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The effect of oral glutamine supplementation on gut permeability and heat shock protein regulation in runners with a history of gastrointestinal distress

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THE EFFECTS OF ORAL GLUTAMINE SUPPLEMENTATION ON GUT PERMEABILITY AND HEAT SHOCK PROTEIN REGULATION AMONG RUNNERS WITH A HISTORY OF GASTROINTESTINAL DISTRESS

BY

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The Effect of Oral Glutamine Supplementation on Gut Permeability and Heat Shock Protein Regulation in Runners with a History of Gastrointestinal Distress

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ABSTRACT

Gastrointestinal (GI) permeability increases during high intensity exercise leading to endotoxin leakage, and a pro-inflammatory immune response. The purpose of this study are to assess whether oral glutamine supplementation (1) reduces exercise induced permeability through up-regulation of the heat shock response resulting in occludin stabilization, and (2) depresses the exercise induced inflammatory response. **Methods.** Eight human subjects (n=8) participated in baseline (PRE) testing, a glutamine (GLN), and placebo (PLA) supplementation trial in a double blind design. After PRE measurements, subjects ingested .9g/kg fat free mass of glutamine per day or a sugar free lemon placebo drink for seven days with a one-month washout period between trials. A 60-min treadmill run at 70% of maximal oxygen consumption was performed at 30°C in an environmental chamber at the end of each supplementation period. Intestinal

permeability was assessed at rest and during each trial through urine concentrations of lactulose and rhamnose. Plasma glutamine, plasma endotoxin, and peripheral blood mononuclear cell levels of heat shock protein 70 (HSP70), and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-α) were measured pre-exercise, post-exercise, 2hr post-exercise, and 4hr post-exercise. Cultured caco-2 human intestinal epithelial cells supplemented with three concentrations of GLN (0, 4, and 6mmol/L) were exposed to heat stress (41ºC) to simulate exercise and control (37ºC) conditions. HSP70, heat shock factor 1 (HSF-1), and occludin were measured from each culture. **Results:** Core temperature was not different between exercise trials (39.40 ± .39 vs. 39.54 ± .22 for PLA vs. GLN, respectively, $p>0.05$). Resting plasma glutamine levels were significantly higher in the GLN trial versus PLA (1.893 ± 0.245mmol.L vs. 0.8285 ± 0.078 mmol.L, $p<0.05$). Permeability as the ratio of lactulose to rhamnose was significantly higher in the PLA trial when compared to PRE (.0604 ± .0470 vs. .0218 ±.0084, respectively, $p<0.05$). Permeability was not statistically different between GLN trial and PRE (.0272 ± .0074 vs. .0218 ± .0084, respectively, $p>0.05$). PBMC expression of IκB-α and HSP70 were higher at the 4hr post-exercise time point in the GLN trial when compared to the 4hr mark in the PLA (.9839 ± .1587 vs. 1.520 ±.2294 and 2.083 ± .6712 vs. 2.895 ± .8444, $p<0.05$ for IKB-α and HSP70, respectively). Plasma endotoxin was higher compared to pre-exercise at the 2hr post-exercise in the PLA trial (2.883 ± 0.4310 pg.ml vs. 4.550 ± 0.3350 pg.ml, $p<0.05$ respectively) and significantly higher when compared to the 2hr post-exercise mark in GLN trial (4.550 ± 0.3350 pg/ml vs. 2.883 ± .4314 pg/ml, $p<0.05$). Results of cell culture: HSP70 expression in Caco-2 cells was higher in the 6mmol 41ºC trial when compared to the 0mmol 41ºC trial (1.973 ±

0.163 vs. 1.133 ± 0.064, $p<0.05$, respectively). HSF-1 was higher in the 4mmol 41 ºC and the 6mmol 41ºC trials when compared to the 0mmol 41ºC (1.649 ± 0.185, 1.785 ± 0.185 vs. 0.6681 ± 0.145, $p<0.05$). Occludin levels were statistically lower in the 0mmol 41ºC when compared to 0mmol 37ºC representing de-stabilization of the TJ protein in response to heat stress (0.7434 ± 0.015 ± 1.0000 ± 0.000, $p<0.05$, respectively). Occludin levels during both 4mmol 41ºC and 6mmol 41ºC trials were statistically higher when compared to 0mmol 41ºC (1.236 ± 0.143 and 1.849 ± 0.143 vs. 0.7434 ± 0.015, $p=0.032$, $p<0.001$, respectively) **Conclusion:** Seven days of oral glutamine supplementation prevents exercise induced intestinal permeability and endotoxin leakage possibly through HSF-1 and HSP70 activation leading to occludin stabilization at the tight junction. In addition, glutamine suppressed the inflammatory response to high intensity exercise through activation of HSP70, reduced IKB-α degradation and possible NFκ-B inhibition.

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SYMBOLS / ABBREVIATIONS

≥: greater than or equal to

>: greater than

≤: less than or equal to

<: less than

±: plus or minus

~: approximately

°C: degrees Celsius

µg: microgram

ml: milliliter

µg/ml: microgram per milliliter

µl: microliter

µmol: micromole

ANOVA: analysis of variance

ATP: adenosine triphosphate

BF%: body fat percentage

bpm: beats per minute

BW: body weight

CD14: cluster of differentiation 14

cm: centimeters

DAG: diacylglycerol

dH_2O: distilled water

EIA: colorimetric enzyme immunoassay

ELISA: enzyme-linked immunosorbent assay

End: endotoxin

FFM: fat free mass

g: gram

g/d: grams per day

GI: gastrointestinal tract

GLN: glutamine

g/kg: grams per kilogram

H_2O: water

HK: hexokinase

HR: heart rate

HRP conjugate: horseradish peroxidase conjugate

HSF-1: heat shock factor-1

HSP: heat shock protein

HSP70: heat shock protein 70

HSP72: heat shock protein 72

Ig: immunoglobin

IKB-α: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IP: intestinal permeability

kg: kilogram

Lac: lactulose

LAL: limulus amebocyte assay

LPS: lipopolysaccharide

M: molar

Mg: milligram

MLC: myosin light chain

MLCK: myosin light chain kinase

mM: millimolar

n: number of subjects

Na: sodium

NaCl: sodium chloride

NAD: nicotinamide adenine dinucleotide

NADP: nicotinamide adenine dinucleotide phosphate

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered saline

PGI: phosphoglucose isomerase

PLA: placebo

PMSF: phenylmethylsulfonyl fluoride

r^2: coefficient of determination

RM ANOVA: repeated measures analysis of variance

RPE: rating of perceived exertion

SD: standard deviation

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SE: standard error

TEA: triethanolamine

Tre: rectal temperature

TNFα: tumor necrosis factor alpha

Tris: tris (hydroxymethyl) aminomethane

VO_2 max: maximal oxygen consumption

VO_2 peak: peak oxygen consumption

CHAPTER 1

Introduction

Gastrointestinal distress is common among endurance athletes with 60% reporting symptoms during competition or training [1, 2]. Commonly reported symptoms include diarrhea, vomiting, nausea, bloating, heart-burn, and cramping [2]. Runners appear to suffer predominately lower GI discomfort where cyclists suffer both upper and lower GI symptoms. Many athletes report distress during their event and up to several hours afterwards [3]. The cause of GI distress is multiplex, where factors that may play roles include dehydration, nutrition, exercise timing, core temperature, and exercise intensity. Our study will focus on GI distress related to elevated core temperature and high intensity exercise in complementary human and cell culture models, and we will determine if oral glutamine supplementation prevents GI dysfunction.

Prolonged high intensity exercise has the potential to induce severe stress to the GI tract. Sixty-minutes of running at 80% of VO2max has been shown to increase gut permeability, which is the breakdown of the epithelial tight junctions [4]. Oktedalen et al (1992) reported gastric erosion, bleeding, and intestinal permeability among long distance runners after competition [5]. Additionally, athletes that have a history of exercise induce GI distress demonstrate greater permeability [6]. Several theories for the cause of exercise induced gastrointestinal permeability have been proposed, and include ischemic injury and high core temperature [7]. This study will address the core temperature theory and be the focus of the investigation.

Direct effect of high temperature theory

When core temperature approaches 40 degrees centigrade in exercising humans the splanchnic temperature may rise to 42 degrees, or approximately two degrees higher than core temperature [8]. The hemodynamic shift to the cutaneous vascular bed for heat dissipation may contribute to the added rise in splanchnic temperature as blood flow can be reduced by 50-60% [8]. A positive relationship has been shown between intestinal permeability and exercise-induced elevation in core temperature, with a threshold of approximately 39 degrees [4]. Rat intestines exposed to heat stress demonstrate membrane breakdown and loss of barrier integrity [9]. In addition, intestinal cells that are exposed physiological temperatures as low as 38.5 degrees show disruption of villi, and greater protein breakdown [10, 11]. Heat-stroke and gut permeability have been correlated with gut derived LPS permeating into the blood stream, and this effect was ameliorated in primates when given gut cleansing antibiotics [12].

Effect on tight junctions

Tight junctions (TJ) form a continuous belt-like ring around epithelial cells at the border between the lateral and apical membranes. The TJs are a complex of proteins that include occludins, claudins, zonula occludens (ZO), and several regulatory proteins that function as a selective paracellular barrier. Ions, solutes, and water move through the TJs while the translocation of luminal antigens and toxins are prevented. The proteins of the TJ are regulated by their phosphorylation state, which can promote barrier stability, or TJ dispersion and complex destabilization [13-15]. Under conditions of heat stress the

protein complex of the tight junction is damaged leading to increased permeability of toxins.

The mechanism for heat induced tight junction permeability is through sloughing of the epithelium off the basement membrane and disruption to the microvilli [10, 16]. In addition heat stress may activate diacylglycerol (DAG) and protein kinase C (PKC), which phosphorylates the occludin protein causing it to move from the tight junction [17]. Mice that reach a core temperature of 41 degrees, and intestinal cells that are exposed to greater than 39 degrees show increased permeability and occludin breakdown [10, 11].

Consequences for the increased gut permeability

Exercise induced heat stress leads to tight junction damage resulting in barrier dysfunction and increased translocation of intestinal endotoxins [18]. Endotoxins are gram negative bacteria that are recognized by the immune system. The most notably is lipopolysaccharide, which is a polysaccharide with a functional lipid (lipid A) that is responsible for the toxic effect. Once LPS moves into the blood stream is binds to lipopolysaccharide binding protein (LBP) that is present in the serum [19]. The lipid A then attaches to the co-receptors CD14 and TLR4 receptors on the innate immune cells leukocytes and macrophages [19]. This causes a cascade of events in immune cells leading to the degradation of IκB-α, translocation of NFκ-B, and pro-inflammatory cytokine synthesis [19, 20]. Pro-inflammatory cytokines have been associated with diarrhea, nausea, vomiting, and flatulence (3).

Endurance athletes who reach a core temperature of 40 degrees during exercise show greater permeability [4], elevated LPS leakage [21], endotoxemia, and NFκ-B activation of peripheral blood mononuclear cells (PBMC) [22]. In addition, marathon runners have shown elevated pro-inflammatory cytokine levels post race [3].

In summary, the stress of exercise in the heat leads to tight junction protein breakdown increasing the permeability of the intestinal epithelial cells to endotoxin. The endotoxin attaches the CD14 and TLR4 receptors on immune cells activating NFκ-B and pro-inflammatory cytokine synthesis and release.

Protective role of HSP

The heat shock protein is a family of proteins that are intracellular molecular chaperones that assist in protein synthesis and cell maintenance [23]. Increased levels of intracellular HSP provides protection to the cell under stressful conditions. During stress heat shock factor one (HSF-1) releases the HSP, which binds to damage proteins. In addition, HSF-1 moves into the nucleus of the cell, and triggers the transcription of additional HSPs, and most notably HSP 72 [24]. In the gastrointestinal tract, an increased level of HSP 72 up-regulates the expression of the tight junction protein occludin leading to lower permeability [11]. HSP 72 has also been shown to reduce the phosphorylation of the MLC actin cytoskeleton by inhibiting the activation of PKC leading to increased barrier function [17]. In addition, HSP 72 inhibits NFκ-B translocation in rat liver epithelial cells, murine liver cells, and human PBMCs in response to LPS induced injury [25, 26].

Intracellular HSP response in PBMCs of athletes are up-regulated after a competitive endurance event [27, 28], and trained athletes show reduce endotoxins and NF-kappa b activation in response to exercise and heat stress [22]. Heat shock proteins have also been associated with lower gut permeability during exercise in humans [29, 30]. Elevating heat shock proteins in response to stress may provide a protective mechanism against exercise induced GI distress.

Recently, researchers have attempted to increase HSP levels through supplementation [29, 31, 32]. Marchbank et al (2010) demonstrated that when subjects ingest bovine colostrum for 14 days they have reduced exercise induced permeability. In a cell model, bovine colostrum induced HSP70 expression [29]. Polaprezinc is an anti-ulcer drug that contains zinc, which is commonly prescribed in Japan, and has been shown to increase HSP 27 and HSP 72 synthesis in intestinal cells [32].

Ability of glutamine to up-regulate HSP

Glutamine is the most abundant amino acid in the human body, and provides protection to many tissues in situations of stress [33-36]. It is the main energy source for immune cells and during illness glutamine levels decline [33, 34]. Glutamine is commonly given intravenously to critically ill patients and burn victims where it has been shown to reduce mortality rates. In addition, it has been used as treatment for irritable bowel and Crohn's disease, which are chronic inflammatory diseases [37]. Glutamine has been given to athletes to boost their immune system to protect against upper respiratory tract infections, and it may help muscle recovery from resistance training [38].

Protective effects of glutamine may be through increasing HSP synthesis in tissue [39-41]. Oral glutamine supplementation in rats has been shown to increase HSP 70 expression in the gut and lower permeability at six and twenty-four hours post heat exposure [40]. This mechanism may be through glutamine activation of heat shock factor one (HSF-1) and specialty protein one (Sp1) [40, 42]. Andreason et al (2009) administered intravenous glutamine in male subjects for 10 hours followed by a bolus of E Coli endotoxin [31], and demonstrated an increase in HSP 72, but the results were not statistically significant. Greater power through increased subject number may have resulted in a statistical difference. This demonstrates that glutamine supplementation may enhance the heat shock protein stress response, which may improve the intestinal barrier and prevent endotoxin leakage. However, this has never been tested in humans.

Study purpose and hypotheses

The purpose of this study is to determine whether seven days of oral glutamine supplementation reduces exercise induced gastrointestinal permeability among a population of endurance athletes who report frequent exercise-induced GI distress. The proposed mechanism is through glutamine mediated increase in HSP70 resulting in a maintained GI barrier, and protection from endotoxemia, and PBMC activation of NFκ-B. An in vitro human intestinal cell model will be used to test the proposed mechanism of glutamine mediated increase in HSF-1 and HSP 70.

Purposes of this Study

1. Demonstrate that high intensity exercise in the heat leads to an elevation in gut permeability, endotoxin, and PBMC NFκ-B levels in susceptible runners.
2. Glutamine supplementation decreases gut permeability, LPS, and NFκ-B levels, along with upregulating the intracellular HSP70 stress response in PBMCs.
3. Glutamine increases HSP 70 through the up regulation HSF-1 in human intestinal cells leading to occludin stabilization in response to heat stress

Hypotheses

In this study we will test the following hypotheses:

1. Oral glutamine supplementation will reduce, or blunt the increase in gut permeability, endotoxin levels, and NFκ-B activation in PBMCs
   In heat stressed rats oral glutamine supplementation has been shown to protect the gut barrier by lowering permeability [40], but has never been tested in humans. A reduction in permeability in humans should reduce endotoxin levels in the blood, and NFκ-B activation in PBMCs [22].

2. Glutamine supplementation will increase HSP 70 levels in human peripheral blood mononuclear cells at baseline and in response to high intensity exercise.

Glutamine is the major nutrient for leukocytes, and may improve immune function during stress by enhancing heat shock protein levels. Intravenous glutamine has been shown increase HSP 70 in critically ill patients and in healthy male subjects when given an E. Coli bolus [31, 39]. The effect of oral glutamine on HSP 70 response has not been tested, and furthermore it has never been tested after high intensity exercise.

3. Glutamine supplementation will increase HSF-1 and HSP 70 levels in human intestinal Caco-2 cells in response to heat stress.

   Glutamine has been shown to increase HSF-1, which mediates HSP 70 levels in rat intestinal cells in response to heat stress [40, 42]. Conversely, Ropeleski et al (2005) demonstrated that HSP 70 up-regulation in response to glutamine is not dependent on HSF-1 activity [43]. HSP 70 has been shown to be higher in Caco-2 cells supplemented with glutamine, but HSF-1 was not measured [44]. This will demonstrate glutamine mediated HSP 70 increase is through HSF-1.

