposal in response to glucose and insulin challenges and following exercise (Henriksen, 2002). Simultaneously, through insulin-independent mechanisms, GLUT4 glucose transporters are upregulated and translocate to the cell surface during and following muscle contractile activity to increase glucose uptake. This represents an important glucoregulatory mechanism in and of itself and is an important adjunct therapy for (and efficacious prevention of) T2Ds (Goodyear & Kahn, 1998), though this effect is short-lived, lasting for ≤2 hours (Borghouts & Keizer, 2000). In sum, acute bouts of exercise improve whole body glucose homeostasis and increase the sensitivity of the periphery, namely the liver and skeletal muscle, to insulin for *at least* 12-16 hours (Borghouts & Keizer, 2000; James, Burleigh, Kraegen, & Chisholm, 1983).

Aerobic exercise training was found to confer marked enhancement in insulin-induced glucose uptake in endurance trained individuals compared to controls (Horton, 1986) and accompanied by lower basal insulin and suppressed insulin response to a glucose challenge (GC), despite greater glucose uptake (Galassetti et al., 1999). Insulin sensitivity improvement with exercise training is controversial, however (Hecksteden et

al., 2013; James et al., 1983; Segal et al., 1991). In accord with this finding, it has been determined that insulin sensitivity, even in trained individuals, is rapidly reversible (Henriksson, 1995) and that acute exercise exhibits primacy with regards to improvements in insulin sensitivity (Borghouts & Keizer, 2000; Fontana et al., 2010; Heath et al., 1983; Kirwan et al., 1991; Magkos et al., 2010, 2008). Various suggestions have been proposed, yet the precise mechanism by which exercise improves insulin sensitivity is not fully understood, and undoubtedly is a multifaceted and multileveled lesson in cellular crosstalk.

Recently, the focus of research regarding insulin sensitivity improvement and exercise-induced enhancement of insulin action has focused on AMPK activity, a promising prospect regarding insulin sensitivity improvement, as AMPK is a central cellular energy homeostasis regulator (Musi & Goodyear, 2006; O'Neill et al., 2013; Richter & Hargreaves, 2013). The consideration of AMPK as a potential player in insulin sensitization is warranted, as it is activated by its upstream AMPKK during exercise, when myocytes achieve high rates of energy turnover (Friedrichsen et al., 2013). Apelin exhibits clear glucose regulation, increasing glucose uptake (Attane et al., 2012; Attané et al., 2011; Dray et al., 2008; Yue et al., 2010; Zhu et al., 2011) and purportedly enhancing insulin sensitivity, ostensibly through an AMPK-dependent mechanism (Almabrouk, Ewart, Salt, & Kennedy, 2014; Xu et al., 2012; Yue et al., 2011). APJ-apelin's effects on insulin sensitivity may be mediated via direct improvement of glucose uptake and by participation in intracellular crosstalk within the insulin signaling cascade or indirect through improvements in energy metabolism,

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including increased mitochondrial biogenesis (Attane et al., 2012; Frier et al., 2009; Röckl, Witczak, & Goodyear, 2008), increased fatty acid oxidation (Attane et al., 2012) or participation at the AMPK level (Attané et al., 2011; Yue et al., 2010, 2011).

At this time, regardless of the mechanism(s) by which apelin exerts its effects, it is necessary to more clearly define APJ-apelin's role on glucose homeostasis and maintenance of insulin sensitivity. Acute aerobic exercise affords an excellent *in vivo* model in which to explore apelin's relationship with each, as these treatments may support or refute the notion that apelin and APJ are necessary for enhancing insulin sensitivity.

Apelin and Chronic Exercise Training

The literature regarding apelin's response to aerobic exercise training in humans is contentious and limited. Table 2 lists these studies' details and outcomes. Not only are these studies few in number, each exhibits multiple limitations that bring into question the veracity of their findings with regards to apelin and its role as a glucoregulatory factor. These studies exhibit the following three major limitations: 1) baseline blood apelin data is not currently well defined, in either healthy or non-healthy populations; 2) these studies report inconsistent mean resting and post-aerobic training values in non-healthy populations (i.e. obese, T2Ds); and 3) no aerobic training study states precisely at what point following training the final blood draw was taken, thus neglecting a critical component to any study assessing circulating factors, particularly those theorized to contribute to glucose homeostasis and alter insulin sensitivity. These blood draws were

stated to have been taken during a resting, fasted state only (Besse-Patin et al., 2013; Kadoglou et al., 2013; Kadoglou et al., 2012; Krist et al., 2013).

Aerobic exercise training in T2D resulted in significantly elevated serum apelin in concert with a decline in blood glucose and insulin, indicating increased insulin sensitivity and a potential role for apelin in this phenomenon (Kadoglou et al., 2012; Kadoglou et al., 2013). Krist et al. (2013) observed, however, that obese subjects with either T2D or impaired glucose tolerance (IGT), following 12 weeks of aerobic exercise (60 minutes/session, 3 sessions/week), had significantly decreased apelin values; yet these post-training values were significantly higher than both pre- and post-training values in obese with normal glucose tolerance (NGT), likely due to the markedly elevated pre- and post-training fasting plasma insulin levels in IGT and T2D subjects, suggesting a strong insulin effect on apelin and APJ mRNA.

Table 2. **Summary of Exercise Training Effects on Apelin Concentration**. - Denotes no specific pre-treatment apelin concentration provided (i.e. only given in illustration). *Significantly different from pre-treatment ($p<0.05$). [a]Study randomized groups into aerobic, resistance, aerobic plus resistance, and control groups.

Study	Subjects	Exercise Training	Duration	Pre-Treatment Apelin (ng/ml)	Post-Treatment Apelin (ng/ml)
Fujie et al. (2014)	Older M/F	Aerobic	8 weeks	2.31 ± 0.20	5.00 ± 0.39*
Besse-Patin et al. (2013)	Obese Males	Aerobic	8 weeks	0.514 ± 0.076	0.490 ± 0.089
Krist et al. (2013)	M/F T2D	Aerobic	12 weeks	-	10-20% reduction in all groups*
Kadoglou et al. (2013)	M/F T2D	Varied[a]	6 months	-	Increased in aerobic and combined groups*
Kadoglou et al. (2012)	M/F T2D	Aerobic	12 weeks	0.209 ± 0.102	0.291± 0.135*

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Both apelin and APJ mRNA were previously shown to be upregulated in adipose

tissue and skeletal muscle in NGT, but only in skeletal muscle in IGT subjects (Dray et

al., 2010). Besse-Patin et al. (2013), however, observed no change in apelin despite

enhanced insulin sensitivity after eight weeks of aerobic training; these authors confirmed

Dray et al.'s (2010) previous findings, observing that apelin and APJ mRNA was

upregulated in skeletal muscle, but not adipose tissue, and that apelin expression and

secretion was significantly increased in human primary myotubes. Increased expression

resulted from cAMP activity and increased Ca^{2+}, corroborating previous studies (Besse-

Patin et al., 2013; Mazzucotelli et al., 2008; Sörhede Winzell et al., 2005). It bears

repeating that these studies suffer from the aforementioned limitations, as well as having

to contend with and make sense of the milieu of inputs regulating the expression of apelin

and APJ, namely via insulin concentration flux and adiposity (Dray et al., 2010;

Heinonen et al., 2009; Sörhede Winzell et al., 2005). Future training studies on this topic

are essential, yet acute exercise studies in healthy populations are a prerequisite.

Apelin and Acute Exercise: A Summary

To our knowledge this was the first study to examine the effects of acute exercise

on plasma apelin, specifically the effects of two bouts of aerobic exercise of varied

intensity on plasma apelin. More importantly, this study contributes to the body of

literature by further defining resting, post-absorptive plasma apelin concentration in

young (18-35 years), apparently healthy individuals, while describing apelin's

contribution to glucose homeostasis and insulin sensitivity alteration following acute

aerobic exercise. Though observed to induce glucose uptake and to improve insulin

sensitivity *in vitro* as an exogenously delivered peptide, apelin's *in vivo*, endogenous contribution has been less well reviewed and is quite contentious, a function of this topic's relative nascence (Tatemoto et al., 1998). In employing exercise protocols known to influence blood glucose and insulin in specific manners, we now better understand and can ascribe the relative contribution of circulating apelin's endocrine effects on insulin sensitive tissues, specifically skeletal muscle.

Apelin is known to be secreted from a variety of tissues, notably adipose tissue and, more recently, as a skeletal muscle-derived myokine (Besse-Patin et al., 2013). Thus, its endocrine effects are likely accompanied by autocrine, paracrine and juxtacrine activity (Charo et al., 2009; Kälin et al., 2007; Ringström et al., 2010). As a result, circulating levels of apelin may not necessarily reflect its *in vivo* activity. The well-documented regulation of apelin and insulin, and their known respective feedback and feedforward interplay, also suggests obligatory circulating endocrine activity. The mechanism(s) by which apelin induces glucose uptake, contributes to metabolic homeostasis and enhances insulin sensitivity have, to this point, been somewhat speculative. Direct or indirect action on the insulin signaling pathway or via AMPK are commonly proposed, with the latter, a powerful energy and metabolic sensor, being increasingly studied given its activation in skeletal muscle. Observing the plasma apelin response to well-typified acute aerobic exercise protocols in healthy adults limits hormonal and pathological signaling milieu present in obese and T2D subjects, thus affording the opportunity to better establish apelin's relevance as an endogenous insulin sensitizer and major glucose homeostatic regulator.

CHAPTER III

METHODS

Subjects

Subjects were consented according to UNCG IRB standards, after which they were pre-screened for participation in the study (See 'Screening Procedures: Inclusion/Exclusion Criteria'; Appendices A and B). Twelve (n=7 male; n=5 female) apparently healthy subjects (22.8 ± 2.9 years), not using any tobacco products or taking medications/supplements that may alter metabolism, oxidation status, blood glucose and/or insulin, were recruited and utilized for this study. Three subjects from the original cohort were excluded from study due to considerable observed resting hypoglycemia, along with severe hypoglycemic responses to GC. A group of twelve subjects was determined to be appropriate to yield a power of 0.80, with an alpha level of 0.05 set. Subjects utilized in this study had their fitness and exercise habits thoroughly reviewed, which were determined to range from moderate to high level of physical activity. Young, apparently healthy individuals were chosen for two reasons: 1) baseline apelin data, at rest and following exercise, has not been firmly established in this cohort, prompting the need to recruit healthy subjects in order to best define baseline apelin concentration and 2) establishing this baseline data and response to exercise may be of great future use in assessing the role of apelin in non-healthy subjects who exercise. No gender differences

have been reported regarding apelin, so the inclusion of both male and female subjects was well justified (Krist et al., 2013).

Study Design

A repeated measures, 3 (two exercise conditions [VO_2max and 70-75% submaximal tests] and one 54g GC) x 4 (4 time points [pre-, immediately post-, 1 hour post- and 24 hours post-condition]) design was employed for this study. After a screening session, at which participation in the first condition (either VO_2max testing or GC) was completed, subjects returned to the lab on five other occasions to attain a 24 hour post-condition blood draw, as well as to complete the final two conditions and accompanying 24 hour post-condition blood draws (See Figure 1 for full detail of study design protocols).

