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Does acute maximal exercise or chronic physical activity affect circulating angiotensin (1-9) concentrations?

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Kinesiology

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Abstract

Introduction: Epidemiological evidence suggests physical inactivity can increase the likelihood of hospitalization from the SARS-COV-2 virus. Further, some data indicate a greater ratio of angiotensin 1-9 to angiotensin I helps prevent severe outcomes during infection. Moreover, related hormones can enhance potentially both physical activity and health. The purpose of this study was to determine whether plasma concentrations of angiotensin (1-9) are modified after a single exhaustive exercise bout and whether sex or chronic physical activity is associated with greater plasma concentrations.

Methods: Participants (n=14) performed a graded exercise test on a cycle ergometer. Antecubital area venipunctures were performed before, immediately following volitional fatigue, and after thirty minutes of recovery. Plasma was analyzed for angiotensin (1-9) using standard ELISA techniques.

Results: No significant changes in concentration of angiotensin (1-9) between time points or between sexes appeared. However, there was a significant correlation between quantity of weekly physical activity and concentration of angiotensin (1-9) immediately post exercise intervention. These results are likely affected by the changes in plasma volume with exercise and not from an increase in production of angiotensin (1-9).

Conclusion: There appears to be no obvious effect of a single dose of exhaustive exercise, sex, or the weekly accumulation of physical activity minutes on the circulating concentrations of angiotensin (1-9).

Keywords

Angiotensin Converting Enzyme 2, Renin Angiotensin System, Graded Exercise Test, Physical Activity, SARS-COV-2

Summary for Lay Audience

When an individual becomes infected with COVID-19, there is a down regulation of certain hormones which are part of a messaging system that controls blood pressure via dilation or constriction of blood vessels. Further, when these hormones become downregulated, complications can arise that can result in fatality. Recent research has indicated that regular physical activity may be beneficial to preventing complications due to COVID-19. Moreover, data suggest that targeting these hormones via supplementation may have a favourable effect on exercise performance. The purpose of this study was to determine whether a single exhaustive bout of exercise affects the circulating concentrations of one of these hormones [angiotensin (1-9)] and whether chronic physical activity alters these conditions. Eight males and six females completed a progressive exercise test to volitional fatigue on a stationary bicycle and had blood drawn from their forearm while resting, immediately following and thirty minutes into their recovery. There were no significant differences between any timepoint or between sexes due to wide variability in the measurements. If more participants were recruited, a difference between the two sexes may be more apparent. There was a positive association with physical activity and concentrations of the target hormone immediately after the exercise test, however, these results are likely an artifact of normal blood volume changes during physical activity. Additional research is required to determine how physically active individuals exhibit a protective effect from complications due to COVID-19 or if there are any performance benefits from targeting this hormone.

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“It is more than one individual that makes a winner”. – Dan Gable

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List of Abbreviations

- (ACE) Angiotensin Converting Enzyme
- (ACE2) Angiotensin Converting Enzyme 2
- (ADAM-17) A Disintegrin and Metalloprotease 17
- (Ang-1-7-Cyd) Angiotensin 1-7 inclusion in Cyclodextrin
- (ANOVA) Analysis of Variance
- (ARB) Angiotensin Receptor Blockers
- (ARDS) Acute Respiratory Distress Syndrome
- (ATR1) Angiotensin Type 1 Receptor
- (ATR2) Angiotensin Type 2 Receptor
- (BMI) Body Mass Index
- (CSEP) Canadian Society for Exercise Physiology
- (COVID-19) Coronavirus Disease 2019
- (EDTA) Ethylenediaminetetraacetic acid
- (ELISA) Enzyme Linked Immunosorbent Assay
- (eNOS) Endothelial Nitric Oxide Synthase
- (GLUT4) Glucose Transporter Type 4
- (hrsACE2) Human Recombinant Soluble Angiotensin Converting Enzyme 2
- (IPF) Idiopathic Pulmonary Fibrosis
- (MAS) MAS1 Oncogene

(mRNA) Messenger Ribonucleic Acid

(RAS) Renin Angiotensin System

(TMPRSS2) Type 2 Transmembrane Serine Protease

(sACE2) Soluble Angiotensin Converting Enzyme 2

(SARS-COV-2) Severe Acute Respiratory Syndrome Coronavirus 2

(VO₂) Oxygen Consumption

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1 Introduction

The renin-angiotensin system (RAS) is a well-established hormonal pathway responsible for maintenance of normal vascular and renal functions. Classically, this system has been targeted as a pharmacological pathway to decrease blood pressure in hypertensive individuals using Angiotensin Converting Enzyme (ACE) inhibitors or Angiotensin Receptor Blockers (ARB) (Azizi et al. 1997). Recently, our understanding has expanded with new knowledge of the counter regulatory axis of the RAS, which offers protective effects such as increased vasodilation and insulin sensitivity through the enzymatic activity of Angiotensin Converting Enzyme 2 (ACE2) (Costa et al. 2010, Munoz et al. 2012). ACE2 receptors are expressed in significant amounts in multiple tissues including the kidneys, lungs, heart, brain, and gastrointestinal tract as well as their associated blood vessels (Hamming et al. 2004). Moreover, studies exploring heart failure indicate that chronic stress elevates ACE2 concentration (Epelman et al. 2008) and that high circulating ACE2 is a strong predictor of cardiovascular disease, diabetes, and risk of death independent of other clinical predictors (Narula et al. 2020). From this work, other factors are also associated with high circulating ACE2 concentration including older age, male sex, high body mass index (BMI), and a history of tobacco smoking.

With the appearance of the severe acute respiratory syndrome coronavirus (SARS-COV-2), there has been an increase in research attempting to understand any factors related to the counter regulatory RAS. On the surface of the cell membrane, ACE2 is used by the SARS-COV-2 virus to enter the cytoplasm (Kuba et al. 2005). A side effect of viral entry is down regulated expression of ACE2 on the host cell's membrane resulting in a reduced production of both angiotensin (1-7) and angiotensin (1-9). This is important because

these peptides are responsible for vasodilation and decreasing inflammation.

Consequently, their absence results in a snowballing inflammatory effect. Associated complications lead to increased hospitalization and mortality due to acute respiratory distress syndrome (ARDS) (Astute and Ysrafil, 2020). Specifically, ARDS is classified as an acute lung injury typically due to aspiration, sepsis, or viral infection, leading to pulmonary oedema from increased vascular permeability, inflammation, and hypoxia (Ware & Matthay, 2000). In studies comparing survivors of ARDS to their non-surviving counterparts, plasma concentration of angiotensin-(1-9) was found to be greater in the survivor group with greater concentration of the precursor molecule, angiotensin-I, in the non-survivor group (Reddy et al. 2019).

While the effect of aerobic exercise on the classical arm of the renin-angiotensin system has been widely reported (Aldigier et al. 1993, Kosunen & Pakarinen, 1976, Fagard et al. 1985), there is far less information on the counter-regulatory arm. However, in rodent models, there is increasing evidence that exercise can be beneficial in restoring balance to the RAS in multiple disease states (Gomes-Santos et al. 2014, Prata et al, 2017, Peng et al, 2019). This might be the physiological mechanism explaining the epidemiological studies indicating that regular physical activity reduces the risk of severe outcomes from the SARS-COV-2 virus (Sallis et al. 2021, de Souza et al. 2021).

For several reasons, it seems reasonable that following an intense bout of exercise, angiotensin (1-9) might increase based on the increasing availability of its immediate precursor, angiotensin I. For example, at the onset of exercise, blood flow to the kidney decreases, resulting in the release of renin into the circulation from the kidneys (Fasola, 1966). The release of renin results in the cleavage of angiotensinogen into angiotensin I

where it can be further broken down. As exercise intensity increases, there is an increase in angiotensin I that is converted to angiotensin II by ACE, and the effects seem to peak after thirty minutes of recovery from exercise (Kosunen & Pakarinen, 1976).

If there is an increase in ACE activity following intense physical activity, there should be an increase in ACE2 activity as these two enzymes share 42% sequence similarity in their catalytic domain (Donoghue et al. 2000). However, current evidence doesn't suggest this is the case, i.e., no changes in plasma concentration of angiotensin (1-7) compared to angiotensin II following high intensity exercise (Maghaleas, 2020). However, it is interesting that the same study did find a greater ratio of plasma concentrations of ACE2/ACE. With increasing concentrations of both enzyme and substrate, there should be an increase in the resulting products unless ACE2 is not as efficient catalytically in its soluble plasma form.