4. Glutamine supplementation will prevent heat induced occludin de-stabilization.

   Increasing HSF 1 levels has been shown to reduced permeability in Caco-2 cells through occludin up-regulation [11]. If glutamine increases intestinal HSF-1 levels then greater occludin expression will be expected.

Scope of the study

Human Experiment

Eight total adult male and female endurance athletes who are susceptible to exercise induced gastrointestinal distress will perform two sixty-minute high intensity exercise bouts in a warm environment. The bouts will be separated by 7-days of glutamine or placebo supplementation. A double blind within subject design will be used with a 4-week washout period between trials. Selection criteria will include no known GI disease, reported symptoms on at least five occasions, and currently performing endurance training. Subjects will be instructed to avoid vitamins, alcohol, and changes in diet and exercise routine during the supplementation trial.

A glutamine or placebo bag, which will contain 7-days of supplement will be given to each subject after the first exercise trial. Subjects will consume .9g/kg fat free mass per day of glutamine or placebo, which is the highest reported intake in young healthy adults without adverse effects [38]. The total quantity per day will be separated into three doses taken in the morning, afternoon, and evening.

The exercise trial will be 60-minutes of treadmill running at 70-80% of VO2max in warm environment. Rectal probes will be used to measure exercise core temperature with a cutoff of 40 degrees during each trial. Blood samples will be taken at several time points that will include: pre-exercise, post-exercise, 2-hours post, and 4-hours post. Endotoxin will be measured in the plasma, and heat shock protein 70 and I-kappa B alpha, a marker of NFκ-B activation, will be measured in the cytosol of PBMCs. In addition, thirty-minutes into exercise subjects will consume a 50ml solution containing the sugar probes lactulose (5g) and rhamnose (2g), which will be used to measure gut

permeability. Urine will be collected every hour for five hours post exercise, and permeability will be determined by the ratio of lactulose to rhamnose.

Caco-2 cell experiment

A line of human carcinoma intestinal epithelial cells (Caco-2) will be cultured, supplemented with glutamine or placebo, and exposed to heat stress. The temperature will be physiologically relevant and range from 39-41 degrees. After heat exposure the cells will be collected, lysed, and tested for HSF-1, HSP70, and occludin.

Limitations

In this study we will measure the effects of glutamine on gastrointestinal distress to test the hypothesis that improvement from glutamine ingestion is through HSP up-regulation. Glutamine is the most abundant amino acid in the body and is important for immune cell health. The gastrointestinal protection from glutamine may not be through HSP, but we will not be able to measure other potential mechanisms in this study.

In the human experiment it will not be possible to measure HSP responses in the intestinal epithelial cells during exercise, and therefore we will use PBMCs and cultured Caco-2 cells to test the effect of glutamine on HSP. The PBMCs will provide an indirect marker of HSP expression, and may not represent up-regulation in the small intestine. Caco-2 is a line of human intestinal carcinoma epithelial cells, which may respond differently to glutamine supplementation than healthy human intestinal cells. The measurements in both PBMCs and Caco-2 cells will be a limitation.

Exercise induced gut permeability has been demonstrated in several studies [4, 29, 30, 45, 46], but has not been consistently shown. It will be important that this occurs during the exercise trial in our study. Research has shown that high intensity exercise and a core temperature greater than 39 degrees are associated with increased gut permeability [4, 29]. For this reason, subjects will run at 70 – 80% of VO2max in a warm environment. In addition, we will use subjects who have a history of exercise induced GI distress. If gut permeability is not demonstrated then this will be a limitation of the study.

Permeability will be measured by quantifying the levels of sugar probes in urine. Several techniques have been used to accurately measure human intestinal permeability, with the most consistent results reported use the lactulose and rhamnose method [4, 29]. In addition, the timing of the probe consumption will be important. If an inappropriate method is chosen this will result in inaccurate permeability measurements and will be a limitation.

We will be testing endurance trained athletes that have a history of GI distress. This population may have blunted HSP responses. Also, subjects may have lower than normal levels of glutamine due to overtraining. These athletes may also suffer from undiagnosed gastrointestinal disorders such as irritable bowel syndrome, or inflammatory bowel disease, or they may already have up regulated HSP.

The study design will be within-subject where each participant will serve as their own control. It will be important that an appropriate washout period is utilized and that the experimental exercise trial does not have a carry-over effect. Subjects will maintain

their current training routine during the supplementation trial, and if they decide to stop training, change their program, or skip a supplement dosage than results could be affected.

Significance of the Study

The prevention of gastrointestinal symptoms during exercise will provide enormous benefit to athletes that compete in endurance exercise events. Over 240,000 people competed in the six largest marathon events in the world in 2010. According to previous reported research that 50-60% of athletes suffers GI distress [1], then 140,000 of these runners experienced one of many symptoms. Supplementing with glutamine seven days prior to competition may reduce the occurrence of GI distress by lowering gut permeability through increased HSP synthesis.

Definitions

Peripheral blood mononucleated cells (PBMC) – A blood cell that has a round nucleus, and includes lymphocytes and monocytes. These cells are important in immune function and contain the T Cells, NK Cells, and B cells.

Nuclear factor kappa beta (NFκ-B) – A nuclear factor that controls the transcription of DNA, and regulates immunological and inflammatory processes.

Endotoxin – toxin that is associated with gram negative bacteria, and is recognized by the immune system.

Lipopolysaccharide (LPS) – a gram negative endotoxin that consists of a polysaccharide and a lipid.

Gut permeability – the paracellular movement of fluid and bacteria from the intestinal lumen into the circulation.

Tight junction protein – a branching network of proteins that connect adjacent epithelial cells, and regulates permeability of fluids.

Core temperature – human body temperature that will be measured using a thermistor probe placed 8-cm beyond the anal sphincter.

Percentage of VO2max – the measurement used to determine the exercise intensity. A VO2max test must be performed to determine this value.

Caco-2 cells – A line of human carcinoma intestinal epithelial cells

Glutamine – the most abundant amino acid in the body

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CHAPTER 2

This chapter presents a review article, entitled “Exercise Regulation of Tight Junction Proteins” which has been accepted for publication by *The British Journal of Sports Medicine*. It is authored by Micah Zuhl, Suzanne Schneider, Kathryn Lanphere, Carole Conn, Karol Dokladny, and Pope Moseley. The manuscript follows the formatting guidelines of the journal. Figures and references are provided at the end of manuscript.

Exercise regulation of intestinal tight junction proteins

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ABSTRACT

Gastrointestinal distress, such as diarrhea, cramping, vomiting, nausea, and gastric pain are common among athletes during training and competition. The mechanisms that cause these symptoms are not fully understood. The stress of heat and oxidative damage during exercise causes disruption to intestinal epithelial cell tight junction proteins resulting in increased permeability to luminal endotoxins. The endotoxin moves into the blood stream leading to a systemic immune response. Tight junction integrity is altered by the phosphoylation state of the proteins occludin and claudins, and may be regulated by the type of exercise performed. Prolonged exercise and high intensity exercise lead to an increase in key phosphorylation enzymes that ultimately cause tight junction dysfunction, but the mechanisms are different. The purpose of this review is to (1) explain the function and physiology of tight junction regulation, (2) discuss the effects of prolonged and high intensity exercise on tight junction permeability leading to GI distress, (4) review agents that may increase or decrease tight junction integrity during exercise.

INTRODUCTION

The intestine is the primary organ for absorption of fluids, nutrients, and electrolytes. The mucosal layer of the intestinal tract is made up of epithelial cells, enterocytes, which are connected to one another by tight junctions consisting of specialized proteins such as occludin, zona-occludens (ZO-1, ZO-2, ZO-3), and claudins.[1, 2] Both tight junctions and the apical membrane of enterocytes constitute the intestinal barrier,[3] which allows absorption of nutrients and water [4] while also preventing the translocation of harmful substances from the gut to the bloodstream.[1] The integrity of the intestinal barrier is regulated by the phosphorylation state of the tight junction proteins where the type of kinase and binding site play a role.[5, 6]

During prolonged exercise that increases core temperature, cardiovascular and thermoregulatory responses compromise intestinal blood flow. As core temperature approaches 39 degrees, intestinal temperature can be as high as 41 degrees [7] leading to epithelial cell damage.[8] In addition, high intensity exercise redirects blood flow away from the splanchnic arteries and to the working muscle leading to an ischemia reperfusion cycle where blood flow returns when exercise intensity is lowered [9], and may result in oxidative damage.[10, 11] Both heat and ischemic/reperfusion stress can influence the phosphorylation state of tight junction proteins resulting in increased permeability,[12, 13] endotoxin leakage, and the provoking of a systemic inflammatory response.[14] These mechanisms may contribute to the high prevalence of gastrointestinal distress reported among endurance athletes, where 60-90% report symptoms, including diarrhea, nausea, stomach problems, bloating, and intestinal

cramps.[15-17] Moseley and Gisolfi in 1993[18] introduced the heat and oxidative pathway leading to gut permeability and endotoxin leakage, and this pathway was further developed by Lambert 2009 [8, 19-21] and additional contributions from Hall [22, 23] However, the underlying molecular mechanisms were not discussed. Therefore, we will build upon the Moseley/Gisolfi model by discussing the mechanisms that regulate the phosphorylation of tight junction proteins dependent on the type of exercise (short high intensity vs. long duration), and the protective effects of intracellular heat shock proteins (HSP). In addition, medications (NSAIDS), or dietary supplements that increase (quercetin) or decrease (glutamine, bovine colostrum) intestinal permeability will be discussed.

TIGHT JUNCTION FUNCTION AND PROTEIN COMPONENTS

Intestinal epithelial tight junctions (TJ) are multiprotein complexes that connect adjacent cells on the apical and lateral membranes forming an extracellular border around the cell (figure 1).[3] The TJs serve as a selective barrier, and regulate bi-directional paracellular movement of ions, water, and other nutrients while providing protection against leakage of luminal toxins into the circulation.[3, 24] Activation of Na+/glucose transporters in response to feeding increases tight junction permeability allowing nutrient absorption.[25-27] An increase in intestinal volume that leads to pressure greater than 4cm H2O enhances paracellular permeability and greater fluid absorption.[28] Conditions of intense physical stress, such as exercise, cause tight junction dysfunction leading to enhanced permeability allowing translocation of luminal toxins into the blood stream.[29-32]

Tight junction integrity is regulated by the assembly of the extracellular loops of the transmembrane proteins occludin and claudins, whereupon aggregation of these proteins at the site of the TJ increases barrier resistance.[33] The intracellular plaque proteins zona occludens (ZO-1, ZO-2, ZO-3) and PDZ link both occludin and claudins to the actin cytoskeleton,[34, 35] which is the transmembrane protein that upon activation shortens, or "contracts" the epithelial cell.[36] Shortening of cytoskeleton is regulated by the state of phosphorylation through binding of myosin light chain kinase (MLCK), and myosin light chain phosphatase (MLCP).[36-39] Similar to vascular smooth muscle contraction, MLCK phosphorylates the myosin light chain of the epithelial actomyosin protein causing shortening and opening of the tight junction, while MLCP dephosphorylates the actomyosin protein leading to closure of the TJ junction.[37] A severe stimulus such as hyperthermia or ischemia will disrupt the interaction between the tight junction proteins zona occluden, occludin, and claudins. The actin cytoskeleton is connected to the TJ proteins via the zona occludens (ZO-1, ZO-2, ZO-3), and when disruption occurs the overall effect is reduced actin cytoskeleton regulation.

REGULATION OF TIGHT JUNCTION PROTEINS

Occludin and claudins (claudin-1, claudin-2, claudin-3) are tetraspanning membrane proteins with 2 extracellular loops, and cytoplasmic N-, and C-terminal domains (Figure 2).[40-42] The extracellular components form a barrier with adjacent epithelial cells, and regulate paracellular permeability. The C-terminal domain is the main site for interaction with the zona-occludens and PDZ proteins, and is required for the assembly at the tight junction.[5, 43]

Initially occludin was thought to be the primary protein responsible in forming the tight junction, as over expression resulted in greater tight junction resistance.[44, 45] However, occludin knock-out mice show normal tight junction resistance, and barrier formation.[46] This has led to the conclusion that occludin is not vital for tight junction formation, but has a regulatory role in TJ assembly. Occludin is required in the ZO-1 and actomyosin cytoskeleton interaction, and through signaling molecules mediates the maintenance of intact TJ complexes, and barrier function.[24, 47, 48] The claudin proteins (claudin-1, claudin-2, claudin-3) are considered to be the primary seal forming protein, and have the ability to polymerize into linear fibrils, which is in contrast to occludin.[39, 49, 50] Overexpression of claudin results in greater tight junction resistance and claudin knock-out mice die within one day of birth.[50]

Both occludin and claudin formation at the tight junction are regulated through phosphorylation by several proteins, including different isoenzyme forms of protein kinase C (PKC),[51] protein kinase A (PKA),[52, 53] tyrosine kinase,[54, 55] MAPK,[6] and several more (figure 3).[6] Occludin phosphorylation by conventional PKC (cPKC) and tyrosine kinases has been shown to decrease tight junction assembly [51, 54, 55] while phosphorylation by novel PKC (nPKC) improves tight junction resistance (Figure 3).[47, 51] The mechanism is through regulating the interaction of occludin with ZO-1, which is required for tight junction formation. Claudin phosphorylation by nPKC promotes fibril formation and tight junction assembly [56] while PKA has opposite effects (Figure 3).[52] Similar to occludin, the phosphorylation state regulates the claudin and ZO-1, ZO-2, and ZO-3 interaction. Research on the interactions between

claudin and occludin is limited, but claudin-1 has been shown to be bound to occludin during tight junction assembly.[57, 58]

HEAT AND LONG DURATION EXERCISE EFFECT ON TJ PROTEINS

Long duration exercise, or exercise in hot environment often results in an increase in core temperature above 39 degrees.[59] To defend core temperature blood flow is diverted from the splanchnic and renal arteries, to the cutaneous vascular bed to increase body heat loss. As core temperature rises intestinal wall temperature also rises, and may be slightly greater than core temp.[7] The reduction in blood flow may be the reason for heightened intestinal wall temperature as heat is not being removed due to vasoconstriction of the splanchnic arteries. Hyperthermia (>40 degree) has been shown to damage intestinal epithelial cells causing cell sloughing, shrinking of the villi, edema, and massive bleeding.[60, 61] Intestinal permeability is increased in runners and cultured human intestinal epithelial cells at temperatures above 39 degrees.[29, 62] The increase in tight junction permeability leads to the translocation of lipopolysaccharide (LPS) into the blood circulation, where it attaches to lymphocyte TLR4, and CD14 receptors, triggering the release of pro-inflammatory cytokines such as TNF-alpha, IFN-alpha, IL-1beta, or IL-6 .[63] TNF-alpha has been shown to damage the Na/K pump on the basolateral membrane of intestinal epithelial cells, resulting in reduced water absorption, fluid secretion from the vasculature into the lumen, and diarrhea.[64] Inflammatory cytokines have been positively correlated with nausea, vomiting, diarrhea, and abdominal cramping among endurance athletes during competition.[16]

Heat stress may increase tight junction permeability through activation of protein kinases resulting in phosphorylation of TJ proteins thus decreasing the interaction of occludin and claudin with the zona-occludins (figure 4).[62, 65] Heat activates phosphorylation enzymes tyrosine kinase and cPKC in epithelial cells, causing a decrease in tight junction resistance.[10, 43, 57] In summary, heat induced intestinal permeability during exercise may be mediated by TJ phosphorylation by several key protein kinases.

Phosphorylation of TJ proteins, and their disassembly may be a result of endotoxin leakage and activation of pro-inflammatory cytokines during heat stress. Physical damage to the intestinal epithelial cells under hyperthermia conditions may cause the increase in intestinal permeability, endotoxin leakage, and the cascade of immune responses (Figure 5). IFN-gamma and TNF-alpha synthesis have been shown to mediate actin cytoskeleton contraction, and tight junction opening through the activation of MLCK, and phosphorylation of MLC.[38, 66] Injection of TNF-alpha into the intestines of rats activates cPKC resulting in tight junction breakdown, inhibition of the Na/K exchanger, and diarrhea.[64] Whether the phosphorylation of TJ proteins, and the loss of barrier integrity is a direct, or indirect result of heat stress is not known.

