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High Altitude Exposures and Intestinal Barrier Dysfunction

Zachary J. McKenna
zmckenna@unm.edu

Zachary John McKenna

Candidate

Health, Exercise, and Sports Sciences

Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Dr. Christine Mermier , Chairperson

Dr. Fabiano Amorim

Dr. Michael Deyhle

Dr. Trevor Gillum

HIGH ALTITUDE EXPOSURES AND INTESTINAL BARRIER DYSFUNCTION

BY

ZACHARY JOHN MCKENNA

B.Sc., Kinesiology
California Baptist University
2015

M.Sc., Kinesiology
California Baptist University
2017

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ABSTRACT

Gastrointestinal complaints are often reported during high altitude ascent (>2500m), though their etiology is unknown. One explanation is hypoxia-mediated intestinal barrier dysfunction. High altitude exposures can result in splanchnic hypoperfusion and hypoxemia causing hypoxic and oxidative stress. Exertion may worsen hypoxia-induced intestinal injury via greater splanchnic hypoperfusion and hypoxemia. We propose that these stressors injure the intestinal barrier leading to increased permeability, bacterial translocation, and local/systemic inflammation which may contribute to gastrointestinal complications or Acute Mountain Sickness (AMS). To test this, we investigated the effects of hypoxia on exercise-induced gastrointestinal symptoms and markers of intestinal injury. Next, we determined the effects of a longer hypoxic exposure on circulating markers of intestinal barrier dysfunction and inflammation. We also determined if these responses were related to AMS development. Finally, we evaluated the effects of ibuprofen on markers of intestinal barrier injury, inflammation, and gastrointestinal symptoms at rest and during exercise in hypoxia.

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SYMBOLS & ABBREVIATIONS

AMS: Acute Mountain Sickness

BLC: B lymphocyte chemoattractant

CD: Cluster of differentiation

CLDN-3: Claudin-3

COX: Cyclooxygenase

CRP: C-reactive protein

d: Cohen's d

EDTA: Ethylenediaminetetraacetic acid

FiO_2: Fraction of inspired oxygen

FITC: Fluorescein isothiocyanate

FOXP3: Forkhead box P3

GI: Gastrointestinal

HACE: High-altitude cerebral edema

HAPE: High-altitude pulmonary edema

HPLC: High performance liquid chromatography

HSF-1: Heat shock factor 1

HSP: Heat shock protein

I-BABP: Ileal bile-acid binding protein

I-FABP: Intestinal fatty-acid binding protein

IFN-γ: Interferon- γ

IFN-γ: interferon-γ

IL-1Ra: Interleukin-1 receptor agonist

IL: Interleukin

L-FABP: Liver fatty-acid binding protein

L/M: Lactulose to mannitol ratio

L/R: Lactulose to rhamnose ratio

LBP: Lipopolysaccharide binding protein

LLS: Lake Louise Score

LPS: Lipopolysaccharides

MCP-1: Monocyte chemotactic protein-1

NF-κB: Nuclear factor-κ light-chain-enhancer of activated B cells

NSAID: Non-steroidal anti-inflammatory drugs

r: Correlation coeffect

RER: Respiratory exchange ratio

ROS: Reactive oxygen species

RPE: Rating of perceived exertion

S-IgA: Secretory immunoglobulin A

SaO_2: Oxygen saturation

sCD14: Soluble cluster of differentiation 14

SD: standard deviation

SDF-1: Stromal differentiation factor-1α

TGF-b: Transforming growth factor-b

Th: T helper

TJ: Tight junction

TLR-4: Toll like receptor-4

TNF-α: Tumor necrosis factor- α

Treg: T regulatory

VO_2max: Maximum oxygen consumption

Δ: Mean difference

η_p^2: Partial eta squared

>: Greater than

<: Less than

≤: Less than or equal to

CHAPTER I: Introduction

Background

Gastrointestinal (GI) complaints are often reported during ascents to high altitude (> 2500 m) (1–3). Indeed, approximately 80% of people suffering from Acute Mountain Sickness (AMS) report at least one symptom of GI distress (e.g., anorexia, nausea, diarrhea, vomiting) (1). The prevalence of diarrhea has been reported to be 30-36% amongst mountaineers attempting to ascend Mt. Everest (3, 4), and gastroenteritis has been reported at a prevalence of 23% in a group of trekkers ascending to 5100 m, despite access to proper hygiene (5). GI lesions, including peptic ulcers have been observed within two to four days following ascent to high altitude (2), and high altitude GI bleeding, a potentially fatal condition, has also been reported amongst a group of manual laborers working at 4905 m (6). Interestingly, the etiologies of these high altitude-associated GI complications are not well understood. One possible explanation is injury to the intestinal barrier which has been implicated in the pathophysiology of several diseases (7, 8).