Another interesting factor to consider is that the proteolytic activity of ACE2 is optimized under a slightly acidic pH of 6.5 (Vickers et al. 2002). Taking into consideration the effect maximal exercise has on blood pH, we can expect that plasma pH will drop from 7.4 to ~7.1, coming closer to the optimal pH for ACE2 activity (Hermansen & Osnes, 1972). It should also be noted that the pH of muscle tissues drops to about 6.4 after maximal exercise, perhaps further improving ACE2 activity at the local tissue level. More studies involving exercise are needed to understand these interactions.

Therefore, the focus of the present study was to assess whether a single exhaustive bout of exercise in young adults alters plasma ACE2 activity. Specifically, we measured the plasma concentrations of angiotensin (1-9) before and after a graded exercise test. We

hypothesized that following exhaustive acute exercise there would be an increase in conversion of angiotensin-I to angiotensin (1-9) and that female participants would have greater concentrations than their male counterparts, because sex is the largest predictor of variations in concentration of ACE2 in local tissues as well as in the circulation. Further, we predicted that following a single bout of exhaustive exercise, individuals with a history of regular physical activity would achieve greater concentrations of angiotensin (1-9) than their sedentary counterparts.

2 Literature Review

2.1 Physiology

The RAS plays a significant role in the maintenance of both electrolyte balance and blood flow (Peach, 1977). When blood flow to the kidney is low, renin is released from the kidneys into the bloodstream. Renin then cleaves 10 N-terminal amino acids from angiotensinogen producing angiotensin-I where the two arms of the RAS begin. The classical arm of the RAS is dependant on the activity of ACE. ACE activity is associated with an increase in blood pressure via vasoconstriction as well as inflammatory, oxidative, cell proliferating and fibrotic effects (Piero and Moncada, 2020). It accomplishes this by converting angiotensin-I to angiotensin-II (Ng & Vane, 1967), followed by the binding of angiotensin-II to the angiotensin type 1 receptor (ATR1). ATR1 activity will then stimulate vasoconstriction as well as the synthesis and release of aldosterone from the adrenal cortex, leading to water and sodium retention in the kidneys and inhibition of the production of renin (Naftilan and Oparil, 1978). Alternatively, angiotensin-II may bind to angiotensin type 2 receptor (ATR2) with the same affinity causing decreases in inflammatory cytokines and vasodilation through the stimulation of bradykinin (Menk et al. 2015, Tsutsumi et al. 1999).

The counter regulatory arm of the RAS involves another membrane bound enzyme, ACE2, which can exert its effect on both angiotensin-I and angiotensin-II. ACE2 is also found in a soluble form in circulation when one of two enzymes, a disintegrin and metalloprotease 17 (ADAM-17) or the type 2 transmembrane serine protease (TMPRSS2), cleaves ACE2 from the cell membrane releasing its enzymatic activity

(Heurich et al. 2014). When angiotensin-I is acted upon by ACE2, it is converted to angiotensin-(1-9) (Donoghue et al. 2000). Angiotensin-(1-9) can activate ATR2 receptors or it can undergo additional catalytic activity from ACE, resulting in angiotensin-(1-7). When angiotensin-II is acted upon by ACE2, it counters many of the effects of angiotensin-II by converting it to angiotensin-(1-7). Angiotensin-(1-7) can then bind to the MAS1 receptor (MAS), resulting in a release of nitric oxide and vasodilation (Santos et al. 2003). The MAS receptor is also responsible for decreased cell proliferation and anti-inflammatory, anti-oxidative and anti-fibrotic effects (Peiro and Moncada, 2020). Moreover, the effects of angiotensin-(1-7) are not isolated to the MAS receptor, because it can also exert an effect on other receptors including ATR1 as a competitive antagonist (Galandrin et al. 2016).

2.2 Genetic Variation

Genetic factors provide a great amount of variability regarding the concentrations of the components of the RAS. For example, plasma ACE activity is influenced by an insertion/deletion polymorphism (Rigat et al. 1990). This polymorphism determines 47% of the total variation of ACE concentrations, with those homozygous for the insertion having the lowest serum ACE concentrations, and those homozygous for the deletion having the greatest concentrations of ACE. This polymorphism has also been shown to have a significant effect on human adaptation to exercise (Montgomery et al. 1999). Those carrying the ACE I/I genotype show greater adaptation to repetitive endurance type exercise. However, individuals carrying the D allele have been shown to respond better to heavy resistance training (Folland et al. 2000). The D allele has also been

associated with increased mortality in individuals who develop ARDS (Marshall et al. 2002).

The greatest variation of circulating ACE2 can be attributed to sex differences, with males showing greater concentrations of circulating ACE2 than females (Sama et al. 2020). This may be explained by the location of the gene encoding for ACE2, because it is on the X chromosome. Further, two genetic loci have been identified as influencing plasma concentrations of ACE2, one of which also belongs on the X chromosome (Gtex Consortium, 2013). As a result, females show greater circulating angiotensin 1-7 concentration than males (Sullivan et al. 2015). Finally, epidemiological evidence suggests that ancestral origin may play a role in additional variation of plasma concentrations, with east Asian and European ancestries demonstrating the highest concentrations and south Asian and African ancestries demonstrating the lowest concentrations (Narula et al. 2020).

2.3 Pathology

Soluble ACE2 (sACE2) has been considered a biomarker for disease because research in chronic heart failure patients showed that greater circulating concentrations of ACE2 are associated with greater severity of disease (Epelman et al, 2009). Additional epidemiological evidence supports these findings, by demonstrating an increased risk of major cardiovascular events with greater concentrations of plasma ACE2 (Narula et al. 2020). This study also found greater plasma concentrations of ACE2 in individuals with diabetes and high blood pressure. Further, ACE2 knockout mice demonstrated significantly reduced contractility of the heart (Crackower et al. 2002) consistent with

findings in other rodent models that show chronic heart failure reduces serum ACE2 activity by 25% (Gomes-Santos et al. 2014).

Research has also demonstrated the effect of ACE2 deficiency in the lungs, specifically regarding ARDS. ACE2 knockout studies demonstrated worsening outcomes following lung injury including pulmonary oedema, decreased oxygenation, lung elastance, increased inflammation, and vascular permeability (Imai et al. 2004). Results from this study also show that lung injury is associated with downregulation of ACE2 while increasing concentrations of angiotensin II. However, ACE knockout mice showed significantly better outcomes, while inhibition of ACE offered protection from ARDS in ACE2 knockout mice. In follow up studies, blocking of ATR1 was also successful in improving pulmonary oedema, while inactivation of ATR2 resulted in greater lung injury (Kuba et al. 2005, Imai et al. 2005).

As the RAS also plays an integral role in the regulation of electrolyte balance, it should be no surprise that ACE2 expression is implicated in the development of kidney disease. In fact, studies have demonstrated an increase in the concentration of ACE2 in the urine of patients with chronic kidney disease (Mizuiru et al. 2011). This evidence contrasts other studies revealing a decreased expression of ACE2 in the glomeruli and tubules of the kidney, possibly due to the cleavage of ACE2 by ADAM-17 (Reich et al. 2008), which is upregulated in kidney disease (Melenhorst et al. 2009).

In diabetic mice, serum and tissue concentrations of ACE2 and production of angiotensin (1-7) decreases (Tikellis et al. 2008). Also, ACE2 knockout studies have demonstrated the potential for angiotensin (1-7) to improve glucose uptake by Glucose transporter type

4 (GLUT4), providing another mechanism for improved insulin sensitivity (Takeda et al. 2013). Further, ACE2 gene knockout studies have shown decreases in muscular strength, bone mass and muscle fibre size, while MAS knockout mice showed no difference from wild type controls (Nozato et al. 2019).

2.4 Pharmacology

It has been shown that greater concentrations of plasma ACE are associated with different diseases including diabetes mellitus, hypertension, and heart failure (Bernstein et al. 2013). This has led to the development of therapeutics targeting ACE to help lower blood pressure, manage left ventricular dysfunction, diabetic nephropathy, and post kidney transplant erythrocytosis. Specifically, there are two classes of drugs that are commonly prescribed, ACE inhibitors and angiotensin receptor blockers (ARBs). ACE inhibitors are a class of drug that inactivate ACE, preventing the conversion of angiotensin-I to angiotensin-II. This causes a decrease in the activation of ATR1 receptors, leading to a decrease in blood pressure via reduced vasoconstriction. In contrast, ARBs work by blocking selectively the ATR1 receptors, allowing ACE to convert angiotensin-I to its product, but preventing angiotensin-II from binding to the receptors on the cell membrane.