ISCHEMIC STRESS AND HIGH INTENSITY EXERCISE EFFECT ON TJ PROTEINS

Intestinal ischemia can occur in as little as 10 minutes of high intensity exercise as measured by gastric tonometry.[9, 67] Van Wijk et al 2010 [9] showed splanchnic hypo-perfusion twenty minutes into a 60 minute bout of cycling at 70% VO2max, and complete reperfusion took place within the first 10 minutes of recovery. Intestinal hypo-

perfusion causes a rapid breakdown of ATP to AMP activating hypoxanthine, and during the reperfusion cycle hypoxanthine is reduced to xanthine by the calcium activated enzyme xanthine oxidase.[12] The increase in calcium may be a result of calcium pump dysfunction during ischemia.[12] The xanthine oxidase reaction then releases hydrogen peroxide, a potent free radical, which causes tissue breakdown, and disruption of tight junction proteins.[12, 68] Hydrogen peroxide levels increase in response to heavy aerobic exercise when measured indirectly through catalase levels and the ratio of glutathione to oxidized glutathione,[69] where both catalyze the breakdown of hydrogen peroxide.

Intestinal permeability is elevated among athletes during high intensity exercise and permeability correlates with markers of oxidant damage.[9] In addition, intestinal permeability increases during exercise among patients with peripheral vascular disease, which is a result of ischemia in peripheral tissue, such as the intestinal tract.[70] The mechanism may be through hydrogen peroxide induced tyrosine phosphorylation of occludin by the tyrosine kinase c-Src causing translocation of occludin into the intracellular membrane and reducing the ZO-1 interaction (Figure 6).[55, 71] In addition, tyrosine kinase inhibition restores tight junction resistance.[72] Hypoxic exposure to epithelial cells has been shown to activate an atypical isoezyme of PKC leading to occludin phosphorylation, and loss of tight junction integrity, but claudin levels were not affected.[73] In summary, high intensity exercise causes intestinal ischemia increasing the production of hydrogen peroxide, which activates protein kinases that phosphorylate TJ proteins leading to hyperpermeability.

Hydrogen peroxide production from ischemic stress has also been shown to activate epithelial cell nuclear factor kappa beta (NF-kappa B), which controls the transcription of pro-inflammatory cytokines (TNF-alpha, IL-6, IFN-gamma, IL-1 beta).[68] The release of TNF-alpha and IL-6 from the rat ileum increase in response to ischemia/reperfusion injury, where the levels cytokine release is related to the magnitude of the ischemic insult.[74] Incubation of intestinal cells with TNF-alpha, IFN-gamma, and IL-1 beta causes reorganization of occludin, claudin, and ZO-1.[66, 75] Ye et al 2006 [66] explained the TNF-alpha molecular mechanism that leads to the decrease in tight junction stability, where NF-kappa B mediates TNF-alpha synthesis leading to upregulation of MLCK promotor activity, and TJ permeability. It is believed that MLCK then phosphorylates the MLC of the actin cytoskeleton leading to the opening of the tight junction.[38]

Therefore, high intensity exercise can cause the production of hydrogen peroxide, which may contribute to the cause of ischemia reperfusion injury. Hydrogen peroxide disorganizes the TJ barrier by two mechanisms, that include (1) phosphorylation of TJ proteins through the activation of protein kinases, and (2) upregulation of NF-kappa B transcription of pro-inflammatory cytokines. It is important to mention that the ischemic pathway leading to gastrointestinal distress has been challenged. Wright et al 2011 [76] showed that splanchnic blood flow was compromised among athletes after a long distance triathlon, but did not differ between those who suffered gastrointestinal symptoms, and those who did not. Measurements taken during the event would better support this argument as hypo-perfusion occurs rapidly [67] and has been associated with GI damage. [9]

HEAT SHOCK PROTEIN PROTECTION AGAINST TJ PHOSPHORYLATION

Heat shock proteins (HSP) are intracellular molecular chaperones that assist in protein synthesis and cell maintenance.[77] Increased levels of intracellular HSP provide protection to the cell under stressful conditions. Intracellular HSP levels in peripheral blood mononuclear cells (PBMCs) of athletes are upregulated after a competitive endurance event,[78] and trained athletes show a greater HSP response to exercise stress.[79] In addition, HSP levels increase in response to heat acclimatization, which provides greater thermotolerance.[2] Heat shock proteins also provide protection against gastrointestinal disease [80] where an increase in heat shock factor 1 (HSF-1), the cytosolic regulator of HSP synthesis, and HSP 70 reduce the levels of gastric lesions and irritable bowel symptoms.[81, 82]

In the gastrointestinal tract, increasing the levels of HSP70 increases the expression of actin fibers and prevents the breakdown of the tight junction protein occludin.[83] Furthermore, HSF-1 mediates the increase in occludin expression during heat stress.[84] HSP70 has also been shown to protect the actin cytoskeleton of intestinal cells from hydrogen peroxide and hypoxia induced damage.[85]

HSP70 protects the intestinal epithelial cells under hyperthermic conditions by preventing the activation of cPKC, and reducing the phosphorylation of both MLC of the actin cytoskeleton, and the occludin protein.[13] HSP 27 reduces tyrosine kinase activation during ischemia reperfusion injury resulting in a stronger occludin and ZO-1 interaction and stabilization of the tight junction.[86] Heat shock proteins also prevent NF-kappa b translocation into the nucleus of intestinal epithelial cells reducing the synthesis of pro-inflammatory cytokines such as TNF-alpha.[83] Over expression of

HSP70 protects epithelial cells from TNF-alpha insult, and maintains TJ stability.[87] This suggests that heat shock proteins may protect the intestinal barrier during both heat and ischemic stress through the decrease in TJ protein phosporlyation and prevention of NF-kappa B activation.

AGENTS THAT PROTECT THE GUT BARRIER

Recently, there has been a surge of research into identifying dietary supplements to upregulate HSPs and protect TJ proteins from stressors such as inflammatory bowel disease and exercise. Traditional methods for increasing HSPs levels are through chronic stress exposures such as heat, or altitude acclimation, or exercise. However, this may not be feasible for some populations. Marchbank et al 2011 [29] demonstrated upregulation of HSP70 in human intestinal cells in response to bovine colostrum supplementation to the cell culture media, along with a reduction in gut permeability in exercising subjects after 14-days of supplementation. Bovine colostrum has also been shown to protect the gut barrier during ischemia reperfusion stress [88] and hyperthermia [89] in rats. Conversely, Buckley et al 2009 [90] showed an increase in exercise induced gut permeability in runners after eight weeks of bovine colostrum supplementation. The explanation for the conflicting results between the Marchbank and Buckley studies is unclear, but because colostrum facilitates small molecule transport prior to gut closure in infants, colostrom may enhance the cellular transport of the lactulose and rhamnose sugar probes.[91] This may have occurred among subjects in the Buckley study because the supplementation period was eight weeks as compared to the 14-day trial in the Marchbank study.

Polaprezinc is an anti-ulcer drug containing zinc and several amino acids, which has been used primarily in Japan. It has been found to increase HSP levels in rat intestines while reducing permeability during hydrogen peroxide injury.[92] In addition, zinc supplementation in humans prevented a rise in gut permeability after NSAID ingestion. In an *in vivo* follow-up study, zinc prevented rat intestinal cell villus shortening and edema.[93] It is thought that zinc is critical for tight junction assembly,[94] but whether or not it upregulates HSP levels is unknown.

Glutamine is the most abundant amino acid in the human body, and provides protection to many tissues in situations of stress.[95-97] It has been used as treatment for patients suffering from irritable bowel and Crohn's disease.[98, 99] Oral glutamine supplementation in rats has been shown to increase HSP 70 expression in the gut in response to heat stress.[100] These rats also demonstrated lower gut permeability 6hrs and 24hrs post heat exposure. In addition, glutamine enhances HSP 70 expression in vitro,[101] and reduces pro inflammatory cytokine release. The mechanism may be through glutamine mediated increase in cytosolic HSF-1 translocation into the nucleus leading to HSP transcription.[100]

There is growing evidence that supports probiotic therapy for improving gut function and enhancing the integrity of the intestinal tight junction.[102] Probiotic supplementation has been shown to prevent phosphorylation of occludin, increasing the ZO-1[103] and actin cytoskeleton interaction [104] in a rat experimental colitis model. However, research in humans is limited, and if probiotics provide protection under conditions of exercise induced heat and ischemic stress is not known. The high

temperature that probiotic bacteria are cultured in may allow it to withstand the rise in core temperature, and provide protection during prolonged exercise.

AGENTS THAT INCREASE INTESTINAL PERMEABILITY

An agent that increases gut permeability in response to stress should increase the susceptibility of gastrointestinal distress. Inhibition of the HSP response to stress increases the breakdown of occludin, ZO-1, and claudin along with reducing barrier integrity. Quercetin, which is an anti-oxidant has been shown to block the rise in HSP70 levels in response to heat stress.[2] In addition, seven days of quercetin supplementation prevented heat acclimation by decreasing thermoregulatory responses, which was mediated through the decrease in HSP levels.[2] Quercetin is also commonly used as an HSP inhibitor in cell culture models,[62] where it inactivates HSF-1.[105] Recently, other antioxidant treatments were found to be ineffective against irritable bowel disease in rats.[106] Conversely, a steady antioxidant infusion slowed gut mucosal damage during ischemic injury in a porcine model.[107] An antioxidant defense mechanism is to increase the levels of protective antioxidative enzymes, however, the capacity of these enzymes may be a limitation, and could be the reason why a constant infusion, but not a bolus, provides gut protection.

Non-steroidal anti-inflammatory drugs (NSAID) have been shown to increase gut permeability in humans during exercise,[108] and induce damage in the intestines of rats.[93] Over expression of HSPs protect intestinal cells from NSAID damage, but the effect of NSAIDs on HSP levels in intestinal cells is not known. In myocardial and nerve tissues, NSAIDS increase HSP 70 levels.[109, 110] If NSAIDs increase the HSP

expression, but also increase intestinal permeability then the effects on the gut may not be mediated through the heat shock protein response, but through another pathway.

Nutrients, such as wheat, lactose, and a bolus of a concentrated glucose solution are shown to damage the intestinal barrier.[111-113] Vigorous exercise combined with poor nutrition habits may enhance gut permeability during exercise.[113] A food allergy, such as wheat intolerance,[111] triggers an immune response causing the release of pro-inflammatory cytokines leading to tight junction breakdown.[66, 114, 115] HSP 70 expression has been shown to protect the integrity of the TJ barrier in children suffering from celiac disease.[116] There is some evidence that the celiac gene is located near the HSP gene cluster, which may cause the silencing of HSP expression. [117] Therefore, food allergies, namely gluten, may cause TJ dysfunction by affecting HSP expression making the cell more susceptible to damage. Research into gut permeability during exercise combined with various nutrients is limited, but very important, and should be a future focus for exercise physiologists.

CONCLUSIONS

Many athletes suffer gastrointestinal problems during training and competition that can affect exercise tolerance, and sport performance. The regulation of tight junction permeability may be the critical mechanism that causes GI distress. Exercise that changes local intestinal temperature, blood flow, and oxidant damage could regulate the phosphorylation of the tight junction proteins and determine the level of interaction between occludin, claudin, zona occludens, and the cytoskeleton. Heat shock proteins protect the intestine from both heat and ischemic stress, and agents that increase the HSP

response may provide benefit to athletes who are susceptible to GI distress. Therefore, a dietary substance that upregulates HSPs may reduce GI symptoms, and improve overall athletic performance.

SUMMARY OF NEW FINDINGS

- Exercise that increases core temperature (prolonged), or high intensity exercise regulate the phosphorylation state of intestinal tight junction proteins leading to disruption and increased permeability
- The increase in permeability allows paracellular movement of endotoxin into the blood stream causing a cascade of immune and inflammatory responses that lead to reduced fluid absorption, fluid secretion, and diarrhea
- Several supplements including, glutamine, bovine colostrum, and prolaprezinc may increase the heat shock protein response during exercise leading to greater tight junction protein stability and lower permeability

Chapter 2 Figures

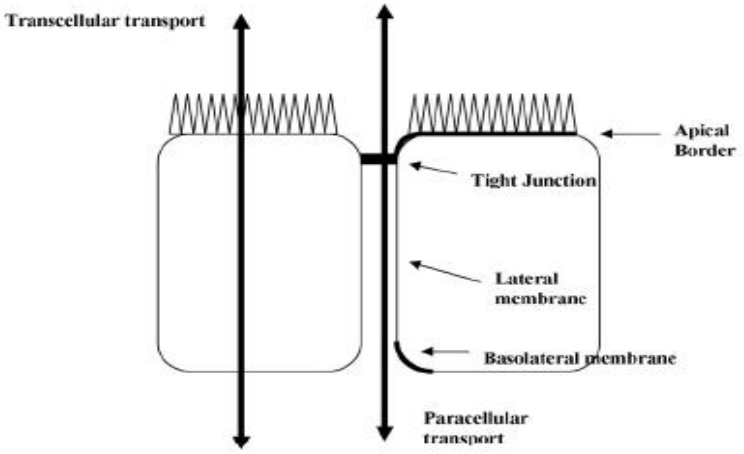

Figure 1:
Tight junctions are located in the extracellular space adjacent to epithelial cells and regulate bi-directional paracellular absorption and secretion

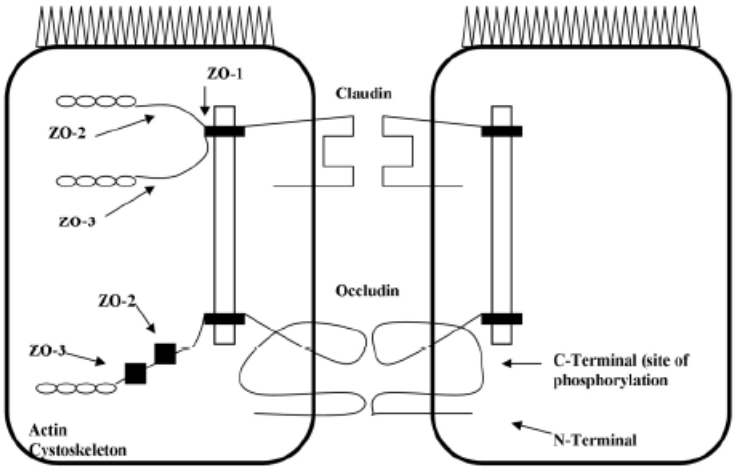

Figure 2. The tight junction barrier is composed of tetraspanning membrane proteins claudins and occludin, and the regulatory proteins ZO-1, ZO-2, and ZO-3

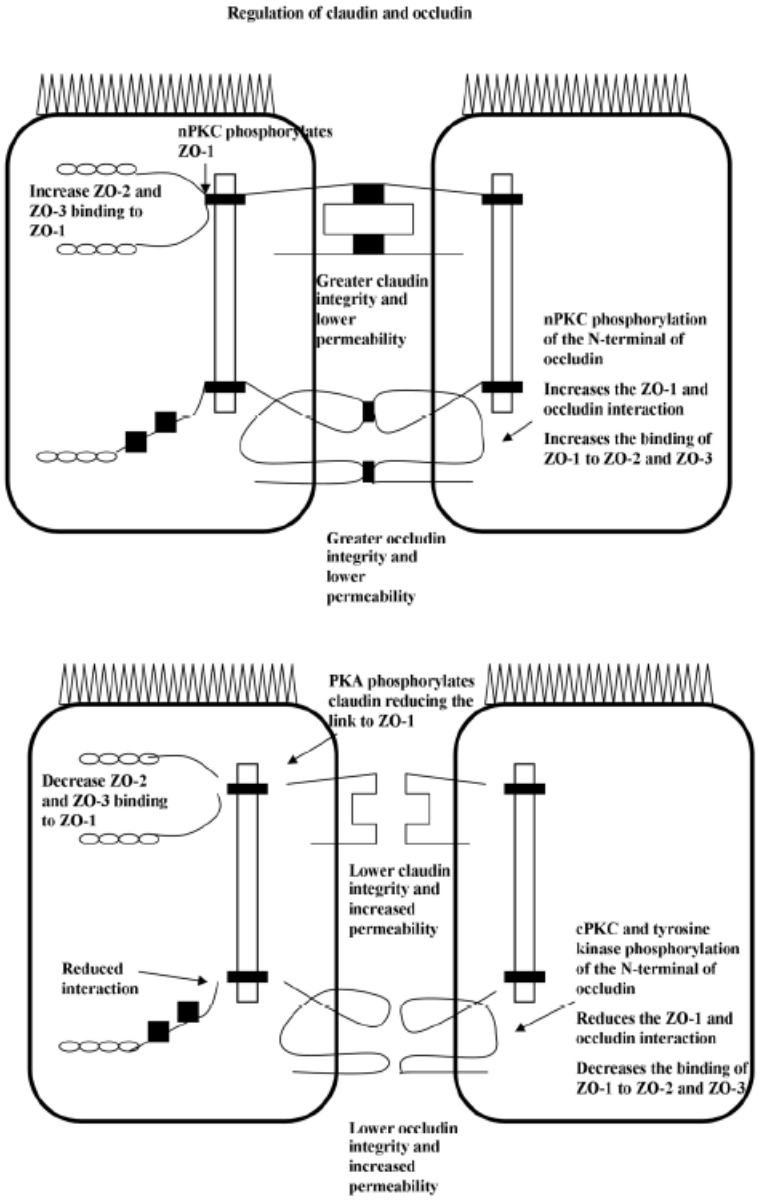

Figure 3. Regulation of tight junction proteins. Top: nPKC phosphorylates both claudin and occludin, increasing the interactions with zona occludens, and decreasing TJ permeability. Bottom: PKA phosphorylates the claudins, and cPKC and tyrosine kinase phosphorylate occludin and decreases the interactions with the zona occludens, and increasing TJ permeability.