Screening Procedures

<u>*Exclusion Criteria*</u>

Prior to accepting volunteers as official subjects, a screening of potential medical, metabolic, cardiovascular, activity and other criteria was completed per approved guidelines (Appendices A and B). Tobacco users and women who were pregnant were excluded. In addition, those volunteers considered obese (per skin folds, who are >2 standard deviations above the norm for their individual age category) or who are not otherwise apparently healthy were excluded from consideration. Volunteer blood pressure and body fat percentage was obtained for inclusion purposes. Volunteers meeting criteria for ≥2 cardiovascular risks per ACSM guidelines (Appendix A) were excluded from consideration. Volunteers on any medicines/supplements known to
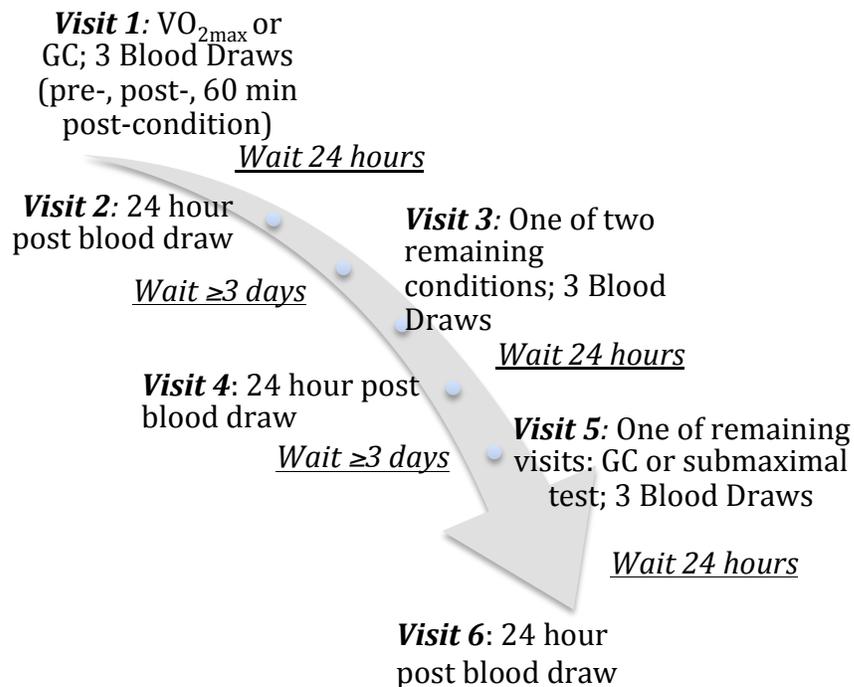
influence metabolism, oxidative stress, and/or inflammation were also excluded, as these factors may have confounded outcome measures (Appendix B). Only those volunteers within the age range who meet all of the inclusion criteria were asked to be included as official subjects.

Screening Session

Consent and screening occurred during the potential subject's first visit to the UNCG Exercise Physiology Laboratory. Upon arrival volunteers were provided all study information and materials, including all assumed subject responsibilities and risks, and allowed to ask questions concerning their involvement in the study. Volunteer informed consent was obtained upon agreeing to participate as subjects in the study. The screening session was performed *after* accepting volunteers into the study as subjects. A Pre-Participation Health/Fitness Facility Screening Questionnaire (Appendix A), as well as a fitness and activity level assessment along with the Adapted Health History Questionnaire (Appendix B), were completed at this time; subjects meeting exclusion criteria per these screening tools were excluded from participation as subjects in the study. Only upon consenting to participate in the study *and* meeting the aforementioned inclusion criteria did volunteers become active subjects. Resting heart rate, blood pressure, weight and height, along with BMI, body fat percentage, WHR and sagittal diameter, were then obtained. Subjects were provided food logs to record all intake three days prior to each visit, as well as during the 24-hour period prior to the fourth blood draw in each condition. A study timeline detailing subject involvement is included below

(Figure 1), which outlines the procedures upon accepting volunteers as active subjects to
be included in the study.

Figure 1. Study Timeline. Study design and subject involvement through six visits to the exercise
physiology laboratory, including consent and pre-screening (visit one) to study completion (visit six).

Visit 1: VO_{2max} or
GC; 3 Blood Draws
(pre-, post-, 60 min
post-condition)
Wait 24 hours

Visit 2: 24 hour
post blood draw
Wait ≥3 days

Visit 3: One of two
remaining
conditions; 3 Blood
Draws
Wait 24 hours

Visit 4: 24 hour post
blood draw
Wait ≥3 days

Visit 5: One of remaining
visits: GC or submaximal
test; 3 Blood Draws
Wait 24 hours

Visit 6: 24 hour
post blood draw

Exercise and Positive Control Procedures

Subjects completed two exercise conditions (treadmill running – graded [VO_2max] or
steady state [70-75% VO_2max]) and one positive control 54g GC condition between 6-9
a.m. in a post-absorptive (10-12 hours) state, without prior caffeine or alcohol
consumption (or otherwise contraindicated substance). Each of these main condition
visits was separated by at least three days, but not more than 14 days, so as to avoid prior
condition effects and to ensure no loss of time-dependent insulin sensitivity, respectively.
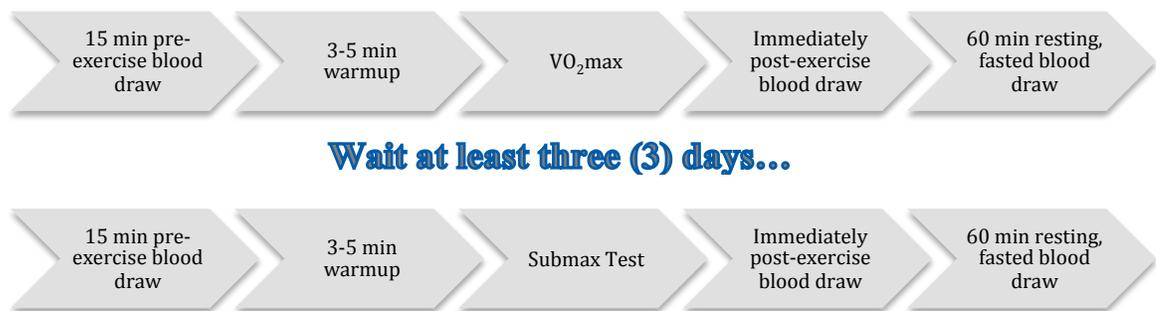
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Each subject also visited the exercise physiology laboratory for a 24-hour post-condition blood draw constituting time point four. Subjects reported to the exercise physiology laboratory having not exercised ≥24 hours prior to testing. The first exercise session consisted of a maximal oxygen consumption graded exercise test (VO_2max) on a treadmill, while the second was a submaximal 30-minute run (at ~70-75% VO_2max), also performed on a treadmill. A 54g GC as a positive control was also performed by each subject. These three conditions were randomized, with the only stipulation being that the VO_2max test must have been performed (for appropriate designation of 70-75% VO_2max submaximal protocol measures) prior to the submaximal 30-minute run. Blood was obtained from an antecubital arm vein as follows:

1. Prior to exercise, after ≥15 minutes of rest in laboratory (post-absorptive).

2. Immediately post-exercise, within one minute of exercise cessation.

3. 60 minutes post-exercise (resting).

4. 24 hours post-exercise, at the same time the following morning (resting, post-absorptive).

Each exercise session began with a short 3-5 minute gradual warm-up on the treadmill and stretching befitting the comfort of each subject. Proper hydration was ensured to maintain appropriate blood volume (BV), with subjects encouraged to intake water ad libitum prior to exercise and up to 1000ml intake during the 60-minute interim period of rest until the third blood draw. Water was not provided *during* the VO_2max test, owing to the placement of headgear and mouthpiece for the duration of exercise to capture real-time gas exchange and respiration for calculation of output variables. Water was

provided *at least* every two minutes during the 70-75% VO$_2$max submaximal test, or

when the subject requested water, as respiration and gas exchange was assessed via

mouthpiece every five minutes for ~30s. Water was provided ad libitum up to 1000ml

during GC positive control to maintain BV. Volume of water intake was closely

monitored and hematocrit measured following each blood draw. Heart rate was

continually monitored throughout exercise and recorded every minute during the

VO$_2$max test and every 5 minutes during the 70-75% VO$_2$max submaximal test.   Subject

rating of perceived exertion (RPE) was assessed in a similar time frame for each exercise

session. Figure 2 outlines the general procedures around each exercise session.

Figure 2. Exercise Protocol Outline. General procedures during exercise protocols to be followed for
each of the two exercise sessions, VO$_2$max and 70-75% VO$_2$max submaximal tests. Both conditions were
followed by a 24-hour post-exercise resting and post-absorptive blood draw.

| 15 min pre-exercise blood draw | 3-5 min warmup | VO$_2$max | Immediately post-exercise blood draw | 60 min resting, fasted blood draw |

Wait at least three (3) days...

| 15 min pre-exercise blood draw | 3-5 min warmup | Submax Test | Immediately post-exercise blood draw | 60 min resting, fasted blood draw |

Blood Handling

Blood was collected in 7 ml K2/K3 EDTA tubes (lavender topped) (2 per time

point = 14 ml whole blood per time point) from an antecubital arm vein and immediately

processed for blood measures. Blood was placed on ice and immediately transported and

centrifuged in a Beckman Allegra swinging bucket centrifuge for 10 minutes at 3000 rpm

(4°C). A small sample of whole blood (<1μl) was retained to assess blood glucose

immediately following each blood draw, in duplicate. Plasma samples were subsequently

aliquoted into microtubes and placed in an -80°C freezer. Assays for plasma apelin and

plasma insulin were completed with batched samples. To assess hematocrit, a small

volume of whole blood (<25μl) was placed into micro-hematocrit capillary tubes, sealed

and centrifuged (American Scientific micro centrifuge) for 5 minutes at 3000 rpm and the

volume ratio determined, measured as red blood cells to total blood volume, in duplicate.

Blood Sampling, Assays and Calculations

1. Blood Glucose – Determined immediately following blood draw utilizing a
   glucose meter (Bayer Contour). The meter was calibrated for use in duplicate
   each morning prior to blood sampling and between subjects.

2. Plasma Insulin: Determined following the ELISA immunoassay (10-1113-01)
   procedures described (Mercodia), absorption read at 450 nm via KC Junior
   microplate reader. Intra-assay variance was <10%. Inter-assay variance was
   eliminated in batching samples from the same subject on common plates.

3. Plasma Apelin: Determined following the ELISA immunoassay (EK-057-15)
   procedures described (Phoenix Pharmaceuticals), measured at 450 nm via KC
   Junior microplate reader. Intra-assay variance was <10%. Inter-assay variance
   was eliminated in batching samples from the same subject on common plates.

4. HOMA-IR: Homeostatic Model of Assessment-Insulin Resistance (HOMA-IR),

   was measured to assess insulin resistance, the reciprocal of insulin sensitivity,

   using the following calculation:

$$\text{HOMA-IR} = [\text{FPG}][\text{FPI}] \div 405,$$

   where FPG is fasting plasma glucose, in mg/dl, and FPI is fasting plasma insulin,

   in mU/L. In this model, a decrease in HOMA-IR represents an improvement in

   insulin sensitivity. The HOMA-IR index has been shown to correlate well with

   studies utilizing glucose clamp methodology (Muniyappa, Lee, Chen, & Quon,

   2008).

Statistical Analyses

Power Analysis

The subject n value was judged appropriate for this study (n=12), yielding a

power >0.80 based on a sample size of n=8 and population mean and standard error of

0.438±0.017 ng/ml for normal, healthy controls in seven previous studies (Table 1),

utilizing a two-tailed test with an α=.05.