High altitude exposures can reduce splanchnic perfusion (9) and lower blood oxygen levels causing hypoxic and oxidative stress (10) which can injure the intestinal barrier. Hypoxia-induced intestinal injury may cause some of the acute GI symptoms reported during high-altitude ascents (e.g., diarrhea) (11). Hypoxia-induced oxidative stress might contribute to the formation of GI lesions such as peptic ulcers (12), and hypoxic stress in the GI tract can damage intestinal microvasculature leading to high altitude GI bleeding (6). A damaged intestinal barrier may impair nutrient absorption which, along with anorexia, could be the reason for some of the weight loss observed during prolonged exposures to high altitude (13–16). In addition, intestinal barrier injury can allow for luminal contents to pass through the

intestinal wall, a process known as increased intestinal permeability. Increases in intestinal permeability can allow for bacterial translocation and the activation of innate immune cells (e.g., resident macrophages, circulating monocytes, Kupffer cells) to initiate a local or systemic inflammatory response (i.e., release of cytokines into the bloodstream). The local release of pro-inflammatory cytokines in the intestinal tract can directly damage the intestinal barrier, increase intestinal permeability, and reduce Na^+/K^+-ATPase activity causing fluid accumulation in the intestinal lumen which may contribute to diarrhea (11, 17). Systemically, the inflammatory response could result in cytokines crossing the blood brain barrier which may have detrimental effects on the central nervous system (18) and contribute to the early onset of fatigue (19). This could partially explain why people suffering from AMS have increased circulating levels of pro-inflammatory cytokines following acute exposure to hypobaric hypoxia (20). In fact, inflammation has been shown to contribute to the development of high-altitude cerebral edema (HACE) (21) which may provide a connection between AMS and HACE.

For people traveling to high altitude, it is prudent to consider the effects of physical work or exertion that may worsen hypoxia-induced intestinal injury. A consequential effect of exercise on the GI tract is profound redistribution of blood flow. During exercise, splanchnic perfusion is reduced which allows for greater perfusion of the skin and contracting skeletal muscle (22, 23). The reduction in blood flow to the GI system during exercise has largely been attributed to an increase in sympathetic tone (24, 25). The effects of exercise on gut blood flow are especially problematic in hypoxic environments because low oxygen also results in greater sympathetic outflow and subsequent vasoconstriction (26). Further, in hypoxic conditions, such as those observed at high altitude, more blood is redistributed away

from the intestinal tract to meet the increased demands for skeletal muscle perfusion (27, 28) which exacerbates intestinal ischemia. Shunting of blood away from the intestinal tract has been shown to cause intestinal cell damage and increased permeability during a bout of exercise (23). Indeed, running (29) and cycling (30) in nomobaric hypoxia has been shown to increase circulating markers of intestinal injury and an increase in the circulation of proinflammatory cytokines when compared to exercise performed in normoxic conditions. Another study showed that exercise in normobaric hypoxia increases circulating endotoxins (31), suggesting increased intestinal permeability and bacterial translocation. However, no study to date has utilized an exercise protocol in hypobaric hypoxia which may present a greater physiological stress than normobaric hypoxia (32). In addition, these previous investigations have not examined the impact of exercise in hypoxia on acute GI symptomology, so it is unclear if the increases in markers of intestinal injury or bacterial translocation relate to GI symptoms.

Ibuprofen is an over-the-counter non-steroidal anti-inflammatory drug (NSAID) which is often used to treat pain, inflammation, and fever (33). Recently, researchers have shown that ibuprofen can prevent symptoms of AMS such as high-altitude headache (34, 35). However, it has been speculated that ibuprofen may worsen symptoms of gastrointestinal (GI) distress or even increase the risk of GI bleeding (36). Ibuprofen inhibits both cyclooxygenase 1 (COX-1) and COX-2 to prevent the formation of prostaglandins (37). In the gut, inhibition of COX enzymes can reduce microvascular blood flow and damage the intestinal barrier (38). In addition to this COX-mediated GI damage, ibuprofen may have COX-independent effects including direct interaction with the phospholipid bilayer and mitochondrial damage which can impair intestinal barrier function (37). Yet, the impact of ibuprofen on the

gastrointestinal system during exercise in high-altitude environments is not well characterized.

Specific Aims

The broader goal of this project was to better understand the impact of high-altitude exposures on the intestinal barrier dysfunction. Accordingly, the project had the following specific aims:

Aim 1 was to determine the effects of hypobaric hypoxia on exercise-induced GI symptoms and markers of intestinal barrier dysfunction. Previous studies have shown that exercise in nomobaric hypoxia increases markers of intestinal injury and inflammation (30, 39), thus we speculated that exercise-induced GI symptoms and markers of intestinal barrier dysfunction would be greater following exercise hypobaric hypoxia compared to normoxia.