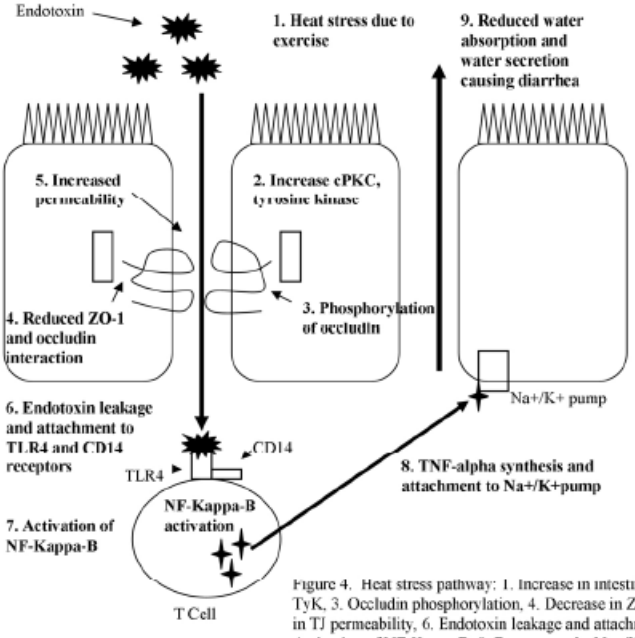

Figure 4. Heat stress pathway: 1. Increase in intestinal wall temperature, 2. Increase in cPKC, TyK, 3. Occludin phosphorylation, 4. Decrease in ZO-1 and occludin interaction, 5. Increase in TJ permeability, 6. Endotoxin leakage and attachment to TLR4 and CD4 receptors, 7. Activation of NF-Kappa B, 8. Damage to the Na+/K+ pump, 9. Diarrhea.

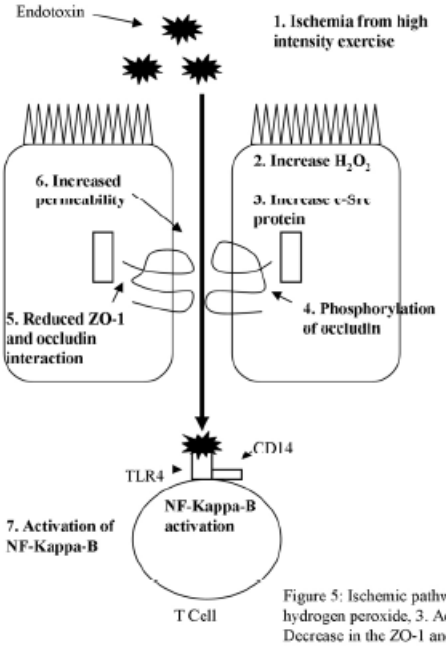

Figure 5: Ischemic pathway. 1. Reduced blood flow and ischemia, 2. Production of hydrogen peroxide, 3. Activation of c-Src protein, 4. Increase occludin phosphorylation, 5. Decrease in the ZO-1 and occludin interaction, 6. Increase TJ permeability, 7. Activation of NF-Kappa-B

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CHAPTER 3

RESEARCH MANUSCRIPT

This chapter presents a research manuscript, entitled “The Effects of Oral Glutamine Supplementation on Exercise Induced Gastrointestinal Permeability and Heat Shock Protein Regulation". This manuscript will be submitted to the British Journal of Sports Medicine. It is authored by Micah Zuhl, Kathryn Lanphere, Len Kravitz, Christine Mermier, Suzanne Schneider, Karol Dokladny, and Pope Moseley. The manuscript follows the formatting and style guidelines of the journal. References are provided at the end of the chapter.

Title: The effects of oral glutamine supplementation on exercise induced gut permeability and heat shock protein regulation among runners with a history of gastrointestinal distress

Running Title: Glutamine lowers exercise induced gastrointestinal permeability

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ABSTRACT

Gastrointestinal (GI) permeability increases during high intensity exercise leading to endotoxin leakage, and a pro-inflammatory immune response. The purpose of this study are to assess whether oral glutamine supplementation (1) reduces exercise induced permeability through up-regulation of the heat shock response resulting in occludin stabilization, and (2) depresses the exercise induced inflammatory response. **Methods.** Eight human subjects (n=8) participated in baseline (PRE) testing, a glutamine (GLN), and placebo (PLA) supplementation trial in a double blind design. After PRE measurements, subjects ingested .9g/kg fat free mass of glutamine per day or a sugar free lemon placebo drink for seven days with a one-month washout period between trials. A 60-min treadmill run at 70% of maximal oxygen consumption was performed at 30°C in an environmental chamber at the end of each supplementation period. Intestinal permeability was assessed pre and during each trial through urine concentrations of lactulose and rhamnose. Plasma glutamine, plasma endotoxin, and peripheral blood mononuclear cell levels of heat shock protein 70 (HSP70), and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκB-α) were measured pre-exercise, post-exercise, 2hr post-exercise, and 4hr post-exercise. Cultured Caco-2 human intestinal epithelial cells supplemented with three concentrations of GLN (0, 4, and 6mmol/L) were exposed to heat stress (41ºC) to simulate exercise and control (37ºC) conditions. HSP70, heat shock factor 1 (HSF-1), and occludin were measured from each culture. **Results:** Core temperature was not different between exercise trials (39.40 ± .39 vs. 39.54 ± .22 for PLA vs. GLN, respectively, $p>0.05$). Resting plasma glutamine levels were significantly higher in the GLN trial versus PLA (1.893 ± 0.245mmol.L vs. 0.8285

± 0.078 mmol.L). Permeability as the ratio of lactulose to rhamnose was significantly higher in the PLA trial when compared to PRE (.0604 ± .0470 vs. .0218 ±.0084, respectively, $p<0.05$). Permeability was not statistically different between GLN trial and PRE (.0272 ± .0074 vs. .0218 ± .0084, respectively, $p>0.05$). PBMC expression of IκB-α and HSP70 were higher at the 4hr post-exercise time point in the GLN trial when compared to the 4hr mark in the PLA (.9839 ± .1587 vs. 1.520 ±.2294 and 2.083 ± .6712 vs. 2.895 ± .8444, $p<0.05$ for IKB-α and HSP70, respectively). Plasma endotoxin was higher compared to pre-exercise at the 2hr post-exercise in the PLA trial (2.883 ± 0.4310 pg.ml vs. 4.550 ± 0.3350 pg.ml, $p<0.05$ respectively) and significantly higher when compared to the 2hr post-exercise mark in GLN trial (4.550 ± 0.3350 pg/ml vs. 2.883 ± .4314 pg/ml, $p<0.05$). Results of cell culture: HSP70 expression in Caco-2 cells was higher in the 6mmol 41ºC trial when compared to the 0mmol 41ºC trial (1.973 ± 0.163 vs. 1.133 ± 0.064, $p<0.05$, respectively). HSF-1 was higher in the 4mmol 41 ºC and the 6mmol 41ºC trials when compared to the 0mmol 41ºC (1.649 ± 0.185, 1.785 ± 0.185 vs. 0.6681 ± 0.145, $p<0.05$). Occludin levels were statistically lower in the 0mmol 41ºC when compared 0mmol 37ºC (0.7434 ± 0.015 ± 1.0000 ± 0.000, $p<0.05$ respectively). Occludin levels during 4mmol 41ºC and 6mmol 41ºC trials were statistically higher when compared to 0mmol 41ºC (1.236 ± 0.143 and 1.849 ± 0.143 vs. 0.7434 ± 0.015, $p<0.001$, respectively). **Conclusion:** Seven days of oral glutamine supplementation prevents exercise induced intestinal permeability and endotoxin leakage possibly through HSF-1 and HSP70 activation leading to occludin stabilization at the tight junction. In addition, glutamine suppressed the inflammatory response to high intensity exercise through activation of HSP70, reduced IKB-α degradation and possible NFκ-B inhibition.

INTRODUCTION

Intestinal permeability and systemic inflammation are associated with gastrointestinal distress, where paracellular endotoxin leakage triggers an immune response causing disruption to intestinal epithelial cell absorption mechanisms (figure 1).(1, 2) In response to the endotoxemia, T-cells release pro-inflammatory cytokines, which inhibit sodium and water absorption in the small intestine.(1) This leads to an imbalance in the absorption and secretion of water and electrolytes (2), fluid build-up in the gut, and possibly diarrhea.(1) During high intensity exercise epithelial cell tight junction (TJ) proteins disassemble resulting in paracellular movement of endotoxin into the blood stream, commonly called "leaky gut"(3, 4), which provokes an inflammatory cascade. This pathway may be responsible for exercise induced gastrointestinal distress, where TJ breakdown is the initial phase of the pathway (figure 1).(5)

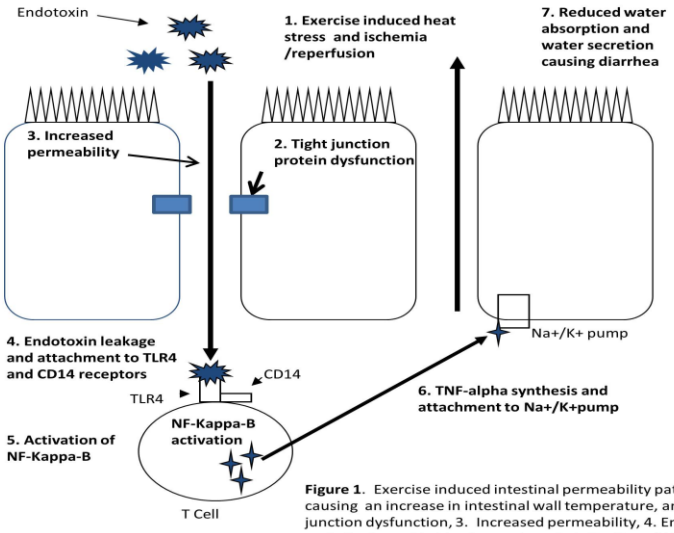

Figure 1. Exercise induced intestinal permeability pathway: 1. High intensity exercise causing an increase in intestinal wall temperature, and ischemia reperfusion cycling 2. Tight junction dysfunction, 3. Increased permeability, 4. Endotoxin leakage into the blood stream and attachment to T-cell receptors, 5. Activation of NFK-β, 6. TNF-α synthesis and causing Na+/K+ pump disruption, 7. Reduced water absorption and secretion. Adapted from Zuhl et al (2012)

Heat shock proteins are intracellular molecular chaperones that protect the cell by increasing protein synthesis and contributing to cell maintenance.(6) Heat shock protein 70 (HSP70) is induced when cells and animals are exposed to heat stress, and protect tissue from subsequent heat exposure.(7, 8) Human skeletal muscle levels of HSP70 increase in response to muscle damaging exercise, prevent damage from further eccentric contractions.(9) Additionally, HSP70 levels in peripheral blood mononuclear cells (PBMC) are up-regulated after endurance exercise, and in response to heat acclimation.(10) Augmenting HSP70 levels in intestinal epithelial cells has been shown to increase tight junction stability and reduce permeability to heat stress.(8) Heat shock factor-1 (HSF-1), the transcription factor for HSP70, has been shown to regulate the TJ protein occludin.(11) In human PBMCs, up-regulation of HSP70 by an adenovirus has been shown to inhibit the nuclear translocation of NFκ-B (12) reducing TNF-α release, which may blunt the inflammatory cascade that leads to GI distress. Kuennen et al (2010) (10) demonstrated that inhibiting HSP70 in PBMCs resulted in an increase in TNF-α, which correlated with a rise in intestinal permeability among exercising humans. This demonstrates that augmenting the heat shock response has a dual mechanisms to protect the gut under conditions of stress. One, through stabilization of the epithelial cell TJ proteins, and secondly, by reducing the inflammatory response in peripheral blood mononuclear cells. We hypothesize that enhancing HSP70 in intestinal cells may reduce exercise induced intestinal permeability, and gastrointestinal distress.

Glutamine is the most abundant amino acid in the human body. It is a major nutrient for enterocytes and immune cells, and reduces mortality when given intravenously to sepsis patients and burn victims.(13, 14) It has been shown to reduce the

symptoms of irritable bowel and Chrohn's disease.(15) The protective effects of glutamine may be through increasing HSP70 synthesis. Oral glutamine supplementation in rats increases HSP70 levels in the gut and reduces intestinal permeability. This mechanism is believed to be due to glutamine activation of heat shock factor one (HSF-1) (16), and stabilization of the TJ protein occludin.(11)

The purposes of this study are to test (1) whether seven-days of glutamine supplementation reduces exercise induced intestinal permeability and endotoxin leakage, (2)the mechanism may be through HSP70 activation, and stabilization of the TJ protein occludin, and (3) glutamine suppresses the pro-inflammatory immune cascade by up-regulating the HSP70 response and inhibiting the translocation of NFκ-B in PBMCs.

MATERIALS & METHODS

Human Protocol

Subjects: The present study was approved by the Human Research Review Committee of the University of New Mexico, Albuquerque, USA. Eight endurance trained adult men (n=5) and women (n=3) 18-45 who experienced exercise induced gastrointestinal distress were recruited from the University population. All subjects completed a health questionnaire, and procedures, discomforts, and risks were discussed before written informed consent was obtained. Subjects were excluded if taking medications (antidepressants or diuretics), or nutritional supplements. All testing was performed in the Exercise Laboratory at the University of New Mexico at 5,200 ft of altitude.