Exercise Variables and Total Work Completed

All exercise variables and total work completed during each exercise condition

were analyzed via repeated measures analysis of variance (RMANOVA). Additionally,

changes in apelin concentration under exercise conditions were assessed as a function of

total work completed.

Assessing Main and Interaction Effects

Plasma apelin, blood glucose, plasma insulin, insulin sensitivity and hematocrit were analyzed via 3 (two exercise conditions [VO_2max and 70-75% submaximal tests] and one 54g GC condition) x 4 (4 time points [pre-, immediately post-, 60 minutes post- and 24 hours post-condition]) RMANOVA to detect main and interaction effects. Bonferroni post hoc tests were employed to identify where main effect significance was noted. RMANOVA was performed separately by condition for simple main effects upon achieving a significant interaction effect.

Plasma Apelin Relationship with Insulin Resistance

A Pearson product-moment correlational coefficient (PPMC) was used to analyze relationships of absolute plasma apelin values with that of blood glucose and plasma insulin, as well as with insulin resistance across conditions by time.

CHAPTER IV

RESULTS

This chapter presents the results of the current study and is organized into the following areas: subject descriptive characteristics, VO_2max and submaximal data, hematocrit and plasma volume response to each session and, finally, blood marker outcomes, including plasma apelin, blood glucose, plasma insulin and insulin resistance, as measured by the HOMA-IR index.

Subjects

Table 3. **Subject Baseline Descriptive Characteristics**. Expressed in overall terms and by gender. Values shown are means ± standard deviation. *Significantly different between genders ($p<0.05$).

Variable	*Overall*	*Males (n=7)*	*Females (n=5)*
Age (yrs)	22.75 ± 2.96	22.43 ± 2.88	23.2 ± 3.35
Weight (kg)	68.56 ± 13.08	72.14 ± 15.11	63.54 ± 8.59
Height (cm)	171.04 ± 7.53	174.29 ± 5.82	166.5 ± 7.8
BMI	23.37 ± 3.55	23.66 ± 3.96	22.96 ± 3.27
BF%	15.32 ± 8.09	11.02 ± 6.11*	21.33 ± 6.84*
RHR (bpm)	65 ± 9.17	63.43 ± 9.07	67.2 ± 9.86
Resting SBP (mmHg)	119.67 ± 9.34	120.29 ± 9.83	118.8 ± 9.65
Resting DBP (mmHg)	77.83 ± 7.29	76.57 ± 8.04	79.6 ± 6.54
Resting MAP (mmHg)	91.78 ± 7.29	91.14 ± 8.51	92.67 ± 6.02
VO_2max (L/min)	3.49 ± 0.95	4.02 ± 0.86*	2.77 ± 0.47*
VO_2max (ml/kg/min)	51.14 ± 11.07	56.17 ± 10.32	44.09 ± 8.42

Each subject (n=12) reported to the UNCG exercise physiology laboratory at a similar time across conditions (between 6-9 a.m.), in a resting, post-absorptive state.

Each subject successfully completed all treatment conditions, as well as achieving a true

VO_2max (51.14 ± 11.07 ml · kg^{-1} · min^{-1}) during maximal oxygen consumption testing,

per established criteria (Midgley, McNaughton, Polman, & Marchant, 2007). In addition,

each subject visited the lab for his or her 24-hour post-treatment visit at precisely the

same time as the previous visit, so as to ensure an accurate 24-hour post blood draw and

resting measures. Each subject participated in a body composition analysis on the first

visit, which included assessment via seven-site skinfold for % body fat determination,

sagittal-abdominal diameter, waist circumference and WHR measures. All baseline and

resting measures were taken ≥ 15 minutes prior to exercise and glucose gel consumption.

Resting measures and blood draws taken during the 24-hour post-treatment visits were

also taken with ≥ 15 minutes rest in a post-absorptive state.

### *VO_2max and Submaximal Test Exercise Outcomes*

Table 4. Exercise Variables and Total Work Completed. Includes VO_2max test and 70-75% VO_2max submaximal run. Heart rate measured in beats per minute (bpm). Values shown are mean ± standard error of the mean. *Significantly different between genders (p<0.05). †Significantly different between conditions (p<0.05).

	HR$_{max}$: VO$_2$max	VO$_2$max Duration (mins)	%VO$_2$max: Submaximal Test	Mean HR: Submaximal Test	Total Work VO$_2$max (Watts)	Total Work Submaximal (Watts)
Overall	187.2 ± 1.77	6.89 ± 0.30	72.71 ± 1.01	166.22 ± 3.36	876.36 ± 105.54[†]	1345.41 ± 173.58[†]
Male	187.86 ± 2.87	7.16 ± 0.42	73.59 ± 1.43	167.19 ± 4.48	1064.5 ± 122.18[*†]	1558.32 ± 164.58[†]
Female	186.2 ± 1.77	6.52 ± 0.42	71.48 ± 1.31	164.87 ± 5.63	612.97 ± 111.53[*]	1047.33 ± 322.35

Total work completed was computed for each subject for both the VO_2max

test and 70-75% VO_2max submaximal test, both of which were completed on a

treadmill with real-time HR monitored throughout with a digital HR monitor (Polar).

Table 4 displays these mean exercise and total work completed data. In addition, to

ensure appropriate exercise intensity for the duration of the submaximal test, subject

HR, RPE (not shown) and $\%VO_2max$ was monitored and recorded every five

minutes. Figure 1 displays the mean $\%VO_2max$ during the 30-minute submaximal

run across time. Total work completed was recorded in both exercise conditions in

order to account for workload- and/or duration-mediated alterations in plasma

apelin, blood glucose, plasma insulin and insulin sensitivity improvements.

Figure 3. 30-Minute Submaximal Exercise Intensity. $\%VO_2max$, as relative exercise
intensity, at five-minute intervals during the 30-minute 70-75% VO_2max submaximal
treadmill test. No significant differences amongst intervals over time were noted.

%VO₂max, Submaximal Treadmill Test

49
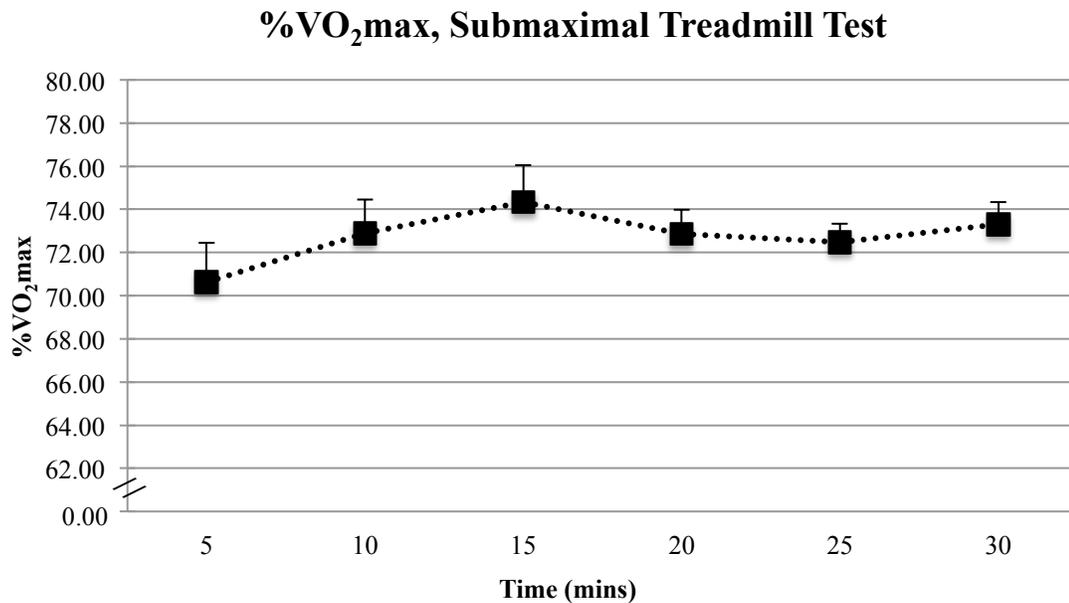
As is evidenced in Figure 3 above, the cohort maintained appropriate intensity – within the prescribed 70-75% VO$_2$max range – throughout the entirety of the 30-minute submaximal test.

Plasma Volume and Hematocrit

To assess the response of plasma volume to acute aerobic exercise in both exercise conditions, as well as during the 54g GC condition, hematocrit was measured at each time point. Table 5 displays these data.

Table 5. **Mean Hematocrit Measures**. Mean values (± SD), as %, across time in each study condition.

Time	VO$_2$max Test	70-75% VO$_2$max	54g Glucose Challenge
Pre (1)	46.33 ± 2.19	46.91 ± 2.23	46.39 ± 1.77
Post (2)	46.37 ± 1.58	46.40 ± 2.10	47.39 ± 1.55
1 hr Post (3)	45.49 ± 1.48	46.06 ± 1.95	46.46 ± 1.69
24 hr Post (4)	45.95 ± 1.25	46.79 ± 1.69	45.72 ± 1.64

No significant differences exist between times one and two (pre- and post-treatment) in any condition, suggesting appropriate control of plasma volume via hydration during exercise and glucose consumption. Table 6 displays the RMANOVA results, revealing no significant interaction or main effects on plasma volume.

Table 6. **RMANOVA Results for Hematocrit Across Condition by Time**.

Effect	df	F-value	Significance
Condition	2	1.087	0.355
Time	3	2.297	0.097
Interaction	6	0.982	0.445

Males exhibited a significantly higher hematocrit than females in conditions B (p=0.016) and C (p=0.029), though there were no differences exhibited by either gender across time.

Blood and Plasma Outcome Markers

Plasma Apelin

RMANOVA results showed no significant interaction or main effects of exercise or GC on plasma apelin (as shown in Table 7). Figure 4 displays the plasma apelin response across each condition over time. No significant differences were noted in plasma apelin by condition or across time. No mean differences or effects were observed for plasma apelin by gender (p=0.616).

Figure 4. Plasma Apelin Response by Condition. The plasma apelin (mean ± SE) response across each condition over time. ●VO$_2$max. ■70-75% VO$_2$max submaximal run. ◆54g glucose challenge.