Aim 2 was to determine if intestinal barrier injury or its inflammatory consequence was related to the development AMS. The pathophysiology of AMS is not well understood, though one prevailing theory is related to dysfunction within the central nervous system (i.e., the brain) (40). Given the high incidence of GI symptoms associated with high altitude exposures, we speculated that the GI system may contribute to the development of AMS as well as other high-altitude associated GI complications (2, 6).

Aim 3 was to evaluate the effect of ibuprofen on symptoms of GI distress, and markers of intestinal barrier dysfunction following exercise in hypobaric hypoxia. Ibuprofen is known to damage the intestinal barrier through inhibition of cyclooxygenase 1/2 and via direct interaction with the phospholipid bilayer (37). Thus, we speculate that ibuprofen will worsen

these effects of exercise-induced intestinal injury and further contribute to high-altitude associated intestinal barrier dysfunction.

We feel that the results of this project will have a broad impact on the field of high-altitude physiology and lead to a deeper understanding of the pathophysiology of AMS as well as other high-altitude related GI complications. In the long term, we hope that the results from these studies inform novel therapeutic targets to treat high-altitude illnesses which will benefit a wide range of groups including mountaineers, military personnel, wildland firefighters, hikers, skiers, and athletes who travel or ascend to high-altitude to perform physical work and/or exercise.

Outline

This dissertation is organized into five subsequent chapters. In chapter II we review the current evidence examining the influence of high-altitude exposures on the gastrointestinal system. In chapter III we test the hypothesis that acute exercise with concurrent hypoxic exposure causes intestinal barrier injury. In chapter IV we determine the effects of a longer hypoxic exposure on markers of intestinal barrier injury and circulating markers of inflammation. In addition, in chapter IV we determine if intestinal barrier dysfunction is related to the development of AMS. In chapter V we examine the impact of ibuprofen on intestinal barrier dysfunction at rest and during exercise in hypobaric hypoxia. Finally, in chapter VI we present a summary of our key findings and presents some avenues for future investigations.

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CHAPTER II: Literature Review

This chapter presents a review manuscript entitled “High altitude exposures and intestinal barrier dysfunction” which has been accepted for publication in *American Journal of Physiology Regulatory and Integrative Physiology*. Tables, figures, and references are provided at the end of the manuscript

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High altitude exposures and intestinal barrier dysfunction

Zachary J. McKenna[1], Felipe Gorini Pereira[2], Trevor L. Gillum[3], Fabiano T. Amorim[1], Michael R. Deyhle[1], Christine M. Mermier[1]

[1] Department of Health, Exercise and Sports Sciences, University of New Mexico Albuquerque, NM, USA

[2] Department of Kinesiology, Indiana University, Bloomington, IN, USA

[3] Department of Kinesiology, California Baptist University, Riverside, CA, USA

Corresponding author: Zachary McKenna

Email: zmckenna@unm.edu

Phone: N/A

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Abstract

Gastrointestinal complaints are often reported during ascents to high altitude (> 2500 m), though their etiology is not known. One potential explanation is injury to the intestinal barrier which has been implicated in the pathophysiology of several diseases. High altitude exposures can reduce splanchnic perfusion and blood oxygen levels causing hypoxic and oxidative stress. These stressors might injure the intestinal barrier leading to consequences such as bacterial translocation and local/systemic inflammatory responses. The purpose of this mini review is to 1) discuss the impact of high-altitude exposures on intestinal barrier dysfunction, and 2) present medications and dietary supplements which may have relevant impacts on the intestinal barrier during high-altitude exposures. There is a small but growing body of evidence which shows that acute exposures to high altitudes can damage the intestinal barrier. Initial data also suggests that prolonged hypoxic exposures can compromise the intestinal barrier through alterations in immunological function, microbiota, or mucosal layers. Exertion may worsen high-altitude related intestinal injury via additional reductions in splanchnic circulation and greater hypoxemia. Collectively these responses can result in increased intestinal permeability and bacterial translocation causing local and systemic inflammation. More research is needed to determine the impact of various medications and dietary supplements on the intestinal barrier during high-altitude exposures.