Experimental Design: Using a double blinded research design each subject participated in baseline (PRE) testing, and both a glutamine (GLN), and placebo (PLA) trial, separated by a 4-week washout period. During the first visit to the lab, baseline measurements (maximal oxygen consumption, body composition, and gut permeability) were performed on each subject. After baseline testing each subject was provided a 7-day supplement bag containing glutamine or placebo along with instructions regarding the proper way to take the supplement. On day seven, subjects returned to the laboratory after an overnight fast to complete the first exercise trial. Height and weight were measured, then a rectal probe was inserted for core temperature measurement. After 20-minutes of seated rest, a 20ml blood sample was taken to measure baseline plasma levels of endotoxin and glutamine, along with peripheral blood mononuclear cell (PBMC) levels of I- Kappa beta-alpha (IκB-α), and HSP 70. Each exercise trial consisted of a 60-minute treadmill run at 70-80% of VO2max in an environmental chamber set at 30°C. Trials were terminated early if subjects reached a core temperature of 40°C. Twenty minutes into the exercise trial, a 50ml sugar probe solution (5g lactulose, 2g rhamnose) was consumed for measurement of intestinal permeability. Venous blood samples were taken immediately post exercise, two hours post, and four hours post exercise for measurements of endotoxin, and PBMC levels of HSP70 and IκB-α. In addition, urine was collected for five hours post exercise for measurement of intestinal permeability by quantifying the levels of lactulose and rhamnose. One month later, subjects returned and performed the second exercise trial using the identical protocol (figure 2)

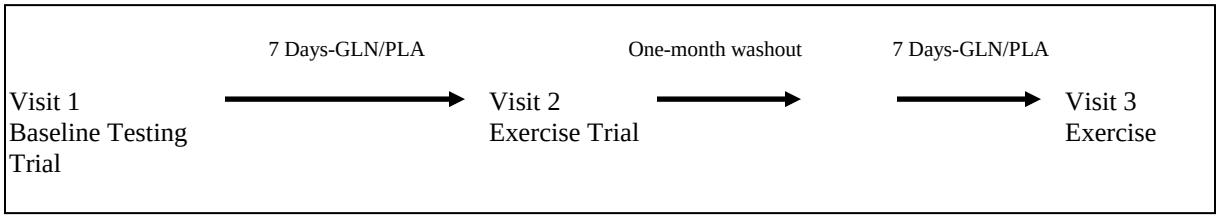

Figure 2. Experimental Protocol

Baseline Measurements: Peak aerobic power (VO_2peak) was measured using open circuit spirometry (Parvomedics, Sandy, UT, USA) and a graded incremental protocol on a motorized treadmill. The highest value for oxygen consumption based on an 11-breath running average was used to assess VO_2peak. Two of the following criteria were used to determine VO_2peak, including VO_2 plateau (<150ml), RER (>1.15), and RPE (>17), and heart rate (+-10bpm of estimated max). Body density was calculated by using the sum of three skin fold sites (men: chest, abdominal, thigh, women: triceps, suprailliac, thigh) and fat free mass was calculated using the Siri equation. Resting gastrointestinal permeability was measured after an overnight fast, and upon early morning arrival to the exercise lab. Subjects ingested 5g lactulose and 2g rhamnose and urine was collected during a five hour fast.(18) Water consumption was allowed during the first 2hrs of the fasting period. Permeability was measured by quantifying the ratio of lactulose to rhamnose in the urine. A 20ml venous blood sample was collected to measure the baseline PBMC levels of HSP70 and IκB-α

Glutamine and Placebo Supplementation: Subjects ingested .9g/kg of fat free mass per day for seven days of glutamine (GLN) mixed with sugar free lemon drink powder or 2g of sugar free lemon drink placebo (PLA). The supplements were separated into three

doses per day, taken in the morning, early afternoon, and evening. Subjects were instructed to mix the GLN or PLA with 12oz of water.

Gastrointestinal Barrier Permeability: The assessment of intestinal permeability was quantified based on the absorption and urinary excretion of two sugar probes. Lactulose is a large disaccharide probe that is a marker of small intestine paracellular permeability. Rhamnose is a smaller monosaccharide that crosses the epithelia via the transcellular pathway (17, 18), and is a marker of intestinal absorption. Lactulose levels will increase while rhamnose stays constant or increases slightly, which provides evidence of stable absorption, and increased paracellular movement. An increase in the ratio of the urinary excretion of the two sugars (lactulose/rhamnose) is a marker for an increase in small intestinal permeability.(16, 17)

Resting baseline (PRE) and exercise intestinal permeability during both trials (PLA and GLN trials) were measured. After an overnight fast, subjects consumed a 50ml solution containing 5g lactulose (L7877 Sigma-Aldrich, St.Louis, MO, USA), and 2 g rhamnose (R3875 Sigma-Aldrich, St. Louis, MO, USA). During the GLN and PLA trials the sugar drink was consumed 20-minutes into each exercise bout to ensure transit of the sugar probe solution when intestinal permeability would likely occur. (3) Urine was collected over a five-hour time frame either during baseline or post exercise. Subjects were allowed to consume water during the first two hours of the five-hour fast to increase urine production. Samples were separated into a 30ml aliquot and stored at -20 degrees for subsequent analysis of the lactulose to rhamnose excretion ratio.

Urinary Lactulose: Lactulose was quantified using a simple enzymatic method developed by Behrans et al 1984. (19, 20) The following materials were used: triethanolamine (TEA), 1M NaOH, ATP magnesium salt > 95% pure, NADP > 95% pure (Sigma Aldrich, St. Louis, MO), $MgSO_4$ (Merck, Darmstadt, Germany), beta-galactosidase 4000U/ml, hexokinase/glucose-6-phosphate dehydrogenase 425U/212U/ml, phosphor-glucoisomerase 1000U/ml (Megazyme, Wicklow, Ireland)

A 200µl sample of urine was added to 100µl triethanolamine buffer (5.6g TEA and 740mg $MgSO_4$ in 50ml dH_20. pH adjusted to 7.5 with 1M NaOH and total volume brought to 100ml). 6.2µl of beta-galactosidase was added to urine and TEA buffer, and incubated at room temperature for 2hrs. After the incubation period, 2.73ml of pre-prepared cocktail was added per sample (1ml TEA, 2g ATP, 2g NADP, .009ml HK/G6PDH, and 1.721ml dH_2O). Absorbance was measured at 340nm (A1) against a blank water sample using a spectrophotometer (Beckman Coulter DU530, Brea, CA). 7µl of phospho-glucoisomerase was added to each sample, and absorbance was measured again at 340nm (A2) against a blank water sample every three minutes until reaction was stable. A blank sample containing water along with the cocktail and enzymes was subtracted from the summed absorbance. Concentration was calculated using Beers law for NADH.

Urinary Rhamnose: Rhamnose was quantified using a colorimetric enzyme immunoassay kit (K-RHAM, Megazyme, Wicklow, Ireland). All materials were supplied by the manufacturer. In a 3ml cuvette, a .100ml urine sample was added to 1.70ml dH_2O, 0.50ml TEA buffer, 0.100ml NAD^+/ATP solution, 0.08ml rhamnulose 1-phosphate aldolase plus lactaldehyde dehydrogenase suspension. Absorbance was read at 340nm

with a spectrophotometer (Beckman Coulter DU530, Brea, CA) after a three-minute incubation at room temperature (A1). After completion of reaction, .02ml rhamnose isomerase suspension was added to the cuvette, and absorbance was read at 340nm after 20-minute incubation at room temperature (A2). All samples were measured against a water blank. Concentration was calculated based on Beers Law for absorbance of NADH.

Blood sampling and analysis: Venous blood was collected pre, post, 2-hr post, and 4-hr post exercise from an antecubital vein. Blood samples were drawn into sterile syringes and immediately transferred into vacutainers containing EDTA (BD Biosciences, Franklin Lakes, NJ). Blood was added to Histopaque (Sigma Aldrich 1077, St. Louis, MO) in a 1:1 ratio (15ml/15ml) and centrifuged at 2,200 rpm for 30-minutes. Plasma was pipetted into 1.5mL microtubes and frozen at -80 for further analysis of endotoxin and glutamine. The buffy coat containing the mononuclear cells was collected, transferred to a clean conical centrifuge tube, and re-suspended with 10 ml of phosphate buffered saline (PBS) (Sigma Aldrich 4417, St. Louis, MO). The mixture was centrifuged at 2,000 rpm for 10-minutes. The supernatant was removed and the mononuclear pellet was stored at -80 for subsequent analysis of HSP70 and IκB-α.

Endotoxin: Endotoxin was assessed in the plasma using a limulus amebocyte lysate chromogenic endpoint assay (HIT302, Hycult Biotech, Plymouth Meeting, PA) sensitive to 1.4 pg/ml. Samples were diluted 1:3 with endotoxin free water then heated to 75 degrees for 5-minutes. The following was added to each well of a 96 well plate; 50µl

plasma sample, 50 µl LAL reagent and incubated for 30-minutes at room temperature. Absorbance was read at 405nm. A logarithmic standard curve was used to calculate concentrations in pg/ml.

Glutamine: Plasma glutamine was assessed with a quantitative colorimetric enzyme assay kit (EGLN-100, BioAssay Systems, Hayward, CA) sensitive to 0.023mM glutamine. All materials and chemicals were provided by manufacturer. Following manufacturer directions, in a 96-well plate a 20µl plasma sample is added to 80µl of working reagent (65µl assay buffer, 1µl enzyme A, 1µl enzyme B, 2.5µl NAD, 14µl MTT solution). After a 40-min incubation at room temperature, 100µl of stop solution was added to each well, and absorbance was read at 565nm. Concentration was calculated based on a standard curve. Glutamate was measured in each sample and subtracted from the glutamine absorbance of the respective sample.

***HSP 70 and IκB-α*:** Mononuclear cells were homogenized for 25-minutes with 200uL of lysis buffer (150mM NaCl, 20mM HEPES, 2mM EDTA, .2% SDS, .5% sodium deoxycholate, 10% Triton X-100, 100uM phenylmethylsulfonyl fluoride (PMSF), 100uM vanadate, 1ug/ml leupeptin, 1ug/ml pepstatin A, 40mM paranitrophenyl phosphate, 1ug/ml aprotinin) and then centrifuged for 10 minutes. Supernatant was collected and protein measurement was performed using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Laemmli gel loading buffer was added to the lysate containing 15-20ug of protein and boiled for 10 minutes. Proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to a

membrane (Trans-blot transfer medium, nitrocellulose membrane; Bio-Rad Laboratories). The membrane was incubated for 1 h in blocking solution (5% dry milk in Tris buffered saline Tween 20 buffer) followed by incubation with HSP 70 (#SPA-810, Clone: C92F3A-5, Stressgen, Victoria, BC, Canada) or IKB-α (Catalog# I0505, Sigma, St.Louis, MO, USA) antibodies in a blocking solution. Each membrane was cut, and the lower half was treated with antibody for β-actin (61-0120, Invitrogen) while the upper half was treated with HSP70 or IKB-α. After incubation the membrane was washed with TBS-Tween then treated with horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). The membrane was developed using Santa Cruz Western Blotting Luminol Reagants (Santa Cruz Biotechnology, Santa Cruz, CA, USA) on the Kodak BioMax MS film (Fisher Scientific, Pittsburgh, PA, USA). Adobe Photoshop (San Jose, CA) was used to quantify protein expression and standardized to β-actin to control for protein loading. Protein levels also were expressed relative to pre time point.

Caco-2 Cell Protocol:

Experimental procedure: An in vitro experimental model was used to determine if glutamine's protection of intestinal cells is mediated through HSF-1, HSP70, and occludin up-regulation. A human carcinoma intestinal epithelial cell line (Caco-2) was supplemented with three concentrations of glutamine (0, 4, and 6mmol/L) and exposed to heat stress. HSF-1, HSP 70, and occludin were measured.

Cell Cultures: Caco-2 cells (American Type Culture Collection, Rockville, MD, USA) were maintained at 37 degrees in a culture medium composed of DMEM (4.5mg/ml

glucose, 50U/ml penicillin, 50U/ml streptomycin, 4mM glutamine, and 25mM HEPES) supplemented with heat-inactivated 10% FBS. Medium was changed every two days. After 80% confluency, Caco-2 cells were subcultured in 2ml plates, and re-supplemented for 7-days with three concentrations of glutamine (0, 4, and 6mmol/L)

Glutamine and heat shock treatment: Supernatants of the confluent Caco-2 cell layers were replaced by DMEM supplemented with GLN in three concentrations (0, 4, and 6mmol). Cells were then incubated in a water bath for 75-minutes at 41.8°C followed by recovery incubation at 37 degrees for 4hrs. The exposure to 41.8°C was to simulate in the increase in core temperature that occurred during human exercise trial.

Assessment of HSF-1, HSP 70, and Occludin protein expression by Western Blot Analysis: At the end of 37°C and 41°C trials, Caco-2 monolayers were immediately rinsed in ice cold PBS, and each plate was scraped for cell collection, and frozen at -70°C. Cell were lysed with a lysis buffer (50mM Tris.HCl, pH 7.5, 150mM NaCl, 500 uM NaF, 2mM EDTA, 100uM vanadate, 100uM PMSF, 1ug/ml leupeptin, 1ug/ml pepstatin A, 40mM paranitrophenyl phosphate, 1ug/ml aprotinin, and 1% Triton X-100), supernatant was collected and protein measurement performed using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Laemmli gel loading buffer was added to the lysate in a 1:1 ratio containing 5-10ug of protein and boiled for 7-min, and afterwards proteins were separated on an SDS-Page gel. Proteins from gel were transferred to a membrane, and incubated for 1h in blocking solution (5% dry milk in TBS-Tween 20 buffer). Each membrane was cut, and the lower half was treated with

antibody for β-actin (61-0120, Invitrogen, Carlsbad, CA, USA) while the upper half was treated with the appropriate primary antibodies (HSF-1, HSP 70, and occludin purchased from Stressgen, Victoria, BC, Canada) and incubated overnight. After incubation the membrane was washed with TBS-Tween then treated with horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). The membrane was developed using Santa Cruz Western Blotting Luminol Reagants (Santa Cruz Biotechnology, Santa Cruz, CA, USA) on the Kodak BioMax MS film (Fisher Scientific, Pittsburgh, PA, USA). Adobe Photoshop (San Jose, CA) was used to quantify protein expression and standardized to β-actin to control for protein loading. Protein levels also were expressed relative to control time point.

Statistical Analysis*:* All results are expressed as means ± SE. In the human experiment, a two-factor repeated measure ANOVA was used to analyze intestinal permeability with condition (glutamine, placebo) and time (pre, post exercise) as the independent variables and a Tukeys test was used for post hoc testing. A two-factor repeated measures ANOVA also was used to analyze plasma glutamine, HSP70 and IκB-α concentrations with condition (glutamine, placebo) and time (pre, post, post 2hr, and post 4hr) as the independent variables. A paired Student t-test was used to compare plasma endotoxin levels in the placebo and glutamine conditions at four time points (pre, post, post 2hr, and post 4hr).

In the Caco-2 intestinal cell experiment, statistical significance was determined for HSF-1, HSP70, and occludin using a two-factor non-repeated measures ANOVA with temperature (37°C and 41°C) and glutamine supplementation (0mmol/L, 4mmol/L, and

6mmol/L) as the independent variables. Significant main effects were identified using a Tukey's post hoc test. Significance is reported at $p < 0.05$. SPSS statistical software was used for data analysis (SPSS, Armonk, NY).

RESULTS

The effect of seven-days of oral glutamine supplementation in exercising humans

Subject physiological characteristics: Eight subjects (5 male, 3 female) completed PRE, PLA, and GLN measurements. Subject characteristics are presented in table 1. All subjects were trained runners, who reported gastrointestinal distress on at least five occasions. Treadmill speed and grade varied during the trials, and intensity was based on oxygen consumption levels. There was no difference between the PLA and GLN trials for exercise intensity reported as percentage of VO_2max (71.35 ± 0.01% vs. 74.34 ± 0.01%, respectively). End exercise core temperature was not different between PLA and GLN trials (39.40 ± 0.13°C vs. 39.54 ± 0.07°C, respectively). One subject did not complete the full 60-minute protocol in both trials due to core temperature reaching termination criteria (40°C).

Table 1: Subject characteristics

	Physiological Characteristics
Sex	Male (5), Female (3)
Age	25 ± 4
Height (cm)	174 ± 5.62
Weight (kg)	72.21 ± 16.39
Body Fat (%)	18.84 ± 9.32
VO_2max (ml.kg.min	51.11 ± 6.58

Data are mean ± SEM, n=8

Oral glutamine supplementation increased plasma glutamine levels (figure 3): A two-way repeated measures ANOVA was used to compare the effects of glutamine supplementation on plasma glutamine levels in pre and post exercise conditions. The main effect was statistically significant, $F(3, 7) = 20.13$, $p < .001$. Post hoc testing using Tukey's showed plasma glutamine levels significantly higher in GLN trial at the pre-exercise time point when compared to pre-exercise in PLA (1.893 ± 0.245mmol/L vs. 0.828 ± 0.078mmol/L, p=0.007, respectively). Plasma glutamine levels significantly declined post exercise in the GLN trial (1.893 ± 0.245mmol/L vs. 0.9265 ± 0.091mmol/L, p=0.012).

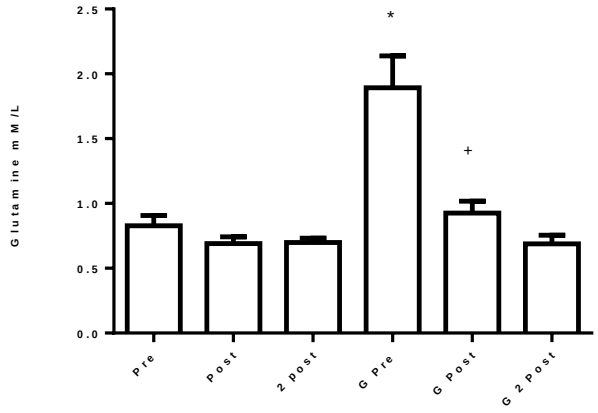

Figure 3. Oral glutamine supplementation increased resting plasma glutamine levels. Plasma glutamine was significantly higher at the pre-exercise (G Pre) time point in the GLN trial when compared to pre-exercise (Pre) in PLA trial. Plasma glutamine was significantly lower at the post-exercise (G Post) in the GLN trial when compared to pre-exercise (G Pre) in the GLN trial. Data are mean ± SEM, n=8. * statistically significant from same time point in PLA trial, $p<0.05$. + statistically significant from pre-exercise in the same trial, $p<0.05$

Oral glutamine supplementation prevented exercise induced intestinal permeability (figure 4): The ratio of urinary lactulose to rhamnose (L/R) was used to measure intestinal permeability. A two factor repeated measures ANOVA was used to compare the effects of oral glutamine supplementation on intestinal permeability in pre and post exercise conditions. Exercise caused an increase in intestinal permeability with the main effect statistically significant $F(2, 7) = 6.060$, $p<0.05$. Tukey's post hoc testing showed

intestinal permeability to be significantly higher in the PLA trial compared to PRE (0.0603 ± 0.0166 vs. 0.0218 ± 0.0029, p=0.019, respectively). In addition, permeability was significantly higher in the PLA trial when compared to the GLN trial (0.0603 ± 0.0166 vs. 0.0272 ± 0.0026, p=0.026).