Recently, attention has shifted from regulating the classical arm of the RAS to looking for novel treatments by modulating the counter regulatory arm. For example, MAS receptor agonists have been developed as a potential therapeutic. Stimulation of the MAS receptor using nonapeptide AVE 0991, increases endothelial nitric oxide production in adult rats (Faria-Silva, Duarte & Santos, 2005). It is also beneficial in the reduction of

oxidative stress and neuronal apoptosis in early brain injury as well as having diuretic effects in the kidney (Mo et al. 2019, Pinheiro et al. 2004).

Infusion of angiotensin (1-7) has been shown to reduce age related declines in muscular strength in ACE2 knockout mice as well as their wild type controls (Takeshita et al. 2018). Additional studies have demonstrated therapeutic effects of angiotensin (1-7) infusion on disuse muscular atrophy through the maintenance of strength, muscle diameter and bone density (Morales et al, 2016). Meanwhile, chronic ingestion of an oral formulation of angiotensin (1-7) reversed autonomic dysregulation, reduced fibrosis of skeletal muscle, and restored spontaneous physical activity in mice exhibiting skeletal muscle dystrophy (Sabharwal et al. 2014).

A selective angiotensin II receptor agonist has been developed to distinguish the effects of the AT₂ receptor. Upon its development, Compound 21 has been shown to increase outgrowth of neurites, duodenal alkaline secretion and lower the mean arterial pressure of spontaneously hypertensive rats (Wan et al. 2004). Further, it was shown that the intravenous infusion of Compound 21 increased sodium excretion in the urine, contributing to the blood pressure lowering effect (Kemp et al. 2014). In addition, studies have evaluated the effects of Compound 21 on stroke prone rats, finding administration delayed brain damage, prolonged survival, and prevented the accumulation of angiotensin I (Gelosa et al. 2009). Studies in human monocytes have also demonstrated an ability of Compound 21 to decrease pro-inflammatory cytokines (Menk et al. 2015).

In recent experiments, human recombinant soluble ACE2 (hrsACE2) has been found to be effective in preventing the SARS-COV-2 virus from infecting other cells by acting as

a competitive inhibitor to the membrane bound ACE2 (Monteil et al. 2020). In clinical trials, hrsACE2 has been found to shift significantly the RAS to the counter regulatory axis, decreasing angiotensin-II concentration and increasing concentrations of angiotensin-(1-7) and angiotensin-(1-9) in patients with severe symptoms from SARS-COV-2 infection (Zoufaly et al. 2020). Once treatment with hrsACE2 concluded, the concentration of angiotensin-(1-9) reversed back to its baseline physiological concentration, while angiotensin-(1-7) showed a slight decline towards baseline. Clinical trials continue to be conducted on the use of hrsACE2 for treatment of SARS-COV-2.

2.5 Exercise Physiology

Acute aerobic exercise results in reduced blood flow to the kidney, producing an increase of plasma renin concentrations (Fasola et al. 1966). As exercise intensity increases above the lactic threshold, there is a large increase in sympathetic nerve activity and a subsequent increase in the production of angiotensin II (Staessen et al. 1987).

Angiotensin II remains elevated during exercise recovery, peaking after 30 minutes, and remaining elevated for up to six hours (Kosunen & Pakarinen, 1976). Other studies have focused on the products of ACE2 metabolism, comparing moderate to intense physical activity to investigate the acute effects. Acute high intensity exercise increases plasma ACE2 concentrations, while moderate intensity exercise increases concentrations of ACE in plasma as well as ACE2 and angiotensin (1-7) in urine samples (Magalhaes, 2020).

Unfortunately, this study did not report on the other molecule directly produced by ACE2, angiotensin (1-9). Consequently, understanding the effect of exercise on other intermediary molecules is important, as angiotensin (1-9) has demonstrated superiority

over angiotensin (1-7) at releasing nitric oxide through the enhancement of bradykinin (Jackman et al. 2002).

Aerobic exercise training has been shown to decrease resting angiotensin II concentration in heart failure patients (Braith et al. 1999). Studies in healthy rodents show an increase in ATR1 and ATR2 receptor mRNA, concentrations of angiotensin-(1-7) and ACE2 activity, and a decrease in angiotensin I, angiotensin II and ACE activity (Fernandes et al. 2011). Inhibition of the ACE enzyme using ACE inhibitors has also been associated with decreases in adaptation to high intensity exercise training, including decreases in lean muscle mass, reduced left atrial volume and a lower total hemoglobin mass (Sjurdarson et al. 2022).

Unfortunately, there are fewer studies investigating the effects of exercise training on the counter regulatory axis of the RAS, however it has become a popular topic recently. For example, Gomes-Santos (2014) found chronic heart failure rats had a decrease in ACE2 activity following left ventricular heart ligation and that exercise training was successful in restoring the deficit back to baseline measures, while simultaneously increasing skeletal muscle gene expression of ATR1, ART2 and MAS receptors. In addition, the ratio of angiotensin (1-7) to angiotensin II increased, demonstrating an overall shift towards the counter regulatory axis.

Aerobic exercise training also presents a mediating role in the balance of the RAS in the kidney. Specifically, in diabetic mice, endurance exercise has been shown to lower the amount ACE2 that is excreted in the urine, by reducing ADAM17 expression in the cortical tubules and cleavage of ACE2 from the cell membrane (Somineni et al. 2014). In

addition, aerobic exercise training downregulates the number of ATR1 receptors in the kidneys of rats, aiding in natriuresis and lowering of blood pressure (Ciampone et al. 2011).

In human skeletal muscle, ACE2 concentrations have been found to increase after four weeks of exercise training, with serum ACE2 concentrations decreasing (Kloting et al. 2020). Also, there are data to show that an individual's VO₂ max may be associated with the expression of ACE2 in skeletal muscle, however, this association disappears as body fat percentage increases (Perez-Valera et al. 2021). Moreover, other studies have shown plasma concentrations of angiotensin (1-7) are correlated inversely with BMI, waist-hip circumference ratio, body fat skinfold thickness, blood pressure, and circulating leptin concentration (Fernandes et al. (2021).

There is also evidence that aerobic exercise training with simultaneous ACE2 stimulation can have beneficial effects on the lungs. Aerobic exercise training has been shown to prevent the progression of idiopathic pulmonary fibrosis (IPF) in rodent models (Prata et al. 2012). IPF is recognised as an additional complication following ARDS, which develops in approximately 40% of COVID-19 patients (Spagnolo et al. 2020). In another study, where bleomycin treatment was given in mice to induce lesions in the lung and followed with the ACE2 stimulating drug diminazine, researchers found that aerobic exercise training significantly reduced fibrous connective tissue from the lung lesions (Prata et al, 2017). While aerobic exercise training alone can improve pulmonary function, quality of life and functional capacity, additional stimulation of the counter regulatory axis of the RAS may further improve these outcomes, at least in patients with IPF (Vainshelboim et al. 2014).

2.6 Nutrition

There is also some evidence supporting the role of nutrition in modulating the RAS. For example, rodent experiments have shown that high fat diets over eight weeks result in decreased insulin sensitivity in skeletal muscles, lipid and glucose intolerance, hypertension, and increased body mass when circulating Angiotensin (1-7) concentrations were low (Santos et al., 2012). Additional studies have demonstrated a sex-related influence in the development of obesity induced hypertension in high-fat diet mice, based on ACE2 deficiencies in adipocytes (Shoemaker et al, 2019). The female mice demonstrated protection from increased systolic blood pressure when compared to their male counterparts, which is believed to be due to the promotion of ACE2 mRNA from estradiol (Wang et al, 2015). High-fat diets in female mice result in greater ACE2 activity in the adipocytes and circulating concentrations of angiotensin (1-7), whereas male mice fed similar diets show a decrease in kidney ACE2 activity and higher plasma concentrations of angiotensin II (Gupte et al, 2012).