Plasma Apelin Response
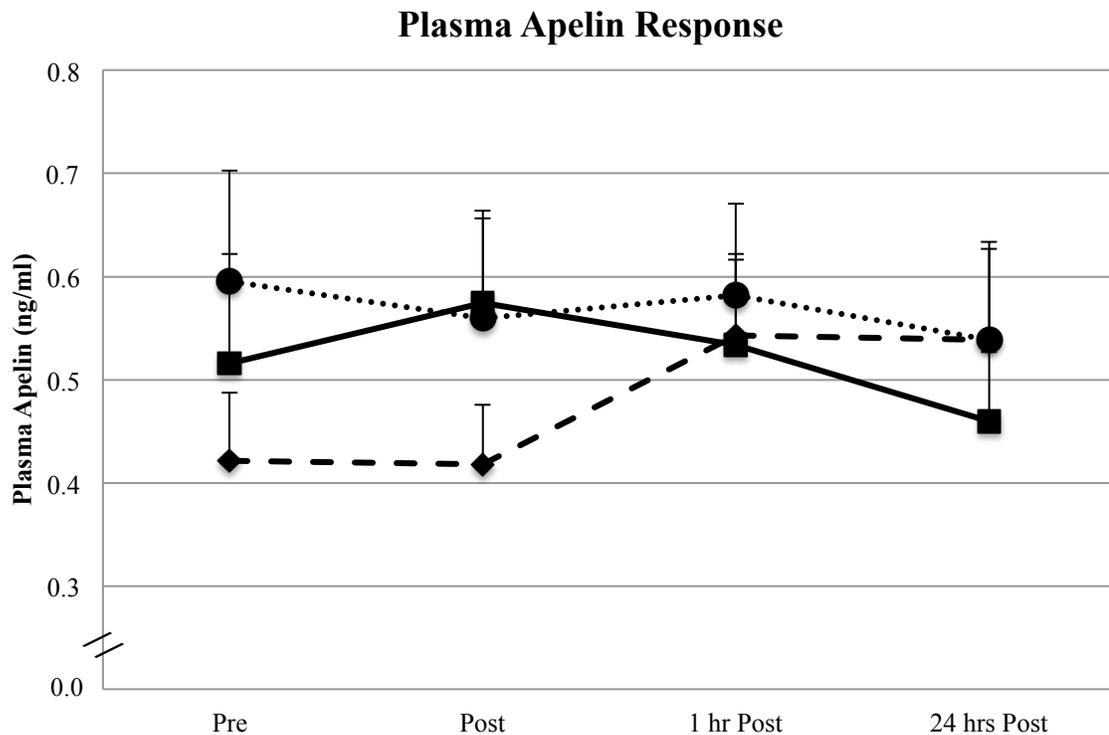
Table 7. RMANOVA Results for Plasma Apelin Across Condition by Time

Effect	df	F-value	Significance
Condition	2	1.187	0.324
Time	3	0.579	0.633
Interaction	6	2.037	0.073

Blood Glucose

Blood glucose was also assessed utilizing 3x4 RMANOVA, with these results expressed below in Table 8. A significant condition by time interaction effect, $F_{(2.307, 25.381)} = 4.783$, $p = 0.014$, was observed.

Table 8. RMANOVA Results for Blood Glucose Across Condition by Time.

Effect	df	F-value	Significance
Condition	1.370	6.36	0.016
Time	3	35.702	<0.001
Interaction	2.307	4.783	0.014

Figure 5 below details the blood glucose response by condition over time. It should be noted that the mean blood glucose response following GC is diluted by three (n=3) subjects who seemed to elicit no response to the challenge. This finding is explained below in the *Plasma Insulin* subsection, as each of these outliers exhibited considerable hyperinsulinemia post-challenge. Omitting these three subjects' post-challenge blood glucose concentration increases the mean (± SE) blood glucose to 116.31 ± 3.3 mg/dl. A Bonferroni post hoc analysis was utilized to assess mean differences over time and by condition. Significant results and comparisons are noted in Figure 5 below. No

significant differences existed between male and female at any time point across

conditions (p=0.896).

Figure 5. Blood Glucose Response by Condition. The blood glucose (mean ± SE) response across each condition over time. *Significantly different from all other times in condition (p<0.05). [†]Significantly different from 70-75% VO_2max Post (p<0.05). ●VO_2max. ■70-75% VO_2max submaximal run. ◆54g glucose challenge.

Blood Glucose Response
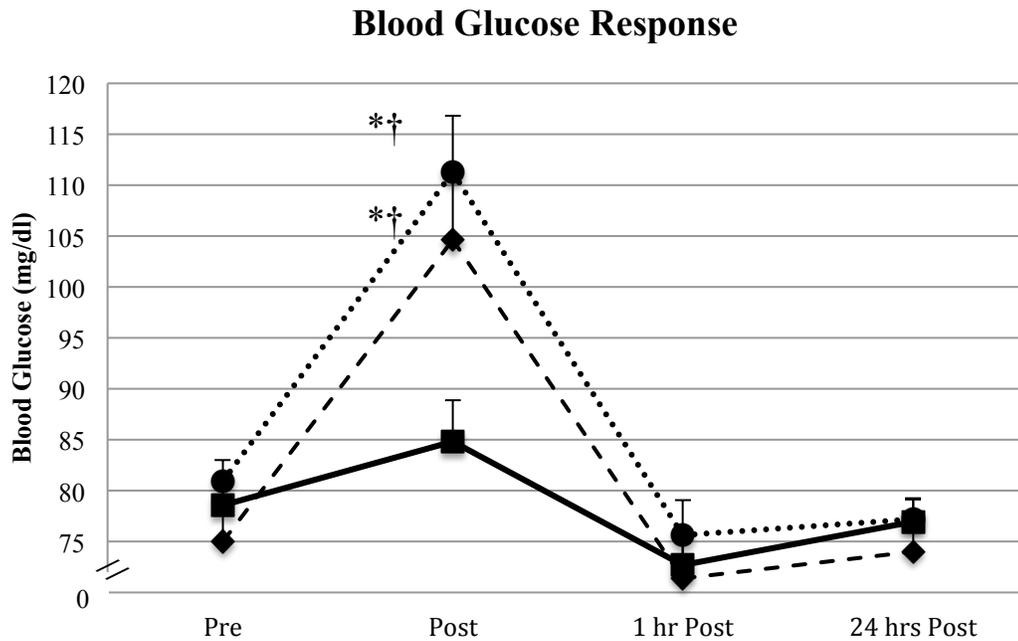
Plasma Insulin

RMANOVA of plasma insulin revealed a significant condition by time interaction

effect, $F_{(1.405, 15.454)}=15.423$, p=0.001. These data are shown in Table 9. Figure 6

illustrates the observed plasma insulin response by condition at each time point. As

significant interaction and main effects were observed regarding plasma insulin's

response by condition, a post hoc analysis utilizing Bonferroni adjustments for pairwise

comparison was performed. Significant results and comparisons are noted in Figure 6

below.

Figure 6. **Plasma Insulin Response by Condition.** The plasma insulin (mean ± SE) response across each condition over time. *Significantly different from all other times in condition ($p<0.05$). [†]Significantly different from 70-75% VO_2max Post ($p<0.05$). ‡Significantly different from VO_2max Post ($p<0.05$). ●VO_2max. ■70-75% VO_2max submaximal run. ◆54g glucose challenge.

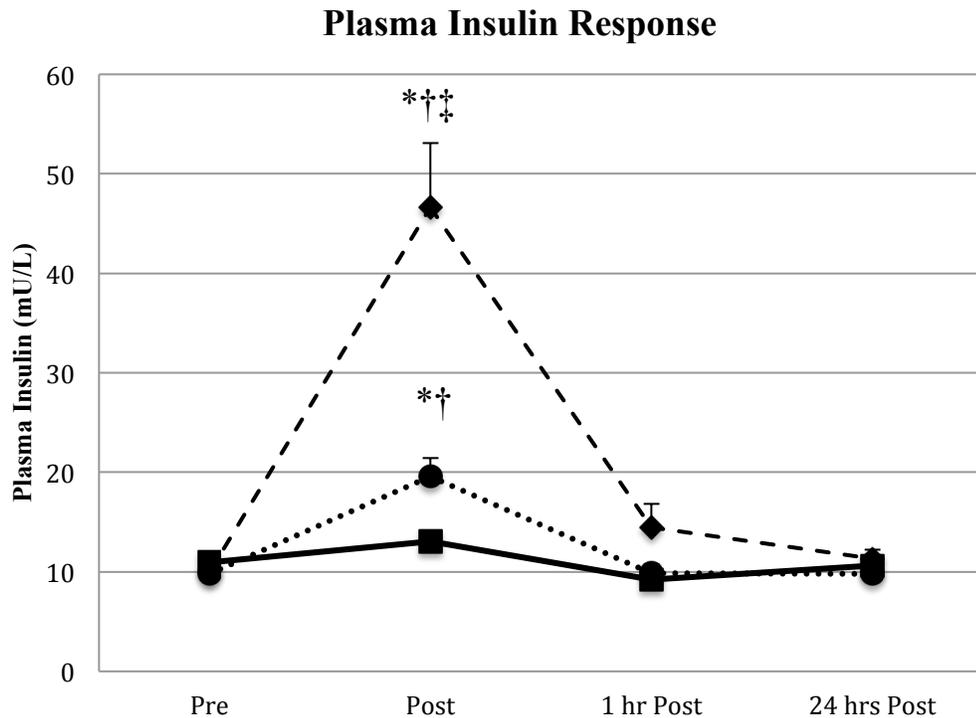Plasma insulin post-GC (time point two) was significantly elevated relative to post-

VO_2max ($p=0.008$) and post-30-min submaximal run ($p=0.001$) plasma insulin, as well as

compared to other time points within the GC condition ($p<0.001$). This extreme insulin

elevation was due to three (n=3) subjects' marked response following GC (mean ± SE

plasma insulin post-challenge: 75.59 ± 4.24 mIU/L); unsurprisingly, these were the same

subjects who demonstrated the aforementioned blood glucose non-response following the

GC. Omitting these three subjects' post-GC plasma insulin concentration reduces the mean (± SE) plasma insulin to 33.98 ± 5.29 mIU/L, which was still significantly elevated relative to both exercise conditions. No significant differences existed between male and female at any time point across conditions (p=0.627).

Table 9. RMANOVA Results for Plasma Insulin Across Condition by Time.

| Effect	df	F-value	Significance
Condition	1.135	24.064	<0.001
Time	1.333	51.167	<0.001
Interaction	1.405	15.423	0.001

Insulin Resistance: HOMA-IR Index

Table 10 displays the 3x4 RMANOVA results comparing HOMA-IR index scores across conditions over time, while Figure 7 illustrates these mean HOMA-IR scores by condition. HOMA-IR scores immediately post-treatment (time point two) are not necessarily reflective of overall resistance to insulin, as this indirect method requires calculation utilizing a constant value and fasting levels of blood glucose and insulin, the concentrations of which rise considerably following a GC and highly intensive aerobic exercise >80% VO_2max (Marliss & Vranic, 2002). A greater appreciation for insulin resistance as it relates to the HOMA-IR index may be gained by assessing ΔHOMA-IR scores over time following treatment (where a decrease in HOMA-IR score represents an improvement in insulin sensitivity), while considering established diagnostic cutoff values based on ethnicity, medical history and risk stratification (Gayoso-Diz et al., 2013). No significant effects by gender were observed for HOMA-IR scores (p=0.736).

Table 10. RMANOVA Results for HOMA-IR Scores Across Condition by Time.

Effect	df	F-value	Significance
Condition	1.075	20.197	0.001
Time	1.291	86.542	<0.001
Interaction	1.301	16.617	0.001

Figure 7. HOMA-IR Response by Condition. HOMA-IR index scores (mean ± SE) across each condition over time. *Significantly greater than all other times in condition ($p<0.05$). [†]Significantly greater than 70-75% VO$_2$max Post ($p<0.05$). ‡Significantly greater than VO$_2$max Post ($p<0.05$). §Significantly lower than Pre and Post measures ($p<0.05$).