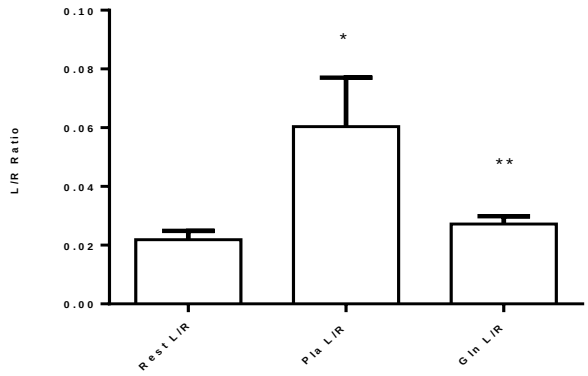

Figure 4. Glutamine prevented a rise in intestinal permeability when compared to rest. Urinary excretion of lactulose (L) and rhamnose (R) were measured among subjects at rest (Rest L/R), after placebo trial (Pla L/R), and after glutamine trial (Gln L/R). * statistically significant from Rest L/R ($p<0.05$). ** statistically significant from Pla L/R ($p<0.05$).

Glutamine prevented the rise in plasma endotoxin in exercising humans (figure 5):

Plasma endotoxin levels increased at the 2hr post-exercise time point compared to resting conditions in the PLA trial (4.550 ± 0.335 pg/ml vs. 3.523 ± 0.232, p=0.01). Plasma endotoxin did not significantly increase from baseline at any time point in the GLN trial. Additionally, plasma endotoxin was significantly lower at the 2hr post-exercise time point in the GLN trial compared to the 2hr post-exercise time point in the PLA trial (2.883 ± 0.431 pg/ml vs. 4.550 ± 0.335 pg/ml. p=0.02).

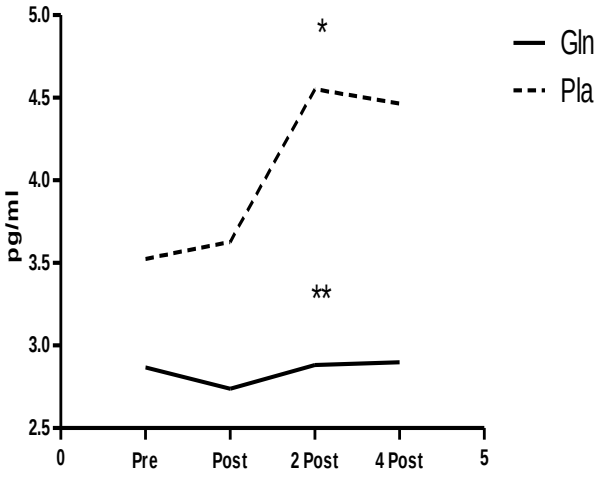

Figure 5. Glutamine prevented the rise in plasma endotoxin levels. Endotoxin was significantly higher at 2hr post-exercise when compared Pre-exercise. Plasma endotoxin was significantly lower at 2hr post-exercise in glutamine trial when compared to 2hr post-exercise in placebo trial. *statistically higher from Pre, $p<0.05$. ** statistically lower from same time point in placebo trial, $p<0.05$.

Glutamine increased PBMC levels of HSP70 and IKB-α in response to exercise stress (figure 6): Glutamine supplementation enhanced the HSP70 response to exercise stress ($F(3,18) = 16.612$, $p<0.05$). HSP70 expression was significantly higher at 4hr post-exercise time point in the GLN trial when compared to the 4hr post-exercise time point in the PLA trial (2.895 ± 0.844 vs. 2.083 ± 0.671, $p=0.007$). In addition, glutamine supplementation enhanced IKB-α levels in response to exercise ($F(3,18) = 10.396$, $p<0.05$). Similar to HSP expression, IκB-α levels were significantly higher in GLN trial at 4hr post-exercise time point in comparison to the 4hr post-exercise time point in the PLA trial (1.411 ± 0.185 vs. $0.933 \pm .121$, $p=0.027$).

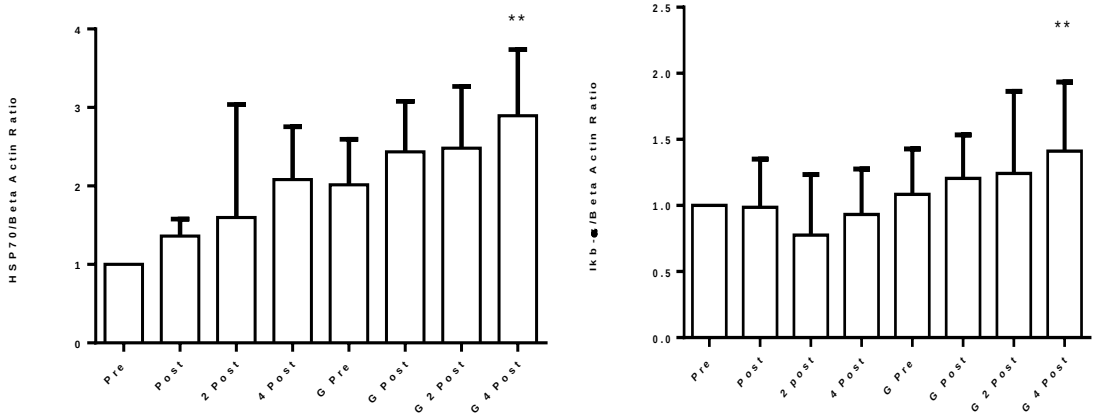

Figure 6. Glutamine supplementation upregulates both HSP70 and IκB-α post exercise at the 4-hour time point when compared to placebo supplementation. HSP70 and IκB-α were measured in peripheral blood mononuclear cells (PBMCs) of subjects after 7-days of placebo (Pla) and glutamine (Gla) supplementation. Measurements were taken before exercise (Pre), post exercise (Post), 2hrs post exercise (2-post), and 4hrs post exercise (4-post). Data are mean ± SEM, n=8 for each time point. ** increased from same time point in Pla trial, $p<0.05$.

The effect of glutamine supplementation in heat stressed Caco-2 intestinal epithelial cells

Glutamine supplementation increased HSF-1 and HSP70 levels in response to heat stress (figures 7 and 8): Glutamine combined with heat stress was required for the increase in HSF-1 levels. $F(2,18)=7.259$, $p<0.05$. Heat stress in the glutamine treated cells caused an increase in HSF-1 expression with statistical significance in the 4mmol 41ºC trial compared to 0mmol 37 ºC (control) (1.649 ± 0.185 vs. 1.000 ± 0.000, $p=0.03$ respectively). HSF-1 was higher in the 4mmol 41 ºC and the 6mmol 41ºC trials when compared to the 0mmol 41ºC (1.649 ± 0.185, 1.785 ± 0.185 vs. 0.6681 ± 0.145, $p=0.001$, $p<.001$, respectively). However, heat stress applied to non-glutamine supplemented cells did not increase HSF-1. HSP70 expression followed a similar trend as HSF-1. Again, the HSP70 response to heat stress was absent without out glutamine supplementation, but significantly increased when heat stress was applied to glutamine-treated cells, $F(2,24) = 4.106$, $p<0.05$. Both 4mmol 41°C and 6mmol 41ºC trials were significantly higher when

compared to the 0mmol 41ºC trial (1.691 ± 0.198 and 1.973 ± 0.163 vs. 1.133 ± 0.064, p=0.011, p<0.001, respectively).

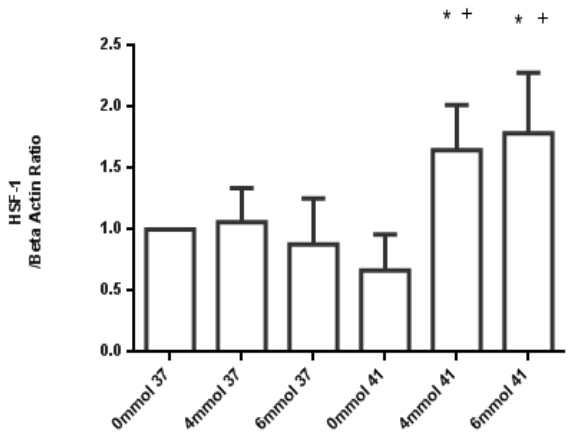

Figure 7. Glutamine supplementation combined with heat stress activated HSF-1 in Caco-2 intestinal cells. HSF-1 was significantly higher in the 4mmol 41 trial compared to 0mmol 37 control. HSF-1 was up-regulated in both 4mmol 41 and 6mmol 41 trials compared 0mmol 41. Data are mean ± SEM, n=4. * increased from 0mmol 37 trial, p<0.05. + increased from 0mmol 41 trial, p<0.05.

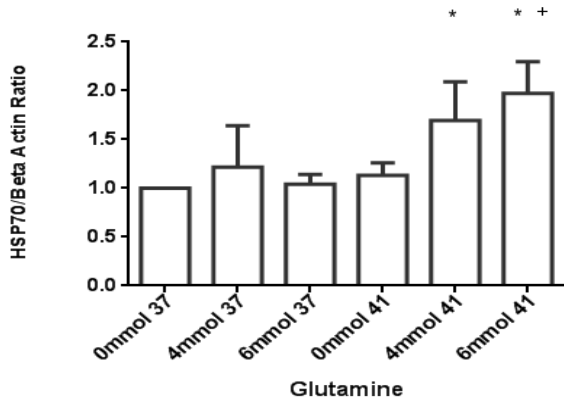

Figure 8. Glutamine supplementation combined with heat stress up-regulated HSP70 expression in Caco-2 intestinal cells. HSP70 was higher in the 4mmol 41 and 6mmol 41 compared to 0mmol 37 (control). HSP70 was higher in the 6mmol 41 trial compared to 0mmol 41. Data are mean ± SEM, n=4. * increased from 0mmol 37 trial, p<0.05. + increased from 0mmol 41 trial, p<0.05.

Glutamine supplementation preserved the stability of occludin at the tight junction (figure 9):

A two-factor ANOVA was used to compare the effects of glutamine supplementation (0mmol, 4mmol, and 6mmol) on occludin expression in control (37ºC) and heat stress (41ºC) trials. Glutamine supplementation was required to increase occludin expression during heat stress. The combined effect of glutamine and heat was statistically significant from when heat was applied to cells without glutamine, $F(2,18) = 7.711$, $p<0.05$. Occludin levels did not increase in control conditions, but were significantly

reduced when cells were exposed to heat in the absence of glutamine (0mmol 41°) when compared to control conditions (0mmol 37°) (0.7434 ± 0.015 ± 1.000 ± 0.000, p=0.003, respectively). Occludin levels were preserved when cells were supplemented with glutamine and exposed to heat stress. Occludin levels during both 4mmol 41ºC and 6mmol 41ºC trials were statistically higher when compared to 0mmol 41ºC (1.236 ± 0.143 and 1.849 ± 0.143 vs. 0.7434 ± 0.015, p=0.032, p<0.001, respectively), indicating stability of occludin at the TJ in response to heat stress.

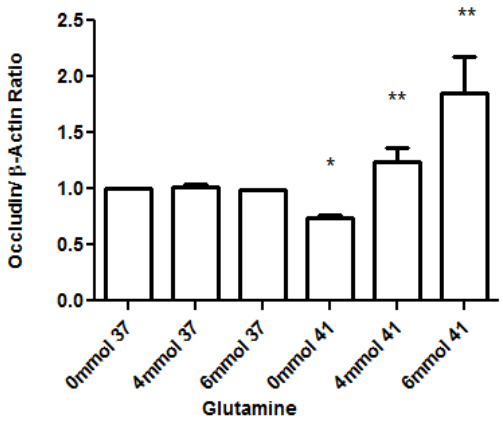

Figure 9. Glutamine supplementation stabilized occludin in Caco-2 intestinal cells. Occludin expression significantly decreased in the 0mmol 41 trial compared to the 0mmol 37 (control) trial. Data are mean ± SEM, n=4. * increased from 0mmol 37 trial, p<0.05. ** statistically different from 0mmol 41, p<0.05.

DISCUSSION

Exercise-induced gastrointestinal distress is a complex process with many variables playing roles. The stress of high intensity exercise has been shown to increase intestinal permeability (3), and lead to elevations in plasma levels of gram negative bacteria (endotoxin) (4) stimulating an immune response and a pro-inflammatory cascade of events (figure 1) (21), eventually causing GI distress. (1) Here, we demonstrate that seven days of oral glutamine supplementation protected the gut during high intensity endurance exercise by reducing intestinal permeability and endotoxin leakage. Through a

total body exercise model combined with a proof of concept in vitro design, we have shown that the mechanism may be through activation of heat shock factor-1 and heat shock protein 70 leading to occludin stabilization at the tight junction. In addition, glutamine supplementation increased the expression of HSP70 and de-phosphorylated IκB-α in human peripheral blood mononuclear cells in response to exercise stress. This indicates a heightened level of protection to a pro-inflammatory response through the suppression of nuclear NFκ-B. Thus, we propose that the protective effects of glutamine on the gut in response to vigorous exercise may be two-fold, through (1) preserving the intestinal TJ barrier reducing paracellular movement of luminal toxins, and (2) suppression of pro-inflammatory mechanisms in immune cells through activation of HSP70 and cytosolic housing of NFκ-B.

Sixty-minutes of high intensity running (70% VO_2max) raised core temperature, and caused an increase in intestinal permeability, and plasma endotoxin levels two hours post-exercise. This effect was completely ameliorated in the GLN trial, and demonstrates the protective effects of glutamine supplementation on the gut. Exercise-induced intestinal permeability has been previously demonstrated during high intensity exercise (3, 22) and in heat stress trials. (10) Furthermore, mild endotoxemia has been induced during exercise in the heat when core temperature exceeds 39.5°C (4, 23), and correlates with gastrointestinal symptoms.(24) In clinical disease states the administration of glutamine has been shown to be effective. For example, enteral glutamine supplementation reduced intestinal permeability among patients undergoing systemic chemotherapy (25) and low birthweight infants with underdeveloped GI tracts.(26) In addition, glutamine administration in a rat jaundice model improved intestinal barrier

function and reduced endotoxin levels.(27) Our study demonstrates that oral glutamine supplementation in humans reduces exercise induced intestinal permeability, and prevents the rise in plasma endotoxin.

The protective effect of glutamine in the gut may be through activation of HSF-1 leading to HSP70 expression. In an in vitro (Caco-2) model, we have shown that glutamine supplementation with 4mmol/L and 6mmol/L up-regulates HSF-1 and HSP70 in response to heat stress. Singleton et al 2006 (28) fed rats oral glutamine for 5-days followed by induced heat stroke and demonstrated elevated HSF-1 and HSP70 expression in the gut, reduced permeability, and lower plasma endotoxin. Several follow-up studies have confirmed that glutamine's action against cellular stress is through the transcriptional activation of HSF-1.(16, 29-31) The process to glutamine induced increase in HSF-1and HSP70 stimulation may be through the hexosamine biosynthetic pathway (HBP). (30, 31) The HBP splits from the glycolytic pathway through fructose-6-phosphate, and glutamine serves as a key substrate leading to the activation of O-linked N-acetylglucosamine (GlcNAc) which plays a critical role in transcription regulation of the stress response.(31). Gong et al 2011(31) demonstrated that inhibition of GlcNAc prevented glutamine induced increase in HSF-1 and HSP70 in lipopolysaccharide (LPS) treated cardiomyocytes. In addition, Wishmeyer's group showed that glutamine induced HSP70 expression is dependent upon activation of HBP in mouse embryonic fibroblasts. (30). It is important to note that glutamine's stimulation of the heat shock protein pathway has only been shown in response to physical (heat, exercise), or chemical (LPS) stress. In other words, glutamine alone does not increase resting HSF-1 and HSP70 levels.