Other rodent models have indicated an increase in ACE concentrations and angiotensin II activity in the lungs with low protein diets during pregnancy (Gao et al., 2016). Food proteins have also been shown to have bio-active peptides, provoking ACE inhibiting effects in vivo but not in vitro along with evidence that certain bioactive peptides can also upregulate ACE2 (Wu et al., 2017). It appears that foods with the greatest bioactivity include beta-casein, a protein found in milk products, and fermented cabbage (Bousquet et al. 2020). Although foods containing these peptides do not have a large enough effect on treating hypertension, a regular diet high in protein may be helpful in preventing cardiovascular diseases or regulation of the RAS (Wu et al., 2017).

Regardless of the type of diet, timing of nutrient intake likely plays a role in the expression of RAS components. Animal studies have revealed that intermittent fasting can increase the expression of ACE2, MAS and ATR2 mRNA measured in left ventricular tissue even when coupled with a high fat or high carbohydrate diet (Camelo et al. 2019). Intermittent fasting was also effective at lowering Renin, ACE and ATR1 gene expression with high fat and high carbohydrate diets, while the control diet was more effective at lowering the expression of these genes and showed no difference in intermittent fasting controls (Camelo, et al 2019).

Oral ingestion of angiotensin-(1-7) is possible through the inclusion of cyclodextrin (Ang-1-7-Cyd) to provide stability of the compound for absorption by the digestive tract (Fraga-Silva, 2011). In spontaneous hypertense mice, this formulation provided an anti-thrombotic effect and increased plasma concentrations of angiotensin-(1-7) with either acute or chronic administration. Other studies have shown that an oral ingestion of angiotensin (1-7) increases insulin sensitivity, reduces circulating lipids and decreases inflammation of the liver of high fat fed rats (Santos et al. 2013).

Moreover, recently, studies by de Moura et al. (2021) have shown that administration of Ang-1-7-Cyd has the potential to also increase the performance of endurance athletes. In their double blinded, cross over study, participants improved their VO₂ max by an average of 10% when compared to their performance with placebo. Additionally, participants reported lower ratings of perceived exertion, as well as greater time to exhaustion and total work completed.

3 Methods

3.1 Participants

Participants were recruited from the Western University campus via in class presentations to undergraduate kinesiology students or via word of mouth from campus recreation clubs and varsity athletic teams. Due to suspected sex variations, males and females were divided into separate groups for comparison and all participants were subject to the same experimental procedures. Based on effect size calculations, sampling continued until there were at least 6 participants for each sex.

The inclusion criteria for this study required participants to be between the ages of 18-39 years and being able to exercise to volitional fatigue. Participants were excluded from the study if they were taking any recreational or prescription drugs or anti-inflammatory medication, had a BMI over 30, consumed >5 alcohol drinks per week, smoked tobacco, had been diagnosed with any chronic health condition, or had a present diagnosis with COVID-19.

The study was approved by the Western University Research Ethics Committee (#117957) and both written informed consent and completion of a medical screening questionnaire were obtained from each participant prior to any data collection.

Participants were instructed to avoid anti-inflammatory medications, alcohol, recreational drugs and smoking for 48 hours prior to the experimental session. Female participants scheduled their visit to the lab within one week of the onset of menstruation to control for cycle variations in estrogen and progesterone. Participants came to the sessions hydrated and fasted for 8 hours. No food was allowed until all blood samples were collected.

3.2 Experimental Procedures

Throughout the study, all COVID-19 measures and guidelines provided by Western University, municipality and provincial jurisdictions pertaining to human research studies were followed and included use of masks and additional cleaning procedures. In addition, upon arrival at the lab, participants were subject to a rapid antigen test for SARS-COV-2 (Flowflex, Hangzhou, China). If the participant tested negative, they were then informed of all study procedures and, after having their questions answered, signed the letter of informed consent. Although no participants tested positive, had any done so, they would have been informed of the test results, instructed to follow proper COVID-19 safety precautions, and asked to reschedule their session the following week.

After giving their written consent, participants completed the Canadian Society for Exercise Physiology (CSEP) Get Active questionnaire to determine their chronic physical activity habits. Then, anthropometric measurements including height, body mass and lean body mass (via densitometry, using a BodPod® [COSMED, Concord, CA]) were completed. The latter uses body mass measured from a weigh scale (Tanita, Japan) and volume of air displacement to determine body density and subsequently fat and lean mass using the Siri equation (Siri, 1961). A baseline (T1) blood sample (10ml) using a BD Vacutainer Eclipse 21G needle (BD, Franklin Lakes, NJ) was collected in K2 EDTA vacutainers (BD, Franklin Lakes, NJ) from a vein in the antecubital area of the forearm. Participants were then fitted with a mouthpiece to collect expired air breath by breath using a Sensormedics Vmax system (Sensormedics Vmax 29, Yorba Linda, CA) and a Polar RST200TM heart rate monitor (Polar Electro Inc., Lachine, Quebec). Next, each participant completed a VO_2 max test (11–22-minute incremental cycle test increasing 15

Watts (W) / minute to volitional fatigue) beginning at 100W on a Veletron cycle ergometer (Racer Mate, Seattle, WA). A second blood draw (T2) was completed upon termination of the VO_2max test, and a third sample 30 minutes later (T3). All whole blood samples were placed on ice before being centrifuged in an Eppendorf 5804R (Eppendorf SE, Hamburg, Germany) at 3000rpm within 15 minutes after collection, transferred into plastic tubes in duplicate, and stored at -80C until analysis. An enzyme linked immune-sorbent assay (ELISA) from Biomatik (Kitchener, Ontario) was then performed and analyzed in duplicate via a Biotek Synergy H1 microplate reader (Santa Clara, CA) to determine plasma concentrations of angiotensin (1-9).

3.3 Measurements

Plasma angiotensin (1-9) was used as the primary outcome measure to identify the effect of a single bout of exhaustive exercise, sex, and weekly physical activity minutes. A competitive inhibition ELISA is a laboratory technique used to quantify the amount of a specific protein in a sample. Antibodies are bound to a plastic plate and, when the sample is applied, will trap the molecule of interest while allowing other molecules to be washed away. A second antibody is then applied to the plate, attaching to the molecule of interest. This allows for a detection reagent to be applied, and a change in colour of the solution. This colour change can then be quantified by a microplate reader by measuring the optical density at 450nm, with the final signal obtained being inversely correlated to the amount of angiotensin (1-9) detected. A four-parameter logistic regression can then be performed from known concentrations of the target molecule, and each sample can be compared to the standard curve. With each sample measured in duplicate, the average

absorbance reading is then used to estimate the plasma concentration of angiotensin (1-9).

Secondary measurements included body fat percentage, VO_2 max, and self reported weekly physical activity minutes. The BodPod Body Composition System uses air displacement to obtain body volume and a weigh scale to measure body mass to achieve a two-compartment model, dividing the body into fat and fat free components. The fat free component consists of tissues comprised of protein, water, bone, glycogen, and other minerals. Using measurements of body mass and volume from the application of Boyle's Law, the proportions of fat and fat free mass can be calculated using the Siri equation (Life Measurement Inc, 1997).

3.4 Statistical Analysis

G*Power 3.1 statistical software (Faul, Erdfelder & Buchner, 2007) was used to calculate the sample size for a repeated measure, mixed effects ANOVA with a power of 0.8, an alpha of 0.05, and a large effect size of $(f) = 0.4$. The total minimal estimates sample size becomes 12, or 6 per group.

Statistical analysis was performed using Blue Sky Statistical software (Version 10.3.1, 2023). Plasma concentrations of angiotensin (1-9) were analyzed using a two-way repeated measures ANOVA and Tukey's HSD as a post hoc analysis for any significant effects that were observed. A simple linear regression was also completed with weekly physical activity minutes recorded from the CSEP Get Active Questionnaire and concentration of angiotensin (1-9) at each timepoint with a Bonferroni correction applied. All data were presented as means \pm standard deviations.

4 Results

4.1 Participant Characteristics

In total, 14 participants were enrolled in the study (8 men and 6 women). One of the male participants was excluded as their results from the ELISA were outside the range of the standard curve and an additional male was excluded due to hemolysis of the plasma samples upon collection. The physical characteristics of the remaining 12 participants are described in Table 1.