HOMA-IR Insulin Resistance Scores
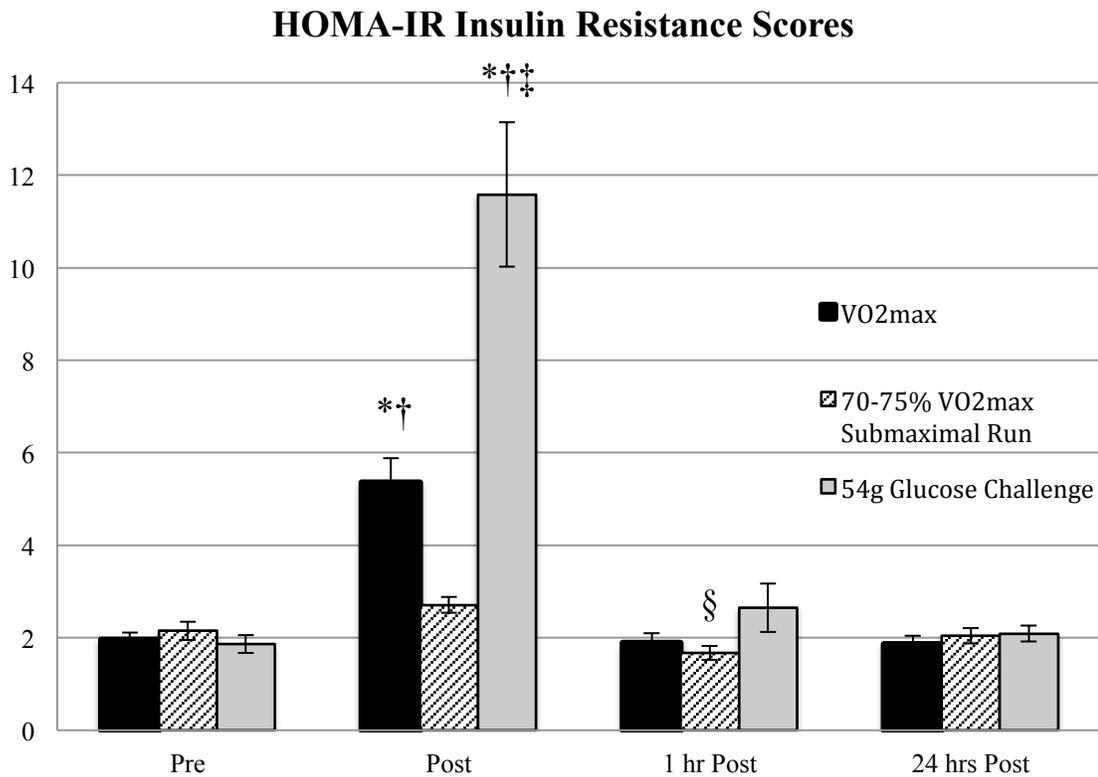
Plasma Apelin and its Relation to Insulin Resistance

To further examine the purported relationship between blood apelin and insulin

concentrations, mean resting plasma apelin concentration was correlated with mean

resting plasma insulin, obtained from pre-treatment resting blood samples across each

condition. Figure 8 displays this relationship, which exhibited a significant correlation

(R=0.702, p=0.011). Furthermore, there was a significant relationship observed between

resting plasma apelin concentration and HOMA-IR score (R=0.738, p=0.006). This

relationship is illustrated in Figure 9 and expounded on at length in the Discussion

section.

To elucidate the importance of plasma apelin as it relates to improvements in

insulin sensitivity, plasma apelin concentration was correlated with insulin resistance at

each time point across conditions, specifically concerning the 70-75% VO_2max

submaximal test, as it was the only condition to elicit a statistically significant decrease in

insulin resistance at one hour compared to pre (p=0.024) and post (p=0.005) measures.

Following VO_2max and GC at 1 hour (VO_2max: R=0.392; GC: R=0.332) and 24 hours

(VO_2max: R=0.29; GC: R=0.307), no significant relationship existed between plasma

apelin and insulin resistance, as measured by HOMA-IR index. However, by one hour

post-70-75% VO_2max submaximal treadmill test, plasma apelin was significantly

correlated with HOMA-IR score (R=0.672, p=0.017), though not at 24 hrs, consistent

with the reversal of insulin ~24 hours post-exercise bout. These data suggest a negative

correlation between plasma apelin and insulin sensitivity, whereby elevated

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concentrations of plasma apelin yield greater insensitivity to insulin in this healthy

cohort. These data are displayed in Figure 10.

Figure 8. Plasma Apelin and Insulin. The relationship between resting plasma apelin and plasma insulin (R=0.702, p=0.011).

Resting Plasma Apelin and Insulin Relationship
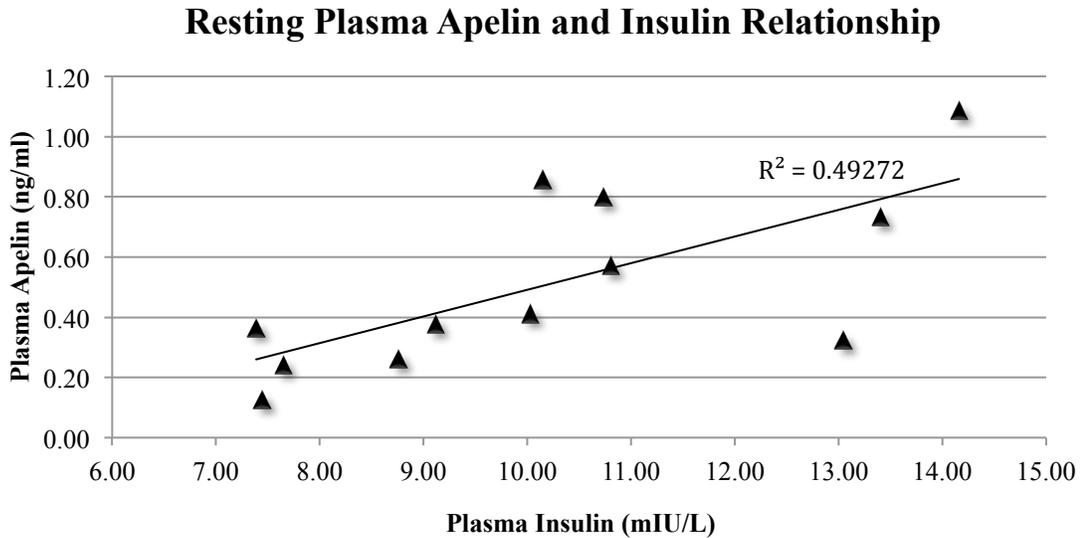
Figure 9. Plasma Apelin and Insulin Resistance at Rest. The relationship between plasma apelin and insulin resistance, as determined by the HOMA-IR index, at baseline (R=0.738, p=0.006).

Baseline Plasma Apelin Relationship with Insulin Resistance
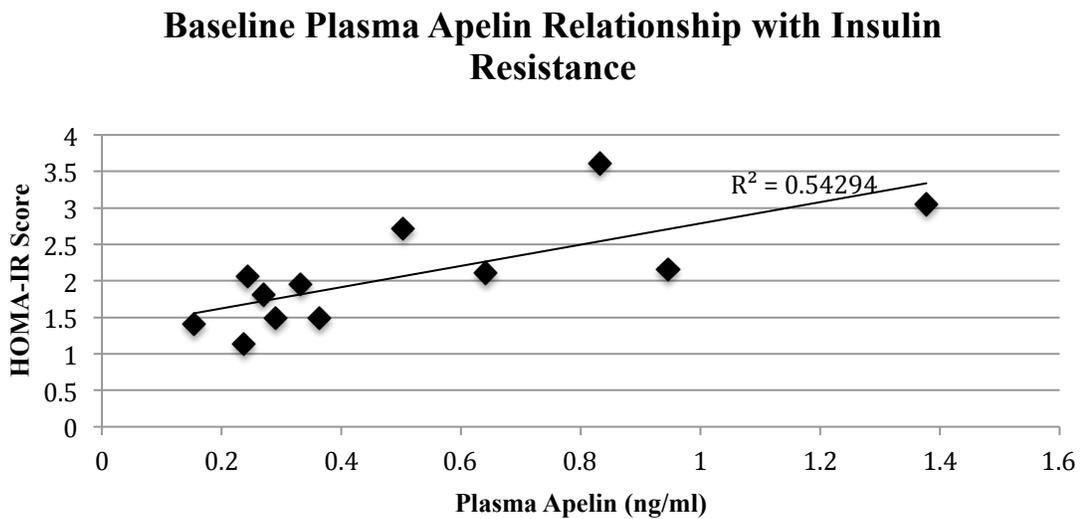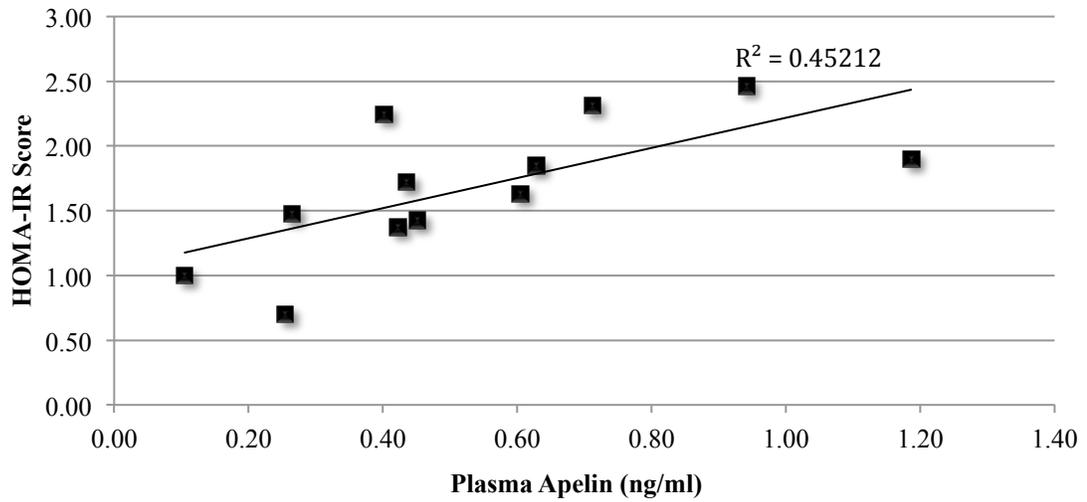
Figure 10. Plasma Apelin and Insulin Resistance Following Sustained Aerobic Exercise.
The relationship between plasma apelin and insulin resistance, as determined by the HOMA-IR
index, following the 70-75% VO_2max submaximal treadmill test at one hour (R=0.672, p=0.017).

**Post-Steady State Exercise Plasma Apelin Relationship
with Insulin Resistance**

CHAPTER V

DISCUSSION

The primary purpose of the current study was to examine the effects of acute

aerobic exercise on plasma apelin, as a function of aerobic exercise intensity, as well as

to a 54g GC serving as a positive control, in young, apparently healthy individuals. This

study sought also to achieve two secondary aims: to define appropriate resting plasma

apelin concentration in apparently healthy adults and to assess the relationship of plasma

apelin to insulin sensitivity.

Both male and female subjects were included in the current study, as previous

literature has shown no significant differences in resting or post-training apelin

concentration between genders (Alexiadou et al., 2012; Krist et al., 2013; Mesmin,

Dubois, Becher, Fenaille, & Ezan, 2010). The current study noted no significant

difference in resting plasma apelin concentration by gender (p=0.141), supporting this

contention in previous literature. In fact, resting plasma apelin amongst all twelve

subjects was quite stable across visits (p=0.990). No significant differences existed by

gender in blood glucose or plasma insulin, nor did subjects differ significantly in other

baseline and resting measures by gender, with the exception of % body fat (11.02 vs.

21.33% in males and females, respectively).