Occludin is a tetraspanning membrane protein, and along with claudins (claudin-1, claudin-2, claudin-3) plays a key role in regulating paracellular absorption and secretion mechanisms in the GI tract.(32, 33) Over-expression of occludin results in improved tight junction resistance, and reduced intestinal permeability. (34) In an in vitro model, we have demonstrated reduced occludin levels in intestinal cells exposed to 75-minutes of heat stress (41°C) followed by five hours at 37°C. This model was chosen in an attempt to simulate exercise stress and recovery. Glutamine supplementation at concentrations of 4mmol/L and 6mmol/L preserved occludin levels under heat stress conditions. The mechanism may be through HSF-1 regulation of occludin. Dokladny et al 2008 (11) demonstrated that HSF-1 plays a central role in mediating heat-induced occludin expression. Caco-2 monolayers were supplemented with quercetin, a known HSF-1 inhibitor, and upon heat stress occludin levels were diminished indicating HSF-1 regulation of occludin.(11) Our results support this mechanism and further show that glutamine activation of HSF-1 increased occludin levels.

Inflammatory cytokines released from lymphocytes have also been linked to intestinal permeability and exercise induced gastrointestinal distress. (24, 35) Tumor necrosis factor alpha (TNF-α), interluekin-1 beta (IL-1 β), interferron-gamma (IFN-γ), and additional cytokines have been shown to disrupt the epithelial cell barrier.(1, 35-37) The mechanism may be through T-cell cytokine release resulting in Na(+)/K(+)-ATPase inhibition leading to intestinal malabsorption and fluid accumulation in the gut. NFκ-B also is the transcription factor for many pro-inflammatory cytokines. Under control conditions it is inactively attached to IκB-α in the cytosol.(21, 38) Upon stimulation with LPS for example, IκB-α is phosphorylated and degraded releasing NFκ-B that

translocates to the nucleus where it activates genes of inflammatory proteins.(21, 38) Exercise induced endotoxemia and tissue exposed to LPS activate NFκ-B in PBMCs promoting pro-inflammatory cytokine release. (4, 12) Dokladny et al 2010 demonstrated that HSP70 over expression suppresses nuclear translocation of NFκ-B and inhibition of TNF-α release in human PBMCs exposed to LPS.(12) Previous work by Moseley's group (12) performed in an in vitro model, demonstrates that HSP70 regulates endotoxin induced nuclear NFκ-B activation. We have further shown that oral glutamine supplementation in humans up-regulates HSP70 and inhibits NFκ-B activation in PBMCs in response to exercise stress. This would indicate an elevated level of protection against an endotoxin insult from exercise by reducing cytokine release and improving gut function. However, we indirectly measured NFκ-B activation through IκB-α levels. A better model would be to measure cytosolic and nuclear fractions of NFκ-B. Future studies are needed to determine role of glutamine on exercise induced plasma cytokine levels.

The effects of glutamine supplementation on HSP70 regulation in PBMCs is not clear. Wischmeyer et al 2003 (39) supplemented human PBMCs in a cell culture model and demonstrated activation of HSP70 and decreased TNF-α levels in response to LPS stimulation. Conversely, Andreasen et al (40) intravenously supplemented men with glutamine for 10 hours followed by an endotoxin insult, but it did not lead to an increase in PBMC levels of HSP70. The conflicting results may be due to the dosage level as the cell model supplemented at a much higher dose. To our knowledge, we are the first to show that oral glutamine supplementation elevates PBMC activation of HSP70 in response to exercise stress. Our results may be due to the longer supplementation period

(7 days), which resulted in a higher resting glutamine level when compared to the Andreasen study (0.765mmol/L vs. 1.893mmol/L).(40) Interestingly, post-exercise plasma glutamine levels in the GLN trials declined by 52% whereas they declined by 21% in the Andreasen study (40) suggesting rapid glutamine uptake by splanchnic and skeletal muscle tissue. (41)

Exercise-induced gastrointestinal distress among recreational endurance athletes is common, with up to 60% reporting symptoms.(42) The pathway leading to gut dysfunction is complicated and multi factorial, but a common cause is damage to the epithelial cell barrier. Glutamine is a primary metabolic fuel for intestinal cells and lymphocytes, and may even exceed glucose and fatty acid metabolism.(43) We have demonstrated that oral glutamine supplementation protects the gut during high intensity exercise in two ways, (1) activation of HSF-1 and HSP70 leading to occludin stabilization lowering intestinal permeability and endotoxin leakage, and (2) stimulation of HSP70 and IκB-α in PBMCs, thus inactivating the NFκ-B pro-inflammatory pathway.

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CHAPTER 4

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Summary

The review manuscript entitled "Exercise Regulation of Intestinal Tight Junction Proteins" added new insights into exercise induced gastrointestinal distress. It is known that high intensity and prolonged exercise that increases core temperature causes intestinal tight junction dysfunction. However, the underlying molecular signals have never been discussed. The review paper is focused on key proteins (PKC, PKA, tyrosine kinase) that are up-regulated during exercise stress, and cause phosphorylation of TJ junction proteins occludin, claudin, and ZO-1. The various phosphorylation states of the TJ proteins control the stability of the intestinal barrier. The manuscript discussed the role that heat shock proteins (HSP) play in regulating these phosphorylation proteins. Intracellular heat shock proteins are induced under conditions of physical (exercise), and chemical (lipopolysaccharide) stress, and may provide protection for gut by regulating phosphorylational proteins. Further, nutritional supplements (glutamine, bovine colostrum, and zinc) that show evidence of inducing HSP were discussed, and potential strategies for protecting the gut during exercise stress.

The research manuscript entitled “The Effects of Oral Glutamine Supplementation on Exercise Induced Gastrointestinal Permeability and Heat Shock Protein Regulation" provides evidence that short term (7 days) oral glutamine supplementation protects the gut by stabilizing the intestinal epithelial tight junction

leading to reduced intestinal permeability, and plasma endotoxin leakage. The mechanism may be through glutamine activated HSF-1, HSP70 resulting in occludin stabilization at the tight junction. In addition, the research shows that glutamine activates HSP70 and IKB-α in peripheral blood mononuclear cells (PBMC), which is evidence of heightened protection against a pro-inflammatory response to exercise. The protective effects of oral glutamine on exercise induced intestinal permeability in humans has never shown. In addition, this is the first study to demonstrate glutamine effects on the HSP response in PBMC's of exercising humans.

Conclusions

The significant findings in this research were (1) seven days of oral glutamine reduced exercise induced gastrointestinal permeability and plasma endotoxin levels in humans, (2) glutamine's effects may be through activation of HSF-1 leading to HSP70 transcription, and occludin expression indicating greater TJ stability, and (3) glutamine suppresses the exercise induced NFK-α inflammatory cascade through stimulation of HSP70 and IKB-α in human PBMCs.

Recommendations

A key measurement that would have improved this research is the analysis of glutamine supplementation on permeability in a Caco-2 cell culture model. This would provide strong evidence about whether the protective effects of glutamine were through HSF-1, HSP70, and occludin. Another supplemental measurement would be to inhibit

HSF-1 through quercetin administration under conditions of glutamine supplementation and heat. This would support glutamine's role as a molecular signal for HSF-1.

It is recommended that future studies examine (1) the acute effects of oral glutamine supplementation on exercise induced intestinal permeability and the HSP response, (2) a cell model that examines glutamine's effect on cytokine activation, and inhibition of intestinal Na(+)/K(+)-ATPase, and (3) the effects of other known HSP regulators on exercise induced intestinal permeability.

APPENDICES

A. HIPAA Form

B. Informed Consent

C. Data and Safety Monitoring Plan

D. Flyer

E. Health History Questionnaire

F. Supplement Guidelines

G. Supplemental Figure 1

H. Supplemental Figure 2

APPENDIX A

UNIVERSITY OF NEW MEXICO HEALTH SCIENCES CENTER
HIPAA[1] AUTHORIZATION TO USE AND DISCLOSE
PROTECTED HEALTH INFORMATION FOR RESEARCH PURPOSES

Title of Study: The effects of oral glutamine supplementation on gastrointestinal permeability and heat shock protein regulation in endurance runners

Principal Investigator: **Len Kravitz, Ph.D.**
UNMHSC Department: **Health, Exercise, and Sport Sciences**
Mailing Address: **Johnson Center, B143**
1 University of New Mexico
Albuquerque, NM 87131

Co-Investigators: Micah Zuhl, Suzanne Schneider, Christine Mermier, Rosie Lanphere, Karol Dokladny, and Pope Moseley.

Sponsor:

1. **What is the purpose of this form?** You have been asked to take part in a research study. The consent form for this study describes your participation, and that information still applies. This extra form is required by the federal Health Insurance Portability and Accountability Act (HIPAA). The purpose of this form is to get your permission (authorization) to use health information about you that is created by or used in connection with this research.

2. **What if I don't want my personal health information (PHI) to be used in this research study?** You do not have to give this permission. Your decision not to sign this form will not change your ability to get health care outside of this research study. However, if you do not sign, then you will not be allowed to participate in the study.

3. **What PHI am I allowing to be used for this research?** The information that may be used includes: Health history questionnaire data, including history of cardiovascular disease, previous injury, medications, history of gastrointestinal distress during exercise, and current exercise program. Height, weight, skin fold caliper, and maximal oxygen consumption will be measured. In addition blood and urine tests will be taken.

4. **Where will researchers go to find my PHI?** We may ask to see your personal information in records at hospitals, clinics or doctor's offices where you may have

[1] HIPAA is the Health Insurance Portability and Accountability Act of 1996, a federal law related to privacy of health information.

received care in the past, including but not limited to facilities in the UNM health care system.

5. **Who will be allowed to use my information for this research and why?** The researchers named above and their staff will be allowed to see and use your health information for this research study. It may be used to check on your progress during the study, or analyze it along with information from other study participants. Sometimes research information is shared with collaborators or other institutions. Your records may also be reviewed by representatives of the research sponsor or funding agency, the Food and Drug Administration (FDA) to check for quality, safety or effectiveness, or the Human Research Review Committee (HRRC) for the purposes of oversight and subject safety and compliance with human research regulations.

6. **Will my information be used in any other way?** Your information used under this permission may be subject to re-disclosure outside of the research study and be no longer protected under certain circumstances such as required reporting of abuse or neglect, required reporting for law enforcement purposes, and for health oversight activities and public health purposes.

7. **What if I change my mind after I give this permission?** You can change your mind and withdraw this permission at any time by sending a written notice to the Principal Investigator at the mailing address listed at the top of this form to inform the researcher of your decision. If you withdraw this permission, the researcher may only use and share your information that has already been collected for this study. No additional health information about you will be collected by or given to the researcher for the purposes of this study.

8. **What are the privacy protections for my PHI used in this research study?** HIPAA regulations apply to personal health information in the records of health care providers and other groups that share such information. There are some differences in how these regulations apply to research, as opposed to regular health care. One difference is that you may not be able to look at your own records that relate to this research study. These records may include your medical record, which you may not be able to look at until the study is over. The HIPAA privacy protections may no longer apply once your PHI has been shared with others who may be involved in this research.

9. **How long does this permission allow my PHI to be used?** If you decide to be in this research study, your permission to access and use your health information in this study may not expire, unless you revoke or cancel it. Otherwise, we will use your information as long as it is needed for the duration of the study.

I am the research participant or the personal representative authorized to act on behalf of the participant. By signing this form, I am giving permission for my personal health information to be used in research as described above. I will be given a copy of this authorization form after I have signed it.

_____ _____ _____

Name of Research Subject Signature of Subject/Legal Representative Date

_____

Describe authority of legal representative

_____ _____ _____

Name of Person Obtaining Authorization Signature Date

APPENDIX B

The University of New Mexico
Consent to Participate in Research

The effects of oral glutamine supplementation on gastrointestinal permeability and heat shock protein regulation in endurance runners

06/07/2012

Introduction

You are being asked to participate in a research study that is being done by Dr. Len Kravitz, who is the principal investigator, and Micah Zuhl, who is a doctoral student from the Department of Health, Exercise, and Sport Sciences. This research is studying the effects of oral glutamine supplementation on gastrointestinal dysfunction in endurance runners who have a history of gastrointestinal symptoms during exercise.

Gastrointestinal (GI) distress is common among endurance athletes with 60% reporting symptoms during competition or training. Commonly reported symptoms include diarrhea, vomiting, nausea, bloating, heartburn, and cramping. Runners appear to suffer predominately lower GI discomfort in areas of the small and large intestines, and many athletes report distress during their event and up to several hours afterwards. The cause of GI distress is complex, and may be caused by the high intensity effort of running, and the increase in core temperature that occurs during long duration endurance events. Our study will analyze GI distress during high intensity running that causes core temperature increase, and we will determine if oral glutamine supplementation prevents GI dysfunction during exercise.

You are being asked to participate in this study because you are an endurance athlete, and suffer from exercise induced gastrointestinal distress. Twelve people will take part in this study at the University of New Mexico.

This form will explain the research study, and will also explain the possible risks as well as the possible benefits to you. We encourage you to talk with your family and friends before you decide to take part in this research study. If you have any questions, please ask one of the study investigators.

What will happen if I decide to participate?

If you agree to participate, the following things will happen:

Your participation will involve three visits to the University of New Mexico Exercise Physiology Lab. During the first visit baseline measurements will be taken after an overnight fast and will include a treadmill maximal oxygen consumption test, body fat

percentage, gastrointestinal permeability, and blood measurements of plasma endotoxins, plasma glutamine, heat shock protein 70 (HSP 70), and nuclear factor kappa beta (NF-kappa B). Women who are pregnant will be excluded from the study, and each female subject will be required to take a urine pregnancy test during during baseline measurements. The treadmill maximal oxygen consumption test is an 8-12 minute running test to fatigue where the amount of oxygen consumed is measured throughout the test, and the highest value of oxygen consumed is used. During this test you will be required to wear a mouthpiece and nose clip. Body fat percentage will be measured using 3-site skinfold caliper test (women: triceps, hip, thigh and men: chest, abdominal, thigh). Gastrointestinal permeability will be measured in the urine after the ingestion of a 50ml (3.3 tbs) sugar drink, and urine will be collected for 5 hours after consuming the drink. During the 5 hour collection period you will carry around a 2000ml urine container that you will void into. Blood measurements will be made by collecting a 20ml (1.3 tbs) venous blood sample by inserting a needle intravenously.

The two additional visits will require 60 minutes of high intensity (70%) treadmill running in a warm environmental chamber (86°F). During each bout heart rate and core temperature will be measured using a heart rate monitor, and a rectal probe placed 13cm past the anal sphincter, respectively. Each trial will be terminated if core temperature reaches 104°F. Gastrointestinal permeability and blood markers (plasma endotoxins, plasma glutamine, HSP 70, and NF-kappa Beta) will be measured after each trial. One exercise bout will be a control, while the remaining two will be after 7-days of glutamine (.9 g/kg/fat free mass) or placebo supplementation. The length of time between each trial will be 4-weeks.

Gastrointesintal permeability after each of the three exercise trials will be measured in the urine after consuming a 50ml (3.3 tbs) sugar drink. The consumption of the drink will occur 20minutes into each exercise bout. Urine will be collected up to 5hours after each trial, and will require fasting during this time. You will be able to drink water during the first hour of the 5-hour fast. Blood measurements will be made by collecting a 20ml (1.3 tbs) venous blood sample at four time points. A resting 20ml (1.3tbs) blood sample will be collected prior to the start of the exercise trial. Immediately after each trial a 20ml (1.3 tbs) blood sample will be collected via venous puncture. Two hours after each exercise trial another 20ml (1.3 tbs) blood sample will be collected via venous puncture. Four hours after each exercise trial the final 20ml (1.3 tbs) blood sample will be collected via venous puncture. In total, 80ml (5.4 tbs) of blood will be collected during each trial (20ml collected at rest, post exercise, 2hrs post exercise, and 4hrs post exercise). The total amount of blood collected will be 180ml (12.17 tbs) over the entire length of the study (20ml at baseline, and 2 x 80ml for exercise trials).

How long will I be in this study?

Participation in this study will take a total of 4 hours over a period of six weeks.

What are the risks or side effects of being in this study?