Table 1

Physical Characteristics of Participants

| | Age | | Body Fat | | Activity | | VO ₂ | |
|--------|----------|--------|----------|--------|----------|----------|----------------------------|---------|
| | (y) | | (%) | | (min/wk) | | (ml/kg•min ⁻¹) | |
| | <i>M</i> | (SD) | <i>M</i> | (SD) | <i>M</i> | (SD) | <i>M</i> | (SD) |
| Sex | | | | | | | | |
| Female | 21.00 | (0.63) | 28.62 | (6.60) | 256.67 | (163.64) | 43.02 | (3.20) |
| Male | 22.17 | (1.17) | 15.38 | (7.39) | 225.00 | (139.10) | 51.37 | (16.32) |

Note: $N = 12$ ($n = 6$ per group). y = years; kg = kilograms; min = minutes; ml = millilitres; wk = week; % = percentage. Data reported as mean (*M*) \pm standard deviation (*SD*).

4.2 Blood Metabolites

Means and standard deviations of circulating angiotensin (1-9) are displayed for males and females at each time point in Table 2. The four-parameter logistic regression used to determine the standard curve of the assay had an acceptable goodness of fit ($r^2 = 0.995$,

MSE = 0.004907). The mean intra-assay coefficient of variation between all samples was < 20%.

Table 2

Concentrations of Plasma Angiotensin (1-9) in pg/ml

| | Baseline | | Post-Intervention | | 30 Minute Recovery | |
|--------|----------|---------|-------------------|---------|--------------------|---------|
| | M | (SD) | M | (SD) | M | (SD) |
| Sex | | | | | | |
| Female | 2.794 | (1.015) | 2.991 | (1.173) | 2.874 | (1.357) |
| Male | 2.517 | (0.625) | 2.599 | (1.042) | 2.525 | (0.815) |

Note: N = 12 (n = 6 per group). Data reported as mean (M) ± standard deviation (SD).

There were no violations of the assumptions for running an ANOVA from Levene's Test for homogeneity of variance, Shapiro-Wilk's test for normality and Mauchly's test of sphericity. The mean plasma concentration of angiotensin (1-9) displayed no significant changes following exercise or after recovery ($p = 0.926$). There appeared to be a difference in the mean of angiotensin (1-9) concentrations between sexes by about 10-15% across all three time points, however due to wide variability of both groups, our ANOVA indicated that there were no significant sex differences ($p = 0.399$). The results of the ANOVA are presented in Figure 1 (See Appendices Table 3 for detailed results).

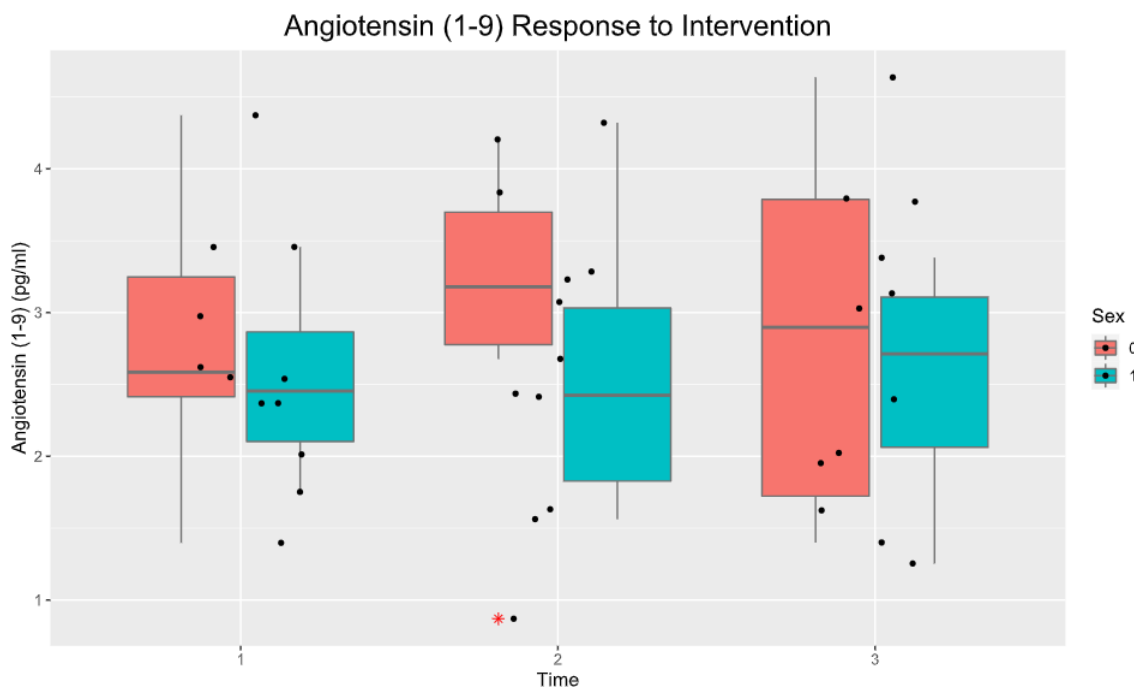


Figure 1: Plasma concentrations of Angiotensin (1-9) in pg/ml at baseline (T1), Immediately Post-Intervention (T2), and after 30 minutes of Recovery (T3). Female sex is indicated in red (0) and male sex in blue (1). First and third quartile ranges are indicated by the box, with the median indicated by the band in the middle. Data plotted outside the whiskers are outliers as indicated by a red asterick.

To determine whether weekly physical activity minutes influence plasma concentrations of angiotensin (1-9), each time point was plotted against the overall self reported weekly physical activity minutes from the CSEP Get Active questionnaire. While there was no significant correlation at baseline or thirty minutes post intervention, there was a strong and statistically significant correlation immediately after the VO_2 max test ($r = 0.71$, adjusted p -value < 0.05). The results of the regression are visualized in Figure 2 (See Appendices Table 5 for detailed results).

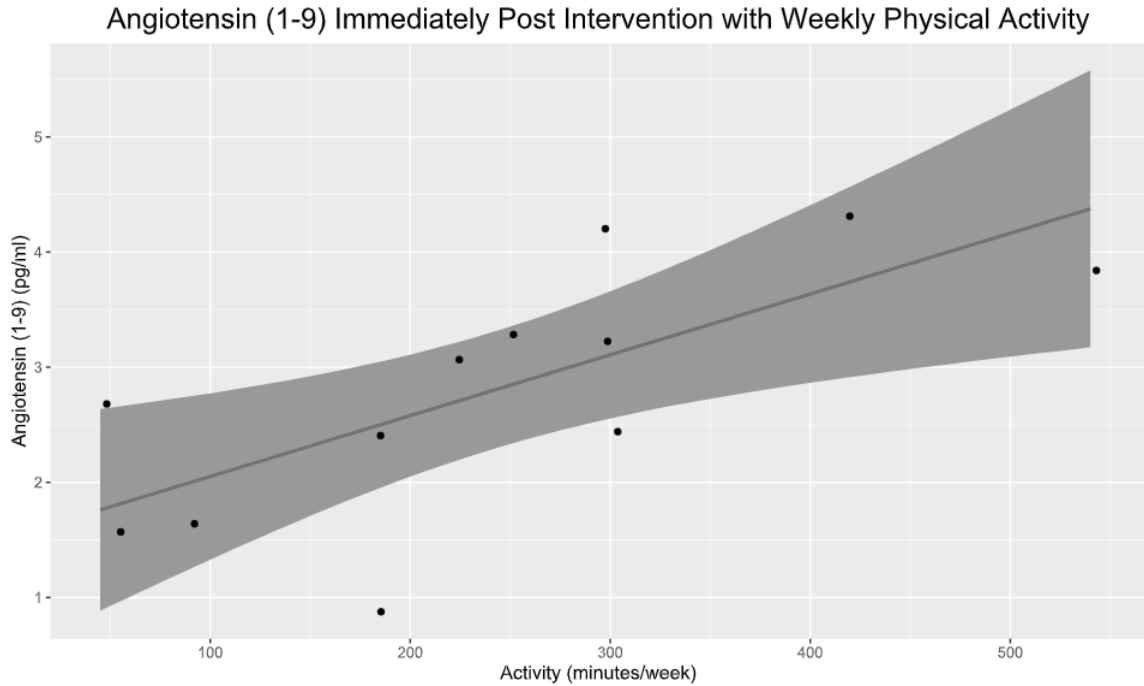


Figure 2: Weekly physical activity minutes from the CSEP Get Active Questionnaire plotted against the plasma concentration of angiotensin (1-9) immediately post-exercise intervention ($r = 0.71$, $p < 0.05$).