Total work performed in the VO_2max test, as well as absolute VO_2max, were

significantly different by gender (Tables 1 and 2), though total work completed and total

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caloric expenditure during exercise did not contribute to any observed differences in our blood markers of interest. A near-significant difference was also observed for relative VO_2max by gender (p=0.057). An abundance of literature exists regarding aerobic fitness and the potential benefits conferred regarding adipokine makeup though, with regards specifically to apelin, data is limited. Study of the effects of fitness on notable adipokines has returned contentious findings. Some maintain that individuals with greater aerobic fitness tend to have favorable adipokine levels (Fontana et al., 2010; Tomaszewski et al., 2003), while others note no significant benefits of fitness on adipokine profile in the absence of weight reduction (Christiansen, Paulsen, Bruun, Pedersen, & Richelsen, 2010; Kelly, Steinberger, Olson, & Dengel, 2007; Kondo, Kobayashi, & Murakami, 2006; Ziccardi, 2002). Subjects utilized in the current study demonstrated good or better aerobic fitness, as described by the American College of Sports Medicine guidelines for fitness and exercise prescription, as well as average or better % body fat. Thus, our inclusion of apparently healthy, aerobically fit subjects limited our ability to fully examine the influence of fitness on plasma apelin concentration. A greater spectrum of aerobic fitness is likely necessary to truly assess this phenomenon. Moving forward, the effects of aerobic fitness on resting plasma apelin concentration should be explored.

  To the authors' knowledge this is the first study to assess the effects of acute aerobic exercise on plasma apelin and, as a corollary, the contribution of apelin in the transient improvement of insulin sensitivity following aerobic exercise. Previous exercise-related studies regarding apelin have focused entirely on the effects of training,

establishing findings related to exercise chronicity, as opposed to the current study's focus on acute alterations in plasma apelin. Despite the burgeoning body of literature in recent years, results regarding apelin's response, activity and contribution to insulin sensitivity are equivocal (Besse-Patin et al., 2013; Fujie et al., 2014; Kadoglou et al., 2010, 2012; Krist et al., 2013). The exclusive selection of obese individuals, T2Ds and/or older adults for inclusion in these studies is but one of their multifactorial limitations. It was, therefore, imperative to address the need for appropriate definition of resting apelin level, as well as the acute effects of exercise on apelin concentration, in apparently healthy young adults.

To this end, we elected to discern the response of plasma apelin to bouts of maximal (VO_2max) and submaximal (30-minute 70-75% VO_2max) aerobic exercise, as compared to a 54g glucose challenge (GC), a stressor known to significantly elevate blood glucose and insulin. The controlled manipulation of glucose and insulin was a key element in the current study of apelin. Highly (>80% VO_2max) intensive aerobic exercise demonstrates empirically characterized and readily reproducible alterations in blood glucose and blood insulin (Marliss & Vranic, 2002), the latter of which is the most potent signal for the synthesis and secretion of apelin from adipose tissue. High-intensity aerobic exercise elevates blood glucose during exercise and causes a surge in insulin immediately following exercise, while moderate intensity aerobic exercise is a model of euglycemia and stable insulinemia both during and following activity. Furthermore, it has been consistently reported that following acute aerobic exercise – particularly after sustained, moderate intensity (50-75% VO_2max) exercise – insulin sensitivity is

enhanced (Bruce & Hawley, 2004; Cartee et al., 1989; Heath et al., 1983; Holloszy, 2005; Magkos et al., 2008; O'Neill, 2013). Whether apelin is implicit in this improvement, irrespective of alterations in blood glucose and insulin, is not currently known.

Understanding these principles, we hypothesized that plasma apelin concentration would be significantly increased from baseline immediately post- and one-hour post-VO_2max, resulting from significant increases in plasma insulin following the surge of blood glucose during maximal exercise. It was also hypothesized that the 54g GC would elicit significant increases in plasma apelin concentration immediately post- and one hour post-challenge, resulting also from significant increases in plasma insulin and blood glucose. In contrast, it was hypothesized that acute aerobic exercise of sustained (30 minutes) moderate (~70-75% VO_2max) intensity would not significantly increase apelin concentration immediately post-exercise or one hour post-exercise; blood insulin has not been shown to change significantly during or following this stressor, as euglycemia is typically maintained at moderate intensities. In addition to these hypotheses, it was also hypothesized that plasma apelin would return to baseline after 24 hours in each condition.

As our data demonstrate, both exercise protocols and the 54g GC elicited anticipated blood glucose and plasma insulin responses. Blood glucose was significantly elevated from baseline following both VO_2max (111.25 ± 5.6 mg/dl, p=0.001) and GC (104.63 ± 5.9 mg/dl, p=0.002), but not following the submaximal bout (84.83 ± 4.1 mg/dl). In addition, plasma insulin was significantly elevated following VO_2max (19.59 ± 1.8 mIU/L, p=0.001) and GC (46.64 ± 6.4 mIU/L, p=0.001). As expected, the

submaximal bout did not elicit significant alterations in plasma insulin (13.08 ± 0.9 mIU/L). Despite these anticipated spikes in insulin, plasma apelin was not significantly altered immediately post- or one hour post-treatment following VO$_2$max; the 54g GC elicited a non-significant elevation of plasma apelin one hour post-challenge (p=0.223). We therefore reject these hypotheses and accept the nulls. Conversely, we accept our hypothesis concluding that submaximal exercise would not significantly alter plasma apelin concentration over time. We also accept our hypothesis that plasma apelin will have resumed baseline, resting concentration at 24 hours post-treatment, as this was observed under each condition.

It is surprising that no significant changes in plasma apelin concentration were observed across conditions or over time, despite both VO$_2$max and GC modalities effecting the specified elevations in blood glucose and plasma insulin. Moreover, we noted a significant correlation between mean resting plasma apelin and plasma insulin (R=0.702, p=0.011), strengthening the foundation of our hypotheses and suggesting that elevated insulin would indeed lead to an increase in plasma apelin. To rationalize these findings contrary to our hypotheses, it is useful to revisit the complex interrelationship that exists between apelin and insulin, and how secretion signaling and its time course, as well as exercise-mediated and postprandial insulin signaling, may have differentially influenced our results.

Firstly, it must be considered that apelin might not be significantly affected acutely *in vivo*, either by exercise or by GC. Rather, changes in apelin may occur only as a result of chronic treatment or conditions. As no previous literature has examined apelin

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following acute exercise, no comparisons can be drawn here; the fact that chronic exercise studies are ambiguous on this topic further complicates our understanding of exercise-related apelin changes. However, regarding glucose consumption, in humans it has been demonstrated that no significant elevation in apelin occurs following OGTT for three hours (Alexiadou et al., 2012). This *in vivo* evidence substantiates our finding that apelin is not acutely affected by GC, provided the challenge in our study (~54g) and the aforementioned study (unspecified; likely 75g) was of sufficient dose. Deficits in the current study's experimental design surely exist herein, as it relates to the proposed effect of insulin on apelin. Apelin has a short circulating half-life, approximately 5-8 minutes, and its degradation could chiefly affect our results (Japp et al., 2008; Yu et al., 2013). More importantly, difficulties pertaining to apelin's half-life, in concert with the relative uncertainty regarding the time course of insulin-apelin signaling and secretion, limits our ability to conclusively interpret apelin changes post-treatment. The lack of blood sampling beyond one hour until 24 hours post-treatment in the current study may have precluded observable, significant findings; more frequent sampling to obtain a detailed plasma apelin profile may have afforded a more accurate glimpse into apelin's response to exercise and effects on insulin sensitivity, particularly given its short circulating half-life.

A few studies have suggested a time course relationship between insulin administration and apelin up-regulation *in vitro*; significant apelin mRNA elevations in these studies occur between three and twelve hours post-insulin administration in isolated adipocytes, though this may not necessarily reflect local or circulating apelin levels and

activity at insulin sensitive tissues *in vivo* (Boucher et al., 2005; Daviaud et al., 2006; Glassford et al., 2007; Wei et al., 2005). Significant findings may have been observed had we sampled blood beyond one hour, per the aforementioned studies' proposed insulin-apelin time course, though these *in vitro* findings may not reflect *in vivo* insulin-apelin synthesis and secretion signaling.

Apelin is known to exert considerable influence on insulin secretion from pancreatic β-cells *in vivo* and *in vitro*, inhibiting glucose-stimulated insulin release (Guo et al., 2009; Ringström et al., 2010; Sörhede Winzell et al., 2005). Consequently, full consideration of the dynamic relationship between apelin and insulin must be given. The present study, in examining alterations only up until one hour post-exercise in the immediately acute period, may have missed apelin alterations between this time point and 24 hours. How this relationship develops and is altered over time should be explored by future studies following both acute and chronic exercise.

Beyond these considerations, it should also be noted that apelin, as an adipokine and ubiquitously released cytokine, has been shown to partake in activity outside of its contribution as a circulating endocrine molecule. It has been well documented that apelin exerts paracrine and autocrine activity (Kälin et al., 2007; Kleinz & Davenport, 2005; Ronti et al., 2006) and that apelin-mediated glucose uptake *in vivo* is a NO-dependent, autocrine/paracrine process (Duparc et al., 2011). It also remains unclear what effect other cytokines exert on cellular release of apelin, and how these effects may vary by tissue. Little research exists on this topic, though it was established that TNF-α up-regulates apelin mRNA and plasma levels (Daviaud et al., 2006). This finding may

suggest a link between the inflammatory state common in obesity and T2D and a

potential protective mechanism, whereby up-regulated apelin contributes to improved

insulin responsiveness. This notion is strengthened further by evidence of apelin up-

regulation following induction of PGC-1α (Mazzucotelli et al., 2008), which has been

shown to improve lipid utilization and glucose disposal in both skeletal muscle and white

adipose tissue obtained from lean and obese T2D subjects (Benton et al., 2010). In short,

exercise-induced cytokine and gene effects on apelin should be explored, as apelin's

response is no doubt partially governed by these influences.

The current study further explored the nature of insulin sensitivity improvement

following both maximal and submaximal bouts of aerobic exercise, as compared with the

54g GC. As we hypothesized, 30 minutes of treadmill running at 70-75% VO$_2$max

elicited a significant improvement in insulin sensitivity at one hour post-exercise

(p=0.024). This sensitization to insulin was observed to have reversed at 24 hours post-

exercise, also as hypothesized. In addition, we hypothesized that the 54g GC would not

elicit significant alterations in insulin sensitivity at one hour and 24 hours post-challenge;

we were able to accept our hypotheses here, as well. Alternatively, we rejected our

hypothesis that maximal aerobic exercise would elicit improvements in insulin sensitivity

one hour post-exercise, as no significant changes were observed. It is likely that the

maximal aerobic exercise bout did not elicit improvements in insulin sensitivity due to its

relatively short duration (6.89 ± 0.30 mins). Submaximal testing likely achieved

significance through sufficient duration of exercise and energy expenditure and total

work required to significantly improve HOMA-IR score from baseline (Bordenave et al.,

2008; Goodyear & Kahn, 1998; Magkos et al., 2008), though Magkos et al. have previously noted that an approximately 900 kcal "threshold" of energy expenditure is necessary to elicit improvements in insulin sensitivity. No subject achieved this threshold energy expenditure even during the 30-minute submaximal run (mean ± SE kcal expenditure, 383.12 ± 30.5), despite observed improvement in insulin sensitivity in our healthy cohort.