Participation during this study may increase your risk for muscle soreness as you will be required to perform three running sessions at 70% of your maximal effort. The risk of soreness is increased at this higher intensity. Blood drawing risks: Drawing blood may cause temporary pain and discomfort from the needle stick, occasional brusing, sweating, feeling faint or lightheaded and in rare case there is risk of infection and tissue damage. The placement of the rectal probe may also cause discomfort. In addition, there is always a minor, very low risk of a cardiovascular event (about 1 in 10,000) when a healthy subject performs exercise. There may be a slight risk of mild GI disturbance (diarrhea, cramping) from the dietary supplements.

Your participation is completely voluntary and you may withdraw at any point whether or not you experience any of these side effects. There are risks of stress, emotional distress, inconvenience and possible loss of privacy and confidentiality associated with participating in a research study. For more information about risks and side effects, ask the investigator.

What are the benefits to being in this study?

There may or may not be any direct benefits for participating in this study. There may be a benefit of learning VO2max.

What other choices do I have if I do not want to be in this study?

Your participation is voluntary, and if you decide to not be in this study then you will not be contacted again.

How will my information be kept confidential?

We will take measures to protect the security of all your personal information, but we cannot guarantee confidentiality of all study data.

Information contained in your study records is used by study staff and, in some cases it will be shared with the sponsor of the study. The University of New Mexico Human Research Review Committee (HRRC) that oversees human subject research and/or other entities may be permitted to access your records. There may be times when we are required by law to share your information. However, your name will not be used in any published reports about this study.

Your information will be stored in a lock cabinet in the UNM Exercise Physiology Lab. In addition, you will only be known as a subject number, which will not be linked to your name.

What are the costs of taking part in this study?

The only cost of participating in this study is your time.

What will happen if I am injured or become sick because I took part in this study?

If you are injured or become sick as a result of this study, UNMHSC will provide you with emergency treatment, at your cost.

No commitment is made by the University of New Mexico Health Sciences Center (UNMHSC) to provide free medical care or money for injuries to participants in this study.

In the event that you have an injury or illness that is caused by your participation in this study, reimbursement for all related costs of care will be sought from your insurer, managed care plan, or other benefits program. If you do not have insurance, you may be responsible for these costs. You will also be responsible for any associated co-payments or deductibles required by your insurance.

It is important for you to tell the investigator immediately if you have been injured or become sick because of taking part in this study. If you have any questions about these issues, or believe that you have been treated carelessly in the study, please contact the Human Research Review Committee (HRRC) at the University of New Mexico Health Sciences Center, Albuquerque, New Mexico 87131, (505) 272-1129 for more information.

Will I be paid for taking part in this study?

You will be paid $50 for your participation, and will be dispersed at the beginning ($25), and completion of the study ($25).

How will I know if you learn something new that may change my mind about participating?

You will be informed of any significant new findings that become available during the course of the study, such as changes in the risks or benefits resulting from participating in the research or new alternatives to participation that might change your mind about participating.

Can I stop being in the study once I begin?

Your participation in this study is completely voluntary. You have the right to choose not to participate or to withdraw your participation at any point in this study.

Whom can I call with questions or complaints about this study?

If you have any questions, concerns or complaints at any time about the research study, Len Kravitz, or his/her associates will be glad to answer them at (505) 277-2658.

If you need to contact someone after business hours or on weekends, please call (269) 767-0003 and ask for Micah Zuhl.

If you would like to speak with someone other than the research team, you may call the UNMHSC HRPO at (505) 272-1129.

Whom can I call with questions about my rights as a research subject?

If you have questions regarding your rights as a research subject, you may call the UNMHSC HRPO at (505) 272-1129. The HRPO is a group of people from UNM and the community who provide independent oversight of safety and ethical issues related to research involving human subjects. For more information, you may also access the IRB website at http://hsc.unm.edu/som/research/hrrc/irbhome.shtml.

CONSENT

You are making a decision whether to participate in this study. Your signature below indicates that you read the information provided (or the information was read to you). By signing this consent form, you are not waiving any of your legal rights as a research subject.

I have had an opportunity to ask questions and all questions have been answered to my satisfaction. By signing this consent form, I agree to participate in this study. A copy of this consent form will be provided to you.

_____ _____ _____

Name of Adult Subject (print) Signature of Adult Subject Date

INVESTIGATOR SIGNATURE

I have explained the research to the subject and answered all of his/her questions. I believe that he/she understands the information described in this consent form and freely consents to participate.

_____

Name of Investigator/ Research Team Member (type or print)

_____ _____

(Signature of Investigator/ Research Team Member) Date

APPENDIX C

UNM HEALTH SCIENCES CENTER
Office of Research

Data and Safety Monitoring Plan (DSMP)
Attachment 6

NOTE: For NMCCA studies, please complete Section 1 and attach the NMCCA DSMP short form.

1. Check the proposed level of risk and provide justification for this determination:

☐ **Minimal Risk:**
The probability and magnitude of harm or discomfort anticipated in the research are not greater in and of themselves than those ordinarily encountered in daily life or during the performance of routine physicals or psychological examinations or tests.
Justify: _____

☒ **Moderate Risk:**
Risks are more than minimal but less than high risk as defined below.
Justify: Subjects will perform an exercise test up to a maximal intensity. The test will take no more than 12 minutes, with only the last 1/3 of the test at higher intensity. In addition, subjects will perform three 60-minute bouts of exercise in a warm environement (30 degrees Celcius). Subjects will be well trained runners who train at high intensity daily and achieve maximal effort in both training and competition.

☐ **High Risk:**
Interventions associated with risk of serious adverse events at high or uncertain frequency; studies in populations associated with very high risk of serious adverse clinical events based on underlying disease or in whom assessment of treatment associated adverse events may be difficult.
Justify: _____

2. Data & Safety Monitoring

A. Is there a Data & Safety Monitoring Board (DSMB) in place for this study? ☐ Yes ☒ No
If YES, provide board name: _____

B. Is there a designated Date Safety Monitor for this study? ☒ Yes ☐ No
If YES, provide monitor name: Dr. Pope Moseley. Dr. Moseley will review exercise trial data after the completion of 6 subjects. During each exercise trial Dr. Moseley will be available to address any safety concerns.

C. Is there a separate Data & Safety Monitoring Plan (DSMP) in place for this study (i.e. NIH grant)? ☐ Yes ☒ No
If YES, submit plan and do not complete the rest of this attachment

D. Please describe the plan to monitor progress and safety: Subject stress will be monitored by heart rate, thermal discomfort scores, and core temperature during all exercise trials. Core temperature of 40°C will be the cutoff as heat related injury does not occur until core temperature increases beyond this point. If subjects show symptoms of dizziness, confusion, extreme nausea, or ataxia the trial will be terminated. Subjects will be monitored up to 5hours after each trial to insure adequate recovery. Subjects are free to terminate the test at any time if they experience undo distress. The exercise laboratory is equipped with emergency medical equipment (crash cart, oxygen, drugs) and emergency procedures in place to respond to an adverse event. The researchers assisting in each trial will be CPR certified. _____

E. What information would result in early termination of the study?
(i.e. unanticipated events; early demonstration of efficacy, inferiority, or futility where results will not confirm nor refute the hypothesis, etc.)
Serious unanticipated events would result in early termination of the study.

APPENDIX D

HRPO# 12-155

The effects of oral glutamine supplementation on gastrointestinal permeability and heat shock protein regulation in endurance runners

We are testing the effects of oral glutamine supplementation on gastrointestinal distress among endurance runners who have a history of this condition.

Looking for endurance runners, who have experienced gastrointestinal distress during training or competition.

If interested in being a subject in our study at The University of New Mexico, please contact Micah Zuhl, M.S. at 269-767-0003, or my email zuhl09@unm.edu.

APPENDIX E
HEALTH HISTORY QUESTIONNAIRE (RESEARCH ONLY)

Subject #______________________________ Date___/___/___

Phone #: home_________________________ cell_______________

Date of Birth ___/___/___ Age____ Gender____ Ethnicity_______ Phone (W)___________________

Address (home)____zip__________
email______________________________________

Primary health care provider and health insurance____

(Only for information/emergency contact)

Person to contact in case of emergency: **name_____________________________phone #**_____________

♦♦

MEDICAL HISTORY

Self-reported: Height______ Weight______

Physical injuries:____
Limitations____
Have you ever had any of the following cardiovascular problems? Please check all that apply.

Heart attack/Myocardial Infarction____ Heart surgery ____Valve problems_____

Chest pain or pressure ___Swollen ankles____Dizziness_____Arrhythmias/Palpitations____

Heart murmur____Shortness of breath _____Congestive heart failure____

Have you ever had any of the following? Please check all that apply.
Hepatitis/HIV_____Depression_____Cancer (specify type)__________

Rheumatic fever_____High blood pressure_____ Thyroid problems_____

Kidney/liver disease_____Obesity_____ Total cholesterol >200 mg/dl_____

Diabetes (specify type)_____Asthma_____ HDL cholesterol <35 mg/dl_____

Emphysema_____Stroke____ LDL cholesterol >135 mg/dl_____

Do immediate blood relatives (biological parents & siblings **only**) have any of the conditions listed above? If yes, list the problem, and family member age at diagnosis.
____

Is your mother living? Y N Age at death______ Cause___________________

Is your father living? Y N Age at death______ Cause___________________

Have you ever been diagnosed with a gastrointestinal disease or disorder? Y N

Have you ever experienced a heat related illness? Y N

Do you currently have any condition not listed that may influence test results? Y N
Details____
____

Indicate level of your overall health. Excellent ____ Good ____ Fair ____ Poor_____
Are you taking any medications, vitamins or dietary supplements now? Y N
If yes, what are they?____
Do you have allergies to any medications? If yes, what are they? ___________________________________
Are you allergic to latex? Y N
Have you been seen by a health care provider in the past year? Y N
If yes, elaborate ____
Have you had a prior treadmill test? Y N. If yes, when?______________ What were the results?
____
Have you ever experienced any adverse effects during or after exercise (fainting, vomiting, shock, palpitations, hyperventilation)? Y N If yes, elaborate.____
____

♦♦

LIFESTYLE FACTORS

Do you now or have you ever used tobacco? Y N If yes: type ________________
How long?______ Quantity____/day Years since quitting______________
How often do you drink the following?
Caffeinated coffee, tea, or soda _______oz/day Hard liquor _______oz/wk Wine _______oz/week
Beer _______oz/wk
Indicate your current level of emotional stress. High____ Moderate ____ Low____

♦♦♦

PHYSICAL ACTIVITY/EXERCISE

Physical Activity
Minutes/Day (*Weekdays*) Minutes/Day (*Weekends*)
______/______ average ______/_____ average
Do you train in any activity (eg. jogging, cycling, swimming, weight-lifting)? Y N
How well trained are you?

Vigorous Exercise (>30 Minute sessions)
_________Minutes/hours a week

♦♦

WOMEN ONLY

Please check the response that most closely describes your menstrual status:
_____ Post-menopausal (surgical or absence of normal menstrual periods for 12 months)
_____ Eumenorrheic – Normal menstrual periods (~every 28 days)
_____ Amenorrheic – Absence of normal menstrual periods for at least 3 months
_____ Oligomenorrheic – Irregular menstrual periods with occasional missed cycles.

APPENDIX F

Supplement Guidelines

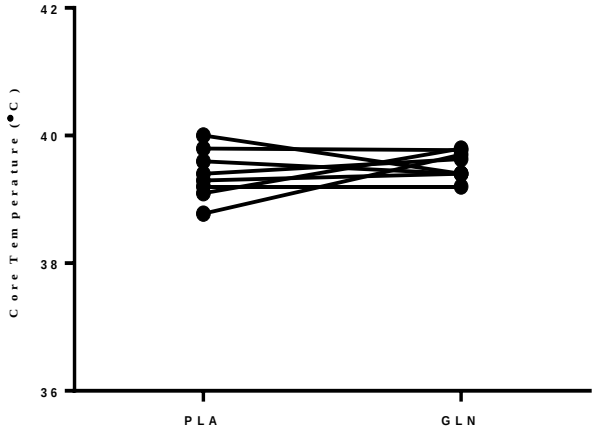
1. Take supplement on empty stomach, at least 4hours after a meal, or before meal time

2. Mix supplement bag with approximately 8oz of water, and be sure all substance is dissolved.

3. Take supplement 3 times per day. In the morning, mid-day, and evening.

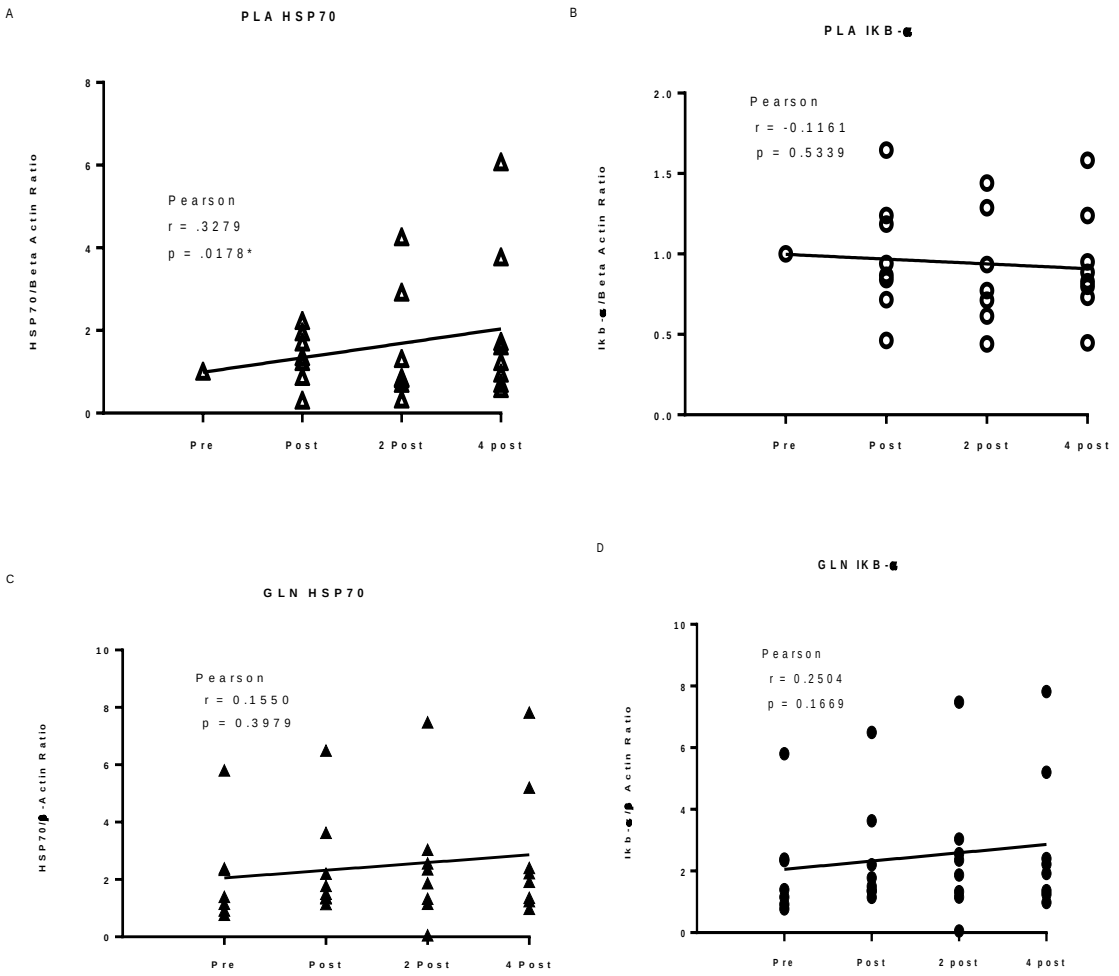
4. Please report any adverse affects to Micah Zuhl, 269-767-0003

APPENDIX G



Supplemental Figure 1: Core Temperature. End exercise core temperature for each subject during the placebo (PLA) and glutamine (GLN) trials. *significant at $p < 0.05$

APPENDIX H



Supplemental Figure 2. The relationship between time and HSP70 was significant in the placebo (PLA) trial only. The relationship between time and IKB-α was not different in both the glutamine (GLN) and PLA trial. Evidence that the HSP70 increase from baseline is greater without glutamine but that GLN enhances the HSP70 and IKB-α overall levels. HSP70 and IKB-α were measured in hu man PBMCs after 7-days of glutamine (GLN) and placebo supplementation. Measurements were made pre-exercise (Pre), post-exercise (Post), 2hrs post-exercise (2 Post), and 4hrs post-exercise (4 Post). * Pearson r statistically significant, $p<0.05$.

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