5 Discussion

The purpose of this study was to determine whether a single bout of exhaustive exercise could modulate the counter regulatory axis of the RAS by converting angiotensin I to angiotensin (1-9). Also, of interest was whether there were any sex related differences and whether weekly physical activity minutes played a role in circulating concentrations.

5.1 Intervention

There was a lack of evidence to support our hypothesis that the graded exercise test would increase concentrations of angiotensin (1-9). In fact, roughly half of the participants demonstrated a decrease in plasma concentration while the other half demonstrated an increase. On average, participants demonstrated an increase of <5% in circulating angiotensin (1-9) concentrations. This is in line with expected increases in hemoconcentration following exercise due to a loss of plasma volume from the vasculature and into the interstitial space and working musculature (Convertino, 1981). Following thirty minutes of recovery, the mean reverted back towards baseline. However, half of the participants demonstrated an increase in plasma concentration following recovery. This demonstrates the large variability of these measurements, requiring more samples to draw any conclusions.

5.2 Sex-Related Differences

Our study did not reveal any statistically significant differences between sex. However, it should be noted that the difference in angiotensin (1-9) concentration between the two-group means was about 10-15% (greater in the female group) for all three time points. However, due to large sample variability, our results were not statistically significant.

The calculated sample size used a power of 0.8, increasing the potential for a Type II statistical error. Future studies with larger sample sizes may refute these findings.

5.3 Chronic Physical Activity

While statistical significance was obtained from the linear regression of weekly physical activity minutes and the plasma concentrations of angiotensin (1-9) following the graded exercise intervention, this correlation does not necessarily mean increased production from ACE2 as circulating concentrations are affected by changes in plasma volume with both acute and chronic exercise.

The link between acute exercise and hemoconcentration has been well documented i.e., with increasing intensity of acute exercise, more plasma is pushed into the working musculature and interstitial space creating a vascular hemoconcentration (Convertino, 1981). Further, a single bout of exercise can increase plasma volume by 10-12% over 24 hours post exercise due to activation of the thirst mechanism and the overall effect of regular exercise is an expanded plasma volume (Convertino, 2007). However, after a few weeks of regular exercise (training), these acute effects plateau somewhat, due to a resulting increase in plasma proteins. While the length of the experimental exercise session studied here was quite short, plasma volume would also be reduced due to sweat fluid losses. Therefore, typically following acute exercise one would expect an increase in the concentration of many plasma proteins. However, individuals with a history of regular physical activity might actually demonstrate a smaller increase post exercise compared to their sedentary counterparts because of their lingering plasma volume expansion. If so, our data are consistent with an increase in the amount of angiotensin (1-9) circulating in those who are physically active.

Alternatively, individuals with a large muscle mass may demonstrate a greater increase in hemoconcentration after exercise due to larger shifts in plasma volume to the working musculature. Therefore, to accurately determine the effects of acute exercise on circulating angiotensin (1-9) in physically trained individuals the degree of plasma volume expansion must be accounted for, or some measure of angiotensin (1-9) production must occur. Further, it is likely important that the exercise intensity and duration of the regular exercise be controlled. Importantly, should physical activity have a significant effect on circulating concentrations of angiotensin (1-9) through catalytic activity of ACE2, this would reinforce the epidemiological evidence of reduced need for hospitalization and supplementary oxygen in physically active individuals following infection of the SARS-COV-2 virus.

5.4 Future Directions

Improvements on the current study protocol could include the use of a catheter to decrease the amount of time between termination of the graded exercise test and sample collection. It may also be worth pursuing a similar study design to investigate the effects of maximal exercise on the RAS using muscle biopsies because with the larger decrease in local muscle pH after maximal exercise, it is reasonable to believe that ACE2 activity could be greater in the working muscle than in circulation. In addition, the use of a more precise biochemical analysis, such as with high performance liquid chromatography, could increase the reliability of the results.

As this study aimed to evaluate changes in components of the RAS in young, healthy, physically active individuals, it would also be beneficial to perform experiments in clinical populations that exhibit dysregulation of the RAS. Specifically, treatment with

ACE inhibitors produces higher circulating concentrations of angiotensin (1-9), opening the possibility of additional modification through exercise (Larouche-Lebel et al. 2019).

Further, with recent research showing the ergogenic effect of supplementing with Ang-1-7-Cyd before aerobic exercise, it would be interesting to investigate whether the same effect would be seen by the inclusion of angiotensin (1-9) with cyclodextrin. As angiotensin (1-9) is a stronger activator of the bradykinin pathway than angiotensin (1-7) (Jackman, 2002), it may show superior results in increasing maximal aerobic capacity on its own or used synergistically with Ang-1-7-Cyd. In addition, inclusion of angiotensin (1-9) with cyclodextrin may show similar therapeutic effects to Compound 21, potentially providing a cost effective and widely available alternative to this designer drug. As recent research has found that Compound 21 can reduce the need for supplemental oxygen during COVID-19 infection (Tornling et al. 2021), an orally available version of angiotensin (1-9) could contribute to the fight against the global COVID-19 pandemic.

5.5 Limitations

This study has several limitations that should be noted. It is important to highlight that the participants recruited were healthy, young individuals. Consequently, these results lack external validity to older or clinical populations including those with a SARS-COV-2 infection.

While all individuals were instructed to come to the lab fasted, variations in diet could lead to variations in the concentration of circulating angiotensin (1-9). Additionally, while individuals were encouraged to hydrate before coming to the lab, changes to hemoconcentration could be a result of hydrations status. Additional measurements of

hematocrit or albumin would aid in determining the effect of hemoconcentration on circulating angiotensin (1-9) concentration.

While the assay used to measure plasma angiotensin (1-9) concentration had a high sensitivity and specificity, large variations were seen in some duplicate samples. This could be due to several factors including collection and preparation of samples or inconsistent laboratory techniques including pipetting or washing of the microplate.

Other than variations due to biological sex, this study does not consider any other genetic considerations of the RAS. While sex is the largest known contributing factor to variations in the counter regulatory axis of the RAS, there is also evidence that ancestral origin plays a role in the determination of the concentration of these peptides. In addition, identification of which ACE allele the participants have could also be a confounding factor as this could affect the amount of substrate available for ACE2 to convert. Finally, it is possible that the same logic could be applied to variations of the gene encoding for ACE2, which is passed along the X chromosome and has the potential of being another confounding variable.

Statistically speaking, this study used a beta probability of 0.8 and a large effect size to calculate the required sample size. As a result, there is an increased possibility of a type 2 error occurring. Larger sample size would be more appropriate to confirm the results.

5.6 Conclusion

The purpose of this study was to determine whether a single exhaustive bout of exercise could induce changes in plasma concentrations of angiotensin (1-9). We also sought to determine whether there were any sex-related differences or changes related to weekly

physical activity minutes in circulating concentrations of the angiotensin (1-9). The results of our study indicate that there is little evidence for changes in plasma concentrations of angiotensin (1-9) following intense aerobic physical activity. In addition, there was no significant sex-related differences in circulating concentrations of angiotensin (1-9) due to high variations in the measurements of each group and a potential for a type 2 error due to the small sample size. Our study did provide some evidence that weekly physical activity minutes could influence the concentration of angiotensin (1-9) immediately following a graded exercise test, but this can be attributed to an increase in hemoconcentration during physical activity.

The results from this study are not conclusive and future studies should investigate further the effect of physical activity on components of the counter regulatory axis of the RAS. Specifically, research into the development of an orally available formulation of angiotensin (1-9) could produce insights into clinical outcomes related to COVID-19 or other cardiovascular diseases.

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Appendices

Appendix A: Human Ethics Approval



Date: 29 April 2022

To: Dr. Peter Lemon

Project ID: 117957

Study Title: The effects of exercise and training status on the counter-regulatory axis of the renin-angiotensin-system.