The secondary objective of this study was to assess the relationship of plasma apelin with insulin sensitivity. That our subjects were all healthy adults, each exhibiting HOMA-IR scores indicating normal glucose tolerance, is a limitation in assessing the overall relationship between apelin and insulin resistance via HOMA-IR; a potential "basement effect" exists herein, given that our healthy subjects may not collectively be able to appreciably improve their glucose tolerance and insulin sensitivity by means of exercise such that indirect scores (i.e. HOMA-IR) are altered and statistical significance achieved. A broader range of glucose tolerances may have yielded better findings, as would have a larger sample size. Nevertheless, a statistically significant relationship was observed between resting plasma apelin concentration and HOMA-IR score at baseline (R=0.738, p=0.006), strengthening the notion from previous literature espousing elevated plasma apelin levels associated with insulin resistance (Boucher et al., 2005; Daviaud et al., 2006; Soriguer et al., 2009). For a more critical assessment, we approached the submaximal bout of aerobic exercise as a clinical tool to evaluate apelin's contribution within the framework of a stressor known to improve insulin sensitivity. In this model, insulin resistance was significantly reduced (HOMA-IR scores of 2.149 vs. 1.675, ~22%)

one hour post-exercise, while insulin resistance was significantly correlated with plasma apelin concentration ($R=0.672$, $p=0.017$). After one hour post-exercise, it is not known when sensitivity returned to baseline, though the relationship was reversed by 24 hours post-exercise in this group. Together, these collective data suggest that elevated levels of plasma apelin may contribute to a greater incidence of insulin resistance or, at the very least, decrements in glucose tolerance within apparently healthy adult populations. The significant relationship observed between plasma apelin concentration and insulin resistance following the submaximal aerobic exercise bout, but not the maximal, implies a duration-, as opposed to intensity-dependent, response, though this effect merits further examination. The fact that plasma apelin concentration remained relatively unchanged following the submaximal bout while demonstrating a similar response to that of the maximal bout over time brings to question the significance of apelin's acute contribution to the improvement of insulin sensitivity. Thus, it remains at question whether apelin contributes to the maintenance or improvement of insulin sensitivity following acute exercise.

The current study suggests that apelin, insofar as it does not respond significantly to acute aerobic exercise of maximal and submaximal intensities, does not significantly contribute to exercise-induced improvements in insulin sensitivity. Various other factors may have contributed to the improvement in insulin sensitivity following sustained, submaximal exercise, such as NO (Roy, Perreault, & Marette, 1998), calcium and calmodulin/Ca^{2+}-dependent protein kinase phosphorylation (Holloszy, 2005; Stanford & Goodyear, 2014), AMPK (O'Neill, 2013), hypoxia and ROS (Sandström et al., 2006),

phosphorylation of downstream signaling proteins, such as MAPK and Akt (Takaishi, Taniguchi, Takahashi, Ishikawa, & Yokoyama, 2003; Vendelbo et al., 2014) or GLUT4 upregulation (Ren et al., 1994), though this latter factor likely does not contribute to enhanced glucose uptake in the immediately acute period.

A number of limitations exist within this study, however, and prohibit a complete view of apelin's relationship with insulin sensitivity. Chief among these limitations is that circulating concentrations of apelin may not necessarily reflect the endogenous, local insulin-sensitizing activity of this adipokine. Moreover, much like exercise itself, it remains uncertain on which molecular targets within the insulin and exercise-induced glucose uptake signaling cascades apelin exerts its effects. Apelin has been consistently shown to exert its glucoregulatory effects and interact with insulin signaling via PI3K/Akt, though it has also been shown to induce glucose uptake *in vivo* and *in vitro* via eNOS activation and AMPK (Dray et al., 2008; Than et al., 2014; Zhang et al., 2014; Zhu et al., 2011), the latter consistently believed to be the central mediator of contraction-induced GLUT4 translocation and glucose uptake. To what extent these targets are modified during exercise remains to be fully determined (Fujii et al., 2005; Maarbjerg et al., 2009; Vendelbo et al., 2014). Further, this study was limited in its scope, having included only apparently healthy, highly fit individuals; a greater breadth of fitness and body composition may have yielded interesting findings. Performance of a true OGTT with an appropriate body weight-based glucose load, up to 75g, would have provided a better means of comparison with previous literature and, perhaps, may have elicited a significant response in plasma apelin. Lastly, it may be that our study was slightly

underpowered as it concerns apelin (observed power 0.699); a greater sample size with

equity amongst gender groups would have greatly enhanced our findings.

Research remains largely equivocal in its interpretation of exercise-mediated

improvements in glucose uptake and insulin sensitivity, particularly where it concerns the

direct effects of exercise on improved insulin responsiveness. The current study's

findings suggest that plasma apelin changes do not significantly contribute to the

transient improvement in insulin sensitivity following acute aerobic exercise, in our

healthy cohort. Likely, apelin requires a greater stimulus for significant response or that

some energy deficit threshold or otherwise significant disruption to glucose homeostasis

must be met, such as the previously outlined threshold suggested by Magkos et al.

(2008).

The specific responses of insulin-sensitive tissues (i.e. skeletal muscle, adipose

tissue, liver) to apelin during exercise also merit examination. Results from the current

study allow the authors to conclude that acute aerobic exercise of both maximal and 70-

75% VO_2max submaximal intensities does not elicit significant alterations in plasma

apelin at the times measured. GC, as well, elicited no significant alteration in plasma

apelin at the times measured. Despite this, the profiles of apelin's response over time, as

it pertains to exercise versus GC, suggest disparate trends. Furthermore, there may exist

an aerobic exercise duration-dependent effect on the metabolic input of apelin and its

supposed role on insulin sensitivity improvement, though the measure of whole body

insulin resistance via HOMA-IR index renders our results and interpretations ultimately

incomplete. The significant relationships observed between plasma apelin concentration

and insulin resistance both at rest and following the 30-minute acute bout of aerobic exercise may well serve as preliminary evidence of apelin's importance regarding acute contribution to glucose homeostasis and insulin sensitivity. The improvement of insulin sensitivity following a single bout of exercise is undoubtedly a multifaceted process, involving a number of key proteins and GLUT4 regulators. Further elucidation of apelin's role in this regard is relevant and necessary, despite the current study's findings. Downstream effector proteins thought to unite insulin- and exercise-mediated GLUT4 transport and glucose uptake – purported to be TBC1D (or AS160) and Rab proteins – must also be further examined through the lens of apelin and exercise response.

Summary and Future Directions

Our study has clearly demonstrated that, because no alterations in plasma apelin were observed in any of the three conditions, *circulating* plasma apelin seems not to be an obligatory regulator of glucose homeostasis or insulin sensitivity, despite each condition effecting known and anticipated alterations in blood glucose and plasma insulin. Future studies should tease apart the exercise-mediated and postprandial apelin trends, however, as this may reveal interesting findings regarding exercise-mediated versus postprandial apelin profiles. For future investigation, it is imperative that a larger and more varied subject pool be utilized so as to increase power as well as enhance our ability to determine plasma apelin's relationship with potential predictors, such as body fat percentage, region of adiposity, fitness level, glucose tolerance, etc. Additionally, a greater appreciation for the effects of exercise on plasma apelin may be gained with more frequent blood draws, as previously mentioned, so as to obtain a working profile of this

important adipokine and how it may affect insulin sensitivity; a profile of up to six hours post-intervention or exercise – with regular blood draws therein – should be examined. As our study ensured, it is essential to consider the effects of hydration and/or certain pharmaceuticals (i.e. ACE inhibitors) on blood pressure and fluid regulation and resulting apelin alterations, as well as appropriate storage and preparation of plasma samples with protease inhibitors when appropriate, as some previous studies have failed to report. Lastly, future efforts must focus on cell- and tissue-specific responses to exercise in animal and human models, with specific focus on apelin's receptor, APJ.

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APPENDIX A

AHA/ACSM HEALTH/FITNESS FACILITY PRE-PARTICIPATION SCREENING
QUESTIONNAIRE

Assess your health status by marking all true statements

History:

You have had:

_____ a heart attack
_____ heart surgery
_____ cardiac catheterization coronary
_____ angioplasty (PTCA)
_____ Pacemaker/implantable cardiac defibrillator
_____ rhythm disturbance
_____ heart valve disease
_____ heart failure
_____ heart transplantation
_____ congenital heart disease

Symptoms:

_____ You experience chest discomfort with exertion
_____ You experience unreasonable breathlessness
_____ You experience dizziness, fainting, or blackouts
_____ You take heart medications

Other health issues:

_____ You have diabetes
_____ You have asthma or other lung disease
_____ You have burning or cramping sensation in your lower legs when
walking short distances
_____ You have musculoskeletal problems that limit your physical activity
_____ You have concerns about the safety of exercise
_____ You take prescription medication(s)
_____ You are pregnant

If you marked any of these statements in this section, consult you physician or other appropriate health care provider before engaging in exercise. You may need to use a facility with a medically qualified staff.

Cardiovascular risk factor:

_____ You are a man older than 45 years
_____ You are a woman older than 55 years, have had a hysterectomy, or are postmenopausal
_____ You smoke, or quit smoking within the previous 6 month
_____ Your blood pressure is >140/90 mm Hg
_____ You do not know your blood pressure
_____ You take blood pressure medication
_____ Your blood cholesterol level is > 200 mg/dl
_____ You do not know your cholesterol level
_____ You have a close blood relative who had a heart attack or heart surgery before age 55 (father or brother) or age 65 (mother or sister)
_____ You are physically inactive (i.e., you get <30 minutes of physical activity on at least 3 per week)
_____ You are >20 pounds overweight

If you marked two or more of the statements in this section you should consult your physician or other appropriate health care provider before engaging in exercise. You will benefit from using our facility with a professionally qualified exercise staff to guide your exercise program.

_____ None of the above

You should be able to exercise safely without consulting your physician or other appropriate health care provider in a self-guided program or almost any facility including our facility that meets your exercise program needs.

Modified from American College of Sports Medicine and American Heart Association. ACSM/AHA Joint Position Statement: Recommendations for cardiovascular screening, staffing, and emergency policies at health/fitness facilities. Medicine and Science in Sports and Exercise 1998: 1018

APPENDIX B

HEALTH HISTORY, DRUG USAGE, AND FITNESS ACTIVITY QUESTIONNAIRE

Subject: _____

ID: _____

Health History

Do you have any musculoskeletal illnesses or injuries that would restrict your participation in either a maximal or submaximal exercise bout (as performed on a treadmill or with use of free weights while performing a squatting motion)?

                                           YES   NO

If yes, please describe._____

_____

_____

_____

Have you ever been diagnosed with cardiovascular disorders (heart problems, high blood pressure, high cholesterol, etc.)?   YES   NO

If yes, please describe._____

_____

_____

_____

Have you ever been diagnosed with metabolic disorders (diabetes, etc.)?

                                             YES   NO

If yes, please describe._____

_____

_____

_____

Could you currently be pregnant?   YES   NO   N/A

Please list any major illnesses, hospitalizations, or surgical procedures within the last two years.

_____

_____

Drug/Supplement Usage

Are you a *current* smoker/Tobacco user?   YES   NO

Have you every smoked/tobacco use in the past?   YES   NO
If yes, please describe history._____

_____

Are you currently taking any medication(s)?   YES   NO

If yes, please list name of medication(s), reason for and length of administration.

_____

_____

Are you currently taking any nutritional/vitamin supplements?
                                                        YES   NO

If yes, please list name of supplement, reason for and length of administration.

_____

Are you taking energy bars or drinks on a routine basis? YES   NO

Fitness Activity

Please describe your current participation in the following types of exercise:

1.   Aerobic (aerobic classes, walking, jogging, stair climbing, hiking, cycling, running swimming etc.) If more than one activity please list separately.