Application Type: HSREB Initial Application

Review Type: Delegated

Meeting Date / Full Board Reporting Date: 21/Dec/2021

Date Approval Issued: 29/Apr/2022

REB Approval Expiry Date: 29/Apr/2023

Dear Dr. Peter Lemon

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above mentioned study as described in the WREM application form, as of the HSREB Initial Approval Date noted above. This research study is to be conducted by the investigator noted above. **All other required institutional approvals and mandated training must also be obtained prior to the conduct of the study.**

Documents Approved:

| Document Name | Document Type | Document Date | Document Version |
|--|-----------------------------------|---------------|------------------|
| GAQ_CSEPPATHReadinessForm_2pages | Paper Survey | | |
| Data collection sheet - Kyle March , 2022 | Other Data Collection Instruments | 07/Mar/2022 | 3 |
| Email Script - March 7, 2022 | Email Script | 07/Mar/2022 | 3 |
| Exercise and the RAS Protocol - Kyle (March 9, 2022) | Protocol | 09/Mar/2022 | 3 |
| Exercise and the RAS - LOIC - April 7 | Written Consent/Assent | 07/Apr/2022 | 5 |

Documents Acknowledged:

| Document Name | Document Type | Document Date | Document Version |
|------------------------|---------------|---------------|------------------|
| References | References | 13/Jul/2021 | 1 |
| Expenses - Mar 7, 2022 | Study budget | 07/Mar/2022 | 1 |

No deviations from, or changes to, the protocol or WREM application should be initiated without prior written approval of an appropriate amendment from Western HSREB , except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the trial.

REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions. Sincerely,

Patricia Sargeant, Ethics Officer (psargean@uwo.ca) on behalf of Dr. Philip Jones, HSREB Chair

Appendix B: Letter of Information and Consent



Letter of Information

Title of Study:

The effects of exercise and training status on the counter-regulatory axis of the renin-angiotensin system.

Principal Investigator: Peter W.R. Lemon (PhD)

Co-investigator: Kyle Weiman (B.Sc.)

Exercise Nutrition Research Laboratory (Room 414 – Health Science Building)
School of Kinesiology, Western University.

LETTER OF INFORMATION AND CONSENT

Invitation to Participate:

You are being invited to participate in a M.Sc student's research study at Western University's Exercise Nutrition Research Laboratory (Room 414, Health Science Building). We will be investigating the effects of aerobic exercise on the renin-angiotensin system in physically active and sedentary young adults. To complete the study, we are looking for 24 individuals with a variety of physical activity levels. You are being invited to the study because you fit the inclusion criteria of the study and have shown interest.

Purpose of the Letter:

The purpose of this letter is to provide you with the necessary information required for you to make an informed decision regarding participation in this research project.

Purpose of the Study:

The purpose of this study is to determine whether aerobic exercise can increase concentrations of counter-regulatory components of the renin angiotensin system (body hormone system which regulates blood pressure, fluid balance, and blood vessel diameter).

Inclusion Criteria:

To be eligible to participate in this study you must be aged 18-39 years old and physically able to exercise to volitional fatigue. In addition, your physical activity level must fall below 75 minutes a week or above 150 minutes a week.

Exclusion Criteria:

You will be excluded from this study if you:

- Have been diagnosed with a chronic health condition (self-reported)
- Have a confirmed diagnosis with COVID-19
- Have a BMI greater than 30 (self-reported)
- Engage in moderate to vigorous exercise between 75-150 minutes a week
- Are a smoker (self-reported)
- Consume more than 5 alcoholic drinks in a week (self-reported)
- Consume any drugs for recreational purposes (self-reported)
- Are taking any prescriptions including anti-inflammatory medications (self-reported)

Study Outline:

You will be required to do the following:

- Complete a physical activity questionnaire at the beginning of the study to determine the group you will be in. Participants will be divided by biological sex and physical activity level according to the CSEP Get Active questionnaire.
- Arrive to the lab in the morning in a fasted state (at least 8 hours since last meal) and refrain from taking any alcohol, recreational drugs or anti-inflammatory medications or smoked tobacco 48 hours prior.
- Have your body composition measured using non-invasive densitometry (BodPod®). This procedure poses no risks and takes only 5 minutes to complete. We record your body mass on a weigh scale and your body volume via air displacement (requires you to sit quietly in a chamber breathing normally for 45 seconds).
- Maximum oxygen consumption will be recorded during an incremental exercise test to volitional fatigue on a bicycle ergometer. This will require the participant to breathe through a mouthpiece with their nose plugged.
- The incremental exercise test will begin with a 5 minute warm up at 30-100 watts. After the warm up is complete, the intensity on the cycling ergometer will increase 15-30 watts every minute. The test will end once you are unable to continue moving the pedals and the mouthpiece and nose plug will be removed. Participants will then be able to continue cycling at 30-100 watts for a cool down period of 2-3 minutes.
- A small blood sample (10ml) will be taken from a vein in the elbow-forearm area prior to and immediately and 30 minutes after the exercise test.

Possible Risks and Harms:

This study has minimal risks with no lasting effects. Fasting may cause patients to feel sensations of hunger, headaches, fatigue and/or light headedness. Should you feel unwell during any portion of the experiment, we will terminate the session for your safety. Bruising may occur because of the venipunctures performed. The exercise sessions may result in sore muscles and fatigue. None of these are of significant risk and resolve after a few days. There is also a risk of breach of privacy of personal information, but all data will be stored on secure university storage drives.

Potential Benefits:

There are no direct benefits to you for participating.

Compensation:

There is no compensation for participation in this study.

Voluntary Participation:

Participation in this study is voluntary. You may refuse to participate, refuse to answer any questions or withdraw from the study at any time without a penalty of any kind.

Rights of a Participant (in the event of a study related injury):

If you suffer any study related injury during your participation in this study, medical care will be provided for the study participant or we will direct you to Campus Health Services or University Hospital, as appropriate.

Confidentiality:

If you agree to join this study, only members of the study team will look at your personal information (e.g., name, experimental results, etc.) and only the information they need for the study will be collected.

Further, all information that is collected for the study will be coded so you cannot be identified. The master list kept in a secure area (Western One Drive). Only the study team or the people or groups listed below will be allowed to look at your records. Identifiable data will be kept for 7 years, whereas de-identified data will be kept indefinitely. These data will be in coded form only. No personal identifiers will be present. These data may be compared to the results of future similar studies.

Representatives of the University of Western Ontario Health Sciences Research Ethics Board may look at the study records and at your personal health information to check that the information collected for the study is correct and to make sure the study followed proper rules and guidelines.

All information collected during this study will be kept confidential and will not be shared with anyone outside the study unless required by law. You will not be named in any reports, publications, or presentations that may come from this study.

Blood samples that have been collected will be stored for up to one year in the Exercise and Nutrition Laboratory allowing researchers to verify results if necessary. After this time biological samples will be disposed of in accordance to Western University protocol.

If you decide to leave the study at any time, the information about you will not be used to answer the research question without your consent. No new information will be collected without your permission.

Contact for Further Information:

If you have any questions about this research project, feel free to call us (Dr. Peter Lemon or Kyle Weiman) for clarification. Further, if you have any questions about the conduct of this study or your rights as a research participant you may contact the Office of Research Ethics at Western University at 519-661-3036 or at ethics@uwo.ca.

Publication

If the results of the study are published, your name will not be used. If you would like to receive a copy of any potential study results, please contact Kyle Weiman.



Consent Form

The effects of training status and intensity on the counter-regulatory axis of the renin-angiotensin system.

Investigators: Peter W.R. Lemon, (PhD) and Kyle Weiman, (BSc)

I have read the accompanying "Letter of Information", have had the nature of the study explained to me and I agree to participate. All questions have been answered to my satisfaction.

If you wish to participate in future studies in the Exercise Nutrition Research Lab, the research team will collect your contact information.

I wish to be contacted for future studies in the Exercise Nutrition Research Laboratory.

Yes _____ (check mark) No _____ (check mark) Date: _____

By signing below, I agree to participate in this study.

Name of Participant (please print): _____

Signature of Participant: _____

Date: _____

Name of Person Obtaining Informed Consent: _____

Signature of Person Obtaining Informed Consent: _____

Date: _____

You will receive a copy of the consent form after it has been signed. You do not waive any legal rights by signing the consent form. This letter is for you to keep for future reference.

Sincerely,

Peter Lemon, PhD

Kyle Weiman, B.Sc

Appendix C: CSEP Get Active Questionnaire



Get Active Questionnaire

CANADIAN SOCIETY FOR EXERCISE PHYSIOLOGY –
PHYSICAL ACTIVITY TRAINING FOR HEALTH (CSEP-PATH®)

Physical activity improves your physical and mental health. Even small amounts of physical activity are good, and more is better.