Frequency (# of days per week):   _____
Duration (time spent per session):   _____minutes
Intensity (difficulty level):   light   somewhat hard   hard   very hard

How long have you been participating in the aerobic activity described above?
Less than three months   3-6 months   6-12 months   greater than 12 months

Additional comments:_____

_____

_____

2.   Anaerobic (weight training, sprinting, boxing, martial arts, interval training, etc.)

Frequency (# of days per week):   _____

Duration (time spent per session): _____ minutes
Intensity (difficulty level):   light   somewhat hard   hard   very hard

How long have you been participating in anaerobic activity as described above?
Less than three months   3-6 months   6-12 months   greater than 12 months

Additional comments: _____

_____

_____

_____

3. Organized or Recreational sports (basketball, soccer, lacrosse, etc.)

Type of sport(s):_____

_____
Frequency (# of days per week):   _____
Duration (time spent per session): _____ minutes
Intensity (difficulty level):   light   somewhat hard   hard   very hard

How long have you been participating in sports activity as described above?
Less than three months   3-6 months   6-12 months   greater than 12 months

Additional comments: _____

_____

4. Other activities

Type of activities (Thai chi, yoga, stretching activities,
Classification (to be assessed by researchers—leave blank)
Need to confirm aerobic capacity by maximal oxygen uptake test.

                Trained   Untrained

APPENDIX C

UNIVERSITY OF NORTH CAROLINA AT GREENSBORO
CONSENT TO ACT AS A HUMAN PARTICIPANT

Project Title: <u>Acute effects of exercise on apelin, glucose, insulin and insulin sensitivity in apparently healthy individuals</u>

Principal Investigator and Faculty Advisor (if applicable): <u>Justin Waller (PI) and Dr. Allan Goldfarb</u>

Participant's Name: _____

What is the study about?

This is a research project. Your participation is voluntary. Your fat cells send signals to other tissues and one of these is known as apelin. The amount of fat you have influences blood apelin level and alters glucose balance and insulin sensitivity. Exercise serves as an excellent model to alter blood glucose levels and enhance insulin sensitivity. Apelin's role on insulin sensitivity and glucose balance in response to acute exercise has not been investigated. We intend to determine how moderate and high intensity acute aerobic exercise influence blood apelin level, along with blood insulin and blood glucose. It is the purpose of this study to see if apelin levels in the blood change to either a high intensity aerobic exercise known to enhance blood glucose levels compared to a moderate intensity exercise known to maintain blood glucose levels.

Why are you asking me?

You are being asked to participate in this study because little research has been done regarding apelin and its effects on glucose homeostasis and insulin sensitivity in apparently healthy human subjects. You also fit our criteria of being between the ages of 18-35. Prior to your participation, you will complete an American Heart Association/American College of Sports Medicine health fitness facility pre-participation screening questionnaire and a physical activity form. This is done in order to screen out individuals who may have musculoskeletal, cardiovascular or metabolic disorders that may interfere with the testing procedures or the measurements obtained, or women who may be pregnant or breast feeding. Only volunteers that do not use tobacco products (minimum 6 months) may be enrolled. Also, if you have been on any antioxidant supplement you must discontinue use for at least two weeks prior to being accepted as a subject. If you are taking any medication or drugs that may influence glucose, insulin or your metabolism/inflammation you will not be allowed to participate in this study. If your

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resting fasting blood glucose is above 100 mg/dl you will be excluded. If your resting blood pressure is elevated (140/90 mmHg) you will also be excluded from participating.

What will you ask me to do if I agree to be in the study?

You will be asked to participate in this study through six (6) visits to our laboratory. Two of these visits will involve exercise and blood sampling. Another visit will involve ingestion of a glucose gel and blood sampling and three visits will only involve blood sampling.

VISIT 1: You will be pre-screened for the information provided in the preceding section of this consent form. Screening will occur on the morning of your first visit. You will also bring a 3-day food record on your first visit listing amounts of food and drink. **The time for this screening should be no longer than 15 minutes**.

After screening you will have your height, weight, blood pressure and body fat percentage (using a skinfold caliper at seven sites) determined. A resting blood sample from an arm vein will then be obtained (approximately 3 teaspoons, or 14 mls); you may feel slight discomfort or pain with the needle stick. You will then perform a graded treadmill run to determine your aerobic capacity (VO$_2$max). The grade and speed of the treadmill will be increased gradually over time as you breathe through a mouthpiece connected to a tube to enable expired air samples to be measured for oxygen content. The test will continue until your aerobic capacity is reached or you indicate you can no longer maintain the pace or want to stop; you may feel some discomfort/fatigue during this test. There are bars on either side of the treadmill to grasp if needed. You will then be given a one-minute cool down/walk, after which another blood sample (3 teaspoons) will be obtained. Another resting, fasted blood sample (3 teaspoons) will be obtained 60 minutes after exercise. You will be provided water throughout this visit. You will press on the needle stick area for 2-5 minutes to avoid bruising after each of these blood samples is taken. **The total time for this part of the visit will be no more than 1 hour and 45 minutes.**

VISIT 2: After 24 hours you will visit the laboratory to have a 24-hr post-exercise blood sample (3 teaspoons) obtained; you must come in after an overnight (8-10 hrs) fast. The procedures for this blood draw will follow the same as the previous blood draws. **The resting, fasted 24-hour blood sample will take less than 15 minutes to collect.**

VISIT 3: You will return to the lab at least 7 days after the initial graded run in the morning (same time and condition) to perform either a 30-minute run at a moderate intensity (75% of your aerobic capacity) or ingest a glucose gel about 50 grams). Your weight, blood pressure and resting heart rate will be determined as before. You will have blood taken at rest (3 teaspoons at each time point), immediately post-exercise (or gel consumption) and 60 minutes post-exercise (or gel consumption). Your heart rate and

oxygen consumption will be monitored every 5 minutes during the run or after the ingestion of the gel. You will also give us feedback as to the difficulty of the exercise. You will be given water throughout this visit. You will also bring a 3-day food record prior to the third visit. **The total time for this visit will be no more than 2 hours.**

VISIT 4: After 24 hours you will visit the laboratory to have a 24-hr post-exercise blood sample (3 teaspoons) obtained; you must come in after an overnight (8-10 hrs) fast. The procedures for this blood draw will follow the same as the previous blood draws. **The resting, fasted 24-hour blood sample will take less than 15 minutes to collect.**

VISIT 5: You will return to the lab at least 7 days after visit 3 in the morning (same time and condition) to perform the treatment you did not complete in visit 3. Your weight, blood pressure and resting heart rate will be determined as before. You will have blood taken at rest (3 teaspoons at each time point)), immediately post-exercise (or gel consumption) and 60 minutes post-exercise (or gel consumption). Your heart rate and oxygen consumption will be monitored every 5 minutes during the run or after the ingestion of the gel. You will also give us feedback as to the difficulty of the exercise. You will be given water throughout this visit. You will also bring a 3-day food record prior to the third visit. **The total time for this visit will be no more than 2 hours.**

VISIT 6: After 24 hours you will visit the laboratory to have a 24-hr post-exercise blood sample (3 teaspoons) obtained; you must come in after an overnight (8-10 hrs) fast. The procedures for this blood draw will follow the same as the previous blood draws. **The resting, fasted 24-hour blood sample will take less than 15 minutes to collect.**

This will conclude your study participation. **Total participation time will be about 6.75 hours.**

All information concerning your records will be kept confidential and you will not be identified in any presentation or published work.

In addition, all blood samples obtained will be kept for possible future analyses and research, though you will not be identified in any way.

You may contact the PI, Justin Waller, at jdwaller@uncg.edu or (336) 613-6899 or Dr. Allan Goldfarb at ahgoldfa@uncg.edu or (336) 334-3029 with any questions concerning the nature of your participation and your consent to participate in this research project.

What are the risks to me?

The specific risks associated with participation in this research project are listed below.

You may feel tired and out of breath after the maximum aerobic capacity test which is normal. There is a very small risk of death as a result of this test (< 0.01%) and this is for all age groups and includes individuals that might have health problems. Since you are young and do not have any known health problems the risks associated with this test are reduced. You may let the researcher know if you are experiencing discomfort during the maximum aerobic capacity test, at which time the test will be discontinued. You may feel tired after the 30 minute run, which is normal.

It is possible that you may feel some symptoms of low blood sugar during either of the exercise sessions. Some of these symptoms are:

- Shakiness or hand tremors
- Ringing in the ears
- Chills or clamminess
- Lightheadedness or dizziness
- Irritability
- Nausea

In the event that you feel these symptoms, you should immediately inform the researcher so that he/she may discontinue the test. To reduce the risk to you, a safety 'spotter' will be in place at all times during the testing period to assist you.

Sterile techniques will be utilized to take the blood and there may be a small sensation of pain associated with taking blood. There may be some slight bruising associated with the blood sampling; placing direct pressure over the area where the blood was taken will minimize this. You must realize that infection can occur with any type of blood drawing but following sterile techniques will reduce this risk.

If you have questions, want more information or have suggestions, please contact: Justin Waller at jdwaller@uncg.edu or (336) 613-6899 or Dr. Allan Goldfarb at (336) 334-3029 or ahgoldfa@uncg.edu.

If you have any concerns about your rights, how you are being treated, concerns or complaints about this project or benefits or risks associated with being in this study, please contact the Office of Research Integrity at UNCG toll-free at (855)-251-2351.

What happens if I am injured during this study?

UNCG is not able to offer financial compensation nor to absorb the costs of medical treatment should you be inured as a result of participating in this research study. However, we will provide a referral to your primary care physician.

Are there any benefits to society as a result of me taking part in this research?

This research project may advance the exercise science literature. Apelin has recently been reported to influence insulin sensitivity and glucose regulation, which carries important implications for the treatment of diabetes. Findings from this research project may advance future research in this area.

Are there any benefits to me for taking part in this research study?

Through your participation in this study, you will learn of your maximal aerobic capacity, percent body fat and blood pressure. Additionally, you will be able to receive information on how you responded to the exercise sessions. You can ask for the blood glucose data to see if you maintained blood glucose balance on the 30 minute run. This information may be important in improving your overall health, and may serve as baseline data related to these measures. Otherwise, the information gathered in this study may not directly benefit you; however, it may advance the exercise science literature.

Will I get paid for being in the study? Will it cost me anything?

There are no costs to you or payments made to you for participating in this study.

How will you keep my information confidential?

All information concerning your records and obtained data will be kept confidential and you will not be identified in any presentation or published work. You will receive a study number, which will not be linked to your name. All records and data related to you, specifically, will be de-identified and coded and will be safely kept in a locked file cabinet. The master list for this study will also be codified with no identifying information. All study data will be destroyed, via document shredding and file erasure, upon publishing of the research data. This consent form will be kept for three years as required by federal law, after which time they will be destroyed to protect your identity. Blood samples will be properly disposed of after manuscripts have been published in accordance with biological hazard codes. All information obtained in this study is strictly confidential unless disclosure is required by law.

What if I want to leave the study?

You have the right to refuse to participate or to withdraw at any time, without penalty. If you do withdraw, it will not affect you in any way. If you choose to withdraw, you may request that any of your data which has been collected be destroyed unless it is in a de-identifiable state.

What about new information/changes in the study?

If significant new information relating to the study becomes available which may relate to your willingness to continue to participate, this information will be provided to you.

Voluntary Consent by Participant:

By signing this consent form/completing this survey/activity (used for an IRB-approved waiver of signature) you are agreeing that you read, or it has been read to you, and you fully understand the contents of this document and are openly willing consent to take part in this study. All of your questions concerning this study have been answered. By signing this form, you are agreeing that you are 18 years of age or older and are agreeing to participate, or have the individual specified above as a participant participate, in this study described to you by either Justin Waller or Dr. Allan Goldfarb.

Signature: _____ Date: _____