For almost everyone, the benefits of physical activity far outweigh any risks. For some individuals, specific advice from a Qualified Exercise Professional (QEP – has post-secondary education in exercise sciences and an advanced certification in the area – see csep.ca/certifications) or health care provider is advisable. This questionnaire is intended for all ages – to help move you along the path to becoming more physically active.

- I am completing this questionnaire for myself.
- I am completing this questionnaire for my child/dependent as parent/guardian.

| YES | NO | PREPARE TO BECOME MORE ACTIVE |
|-----|----|--|
| ✓ | ✓ | The following questions will help to ensure that you have a safe physical activity experience. Please answer YES or NO to each question <u>before</u> you become more physically active. If you are unsure about any question, answer YES . |
| • | • | 1 Have you experienced ANY of the following (A to F) within the past six months ? |
| • | • | A A diagnosis of/treatment for heart disease or stroke, or pain/discomfort/pressure in your chest during activities of daily living or during physical activity? |
| • | • | B A diagnosis of/treatment for high blood pressure (BP), or a resting BP of 160/90 mmHg or higher? |
| • | • | C Dizziness or lightheadedness during physical activity? |
| • | • | D Shortness of breath at rest? |
| • | • | E Loss of consciousness/fainting for any reason? |
| • | • | F Concussion? |
| • | • | 2 Do you currently have pain or swelling in any part of your body (such as from an injury, acute flare-up of arthritis, or back pain) that affects your ability to be physically active? |
| • | • | 3 Has a health care provider told you that you should avoid or modify certain types of physical activity? |
| • | • | 4 Do you have any other medical or physical condition (such as diabetes, cancer, osteoporosis, asthma, spinal cord injury) that may affect your ability to be physically active? |
| • | • |▶ NO to all questions: go to Page 2 – ASSESS YOUR CURRENT PHYSICAL ACTIVITY▶ |
| • | • | YES to any question: go to Reference Document – ADVICE ON WHAT TO DO IF YOU HAVE A YES RESPONSE - - -▶ |

Get Active Questionnaire

ASSESS YOUR CURRENT PHYSICAL ACTIVITY

Answer the following questions to assess how active you are now.

- 1 During a typical week, on how many days do you do moderate- to vigorous-intensity aerobic physical activity (such as brisk walking, cycling or jogging)? DAYS/WEEK
- 2 On days that you do at least moderate-intensity aerobic physical activity (e.g., brisk walking), for how many minutes do you do this activity? MINUTES/DAY
- For adults, please multiply your average number of days/week by the average number of minutes/day: MINUTES/WEEK

Canadian 24-Hour Movement Guidelines recommend that adults accumulate at least 150 minutes of moderate- to vigorous-intensity physical activity per week. For children and youth, at least 60 minutes daily is recommended. Strengthening muscles and bones at least two times per week for adults, and three times per week for children and youth, is also recommended (see csep.ca/guidelines).

GENERAL ADVICE FOR BECOMING MORE ACTIVE

Increase your physical activity gradually so that you have a positive experience. Build physical activities that you enjoy into your day (e.g., take a walk with a friend, ride your bike to school or work) and reduce your sedentary behaviour (e.g., prolonged sitting).

If you want to do **vigorous-intensity physical activity** (i.e., physical activity at an intensity that makes it hard to carry on a conversation), and you do not meet minimum physical activity recommendations noted above, consult a Qualified Exercise Professional (QEP) beforehand. This can help ensure that your physical activity is safe and suitable for your circumstances.

Physical activity is also an important part of a healthy pregnancy.

Delay becoming more active if you are not feeling well because of a temporary illness.

DECLARATION

To the best of my knowledge, all of the information I have supplied on this questionnaire is correct.
 If my health changes, I will complete this questionnaire again.

I answered **NO** to all questions on Page 1

I answered **YES** to any question on Page 1

Sign and date the Declaration below

Check the box below that applies to you:

- I have consulted a health care provider or Qualified Exercise Professional (QEP) who has recommended that I become more physically active.
- I am comfortable with becoming more physically active on my own without consulting a health care provider or QEP.

| | | |
|---|---|----------------------|
| <input type="text"/> | <input type="text"/> | <input type="text"/> |
| Name (+ Name of Parent/Guardian if applicable) [Please print] | Signature (or Signature of Parent/Guardian if applicable) | Date of Birth |
| <input type="text"/> | <input type="text"/> | <input type="text"/> |
| Date | Email (optional) | Telephone (optional) |

With planning and support you can enjoy the benefits of becoming more physically active. A QEP can help.

- Check this box if you would like to consult a QEP about becoming more physically active.
 (This completed questionnaire will help the QEP get to know you and understand your needs.)

Appendix D: Supplementary Tables

Table 3

ANOVA results

| Predictor | df_{Num} | df_{Den} | SS_{Num} | SS_{Den} | F | p | η^2_g |
|------------|------------|------------|------------|------------|------|------|------------|
| Sex | 1 | 10 | 1.04 | 13.38 | 0.78 | .399 | .04 |
| Time | 1 | 10 | 0.01 | 12.62 | 0.01 | .926 | .00 |
| Sex x Time | 1 | 10 | 0.01 | 12.62 | 0.01 | .938 | .00 |

Note: df_{Num} indicates degrees of freedom numerator. df_{Den} indicates degrees of freedom denominator. SS_{Num} indicates sum of squares numerator. SS_{Den} indicates sum of squares denominator. η^2_g indicates generalized eta-squared.

Table 4

Regression results using Angiotensin (1-9) concentration at T1 as the criterion

| Predictor | β | Beta 95% CI [LL, UL] | r | Fit |
|-----------|---------|-------------------------|-----|------------------|
| Activity | 0.42 | [-0.23, 1.06] | .42 | |
| | | | | $R^2 = .172$ |
| | | | | 95% CI [.00,.50] |

Note. β indicates the standardized regression weights. r represents the zero-order correlation. LL and UL indicate the lower and upper limits of a confidence interval, respectively. * indicates $p < .05$. ** indicates $p < .01$.

Table 5

Regression results using Angiotensin (1-9) concentration at T2 as the criterion

| Predictor | <i>beta</i> | Beta 95% CI [LL, UL] | <i>r</i> | Fit |
|-----------|-------------|-------------------------|----------|-------------------|
| Activity | 0.71 | [0.22, 1.21] | .71** | |
| | | | | $R^2 = .511^{**}$ |
| | | | | 95% CI [.05,.72] |

Note. *beta* indicates the standardized regression weights. *r* represents the zero-order correlation. *LL* and *UL* indicate the lower and upper limits of a confidence interval, respectively. * indicates $p < .05$. ** indicates $p < .01$.

Table 6

Regression results using Angiotensin (1-9) concentration at T3 as the criterion

| Predictor | <i>beta</i> | Beta 95% CI [LL, UL] | <i>r</i> | Fit |
|-----------|-------------|-------------------------|----------|------------------|
| Activity | -0.03 | [-0.73, 0.68] | -0.03 | |
| | | | | $R^2 = .001$ |
| | | | | 95% CI [.00,.15] |

Note. *beta* indicates the standardized regression weights. *r* represents the zero-order correlation. *LL* and *UL* indicate the lower and upper limits of a confidence interval, respectively. * indicates $p < .05$. ** indicates $p < .01$.

Curriculum Vitae

Name: Kyle Weiman

Post-secondary Education and Degrees: University of Saskatchewan
Saskatoon, Saskatchewan, Canada
2007-2013 B.Sc.

University of Alberta
Edmonton, Alberta, Canada
2015-2017 B.Ed.

The University of Western Ontario
London, Ontario, Canada
2020-2023 M.Sc.

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2020-2023

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Publications:

Jin, J., Weiman, K., Bremault-Phillips, S., & Vermetten, E. (2022). Moral Injury and Recovery in Uniformed Professionals: Lessons From Conversations Among International Students and Experts. *Frontiers in psychiatry, 13*, 880442. <https://doi.org/10.3389/fpsy.2022.880442>

Hamilton, T., Burbach, L., Smith-MacDonald, L., Jones, C., Brown, M. R. G., Mikolas, C., Tang, E., O'Toole, K., Vergis, P., Merino, A., Weiman, K., Vermetten, E. H. G. J. M., & Brémault-Phillips, S. (2021). Moving Toward and Through Trauma: Participant Experiences of Multi-Modal Motion-Assisted Memory Desensitization and Reconsolidation (3MDR). *Frontiers in psychiatry, 12*, 779829. <https://doi.org/10.3389/fpsy.2021.779829>