Introduction

Gastrointestinal (GI) complaints are often reported during ascents to high altitude (> 2500 m) (1–3). For example, approximately 80% of people suffering from acute mountain sickness (AMS) report at least one symptom of GI distress (e.g., anorexia, nausea, diarrhea, vomiting) (1). The prevalence of diarrhea has been reported to be 30-36% amongst mountaineers attempting to ascend Mt. Everest (3, 4), and gastroenteritis has been reported at a prevalence of 23% in a group of trekkers ascending to 5100 m, despite access to proper hygiene (5). GI lesions, including peptic ulcers have been observed within two to four days following ascent to high altitude (2). Further, high altitude GI bleeding, a potentially fatal condition, has also been reported amongst a group of manual laborers working at 4905 m (6). Interestingly, the etiologies of these high altitude-associated GI complications are not well understood. One possible explanation is injury to the intestinal barrier which has been implicated in the pathophysiology of several diseases (7, 8).

Hypoxia-induced intestinal injury could explain some of the acute GI symptoms reported during high-altitude ascents (e.g., diarrhea) (9). Hypoxia-induced oxidative stress might contribute to the formation of GI lesions such as peptic ulcers (10), and hypoxic stress in the GI tract can damage intestinal microvasculature leading to high altitude GI bleeding (6). A damaged intestinal barrier may impair nutrient absorption which could explain some of the weight loss observed during prolonged exposures to high altitude (11–14). In addition, intestinal barrier injury can allow for luminal contents to pass through the intestinal wall, a process known as increased intestinal permeability. Increases in intestinal permeability can allow for bacterial translocation and the activation of innate immune cells (e.g., resident macrophages, circulating monocytes, Kupffer cells) to initiate a local or systemic

inflammatory response (i.e., release of cytokines into the bloodstream). This could partially explain why people suffering from AMS have increased circulating levels of interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor- α (TNF-α) following acute exposure to hypobaric hypoxia (15). In fact, inflammation has been shown to contribute to the development of high-altitude cerebral edema (HACE) (16) which may provide a connection between AMS and HACE.

Despite this evidence, research investigating the link between intestinal injury, increased intestinal permeability, or bacterial translocation and high-altitude related GI complications is limited. Hypoxia-induced damage to the intestinal barrier is relevant for several populations including mountaineers, military personnel, wildland firefighters, hikers, skiers, athletes, and other people who might ascend to high-altitude to work or recreate. Therefore, the purpose of this review is to discuss the impact of high-altitude environments on intestinal barrier dysfunction. In addition, we will discuss the role of physical exertion which may worsen hypoxia-induced intestinal injury. Finally, we will present some medications and dietary supplements that are commonly used during high altitude ascent which may have relevant impacts on the intestinal barrier during high altitude exposure.

Overview of the Intestinal Barrier

A fundamental function of the GI system is to allow for the transport of essential nutrients across the intestinal epithelium while prohibiting the translocation of potentially harmful substances, such as endotoxins which reside on the outer membrane of gram-negative bacteria, that exist within the intestinal tract. This process is regulated by the intestinal barrier which is made up by a continuous monolayer of intestinal epithelial cells (e.g., enterocytes, goblet cells, Paneth cells, enteroendocrine cells, etc.) that are adjoined by

junctional complexes (i.e., tight junctions, adherens junctions, and desmosomes) (see Figure 1). Adherens junctions and desmosomes serve to maintain the structural integrity of the intestinal barrier, while tight junction complexes located near the apical border of the cell are responsible for the transport of small molecules across the intestinal wall. Goblet cells secrete mucins to produce the mucosal layers which overlay the cell monolayer and contribute to the intestinal barrier by preventing many substances from making direct contact with the epithelial cells (17).

In addition to these physical components, the intestinal tract is home to various immunological factors which contribute to the intestinal barrier. For example, enterocytes and Paneth cells secrete antimicrobial proteins that can kill or inactivate microorganisms within the intestinal lumen (18, 19). Plasma cells in the GI tract produce secretory immunoglobulin A (S-IgA) which helps maintain intestinal homeostasis by neutralizing pathogens, downregulating inflammatory responses, and regulating gut microbiota composition (20). Resident immune cells including macrophages, dendritic cells, and T-cells residing in the lamina propria and Peyer's patch play vital roles in responding to luminal contents and maintaining immune homeostasis.

For example, dendritic cells capture and present antigens to the adaptative immune system supporting the differentiation of naïve T-cells. T-cells are a diverse class of immune cells that are broadly characterized based on the expression of cluster of differentiation (CD) 4 or CD8. The former, CD4-positive T-cells are called T helper (Th) cells. These cells are further classified into subsets including Th1, Th2, Th17, and T regulatory (Treg) cells based of the functional phenotype (e.g., their secretory repertoire) and immunological niche they fill (e.g., combating extracellular or intracellular pathogens, etc.). While some CD4-positive

T cells exit the thymus already committed to a particular subset (e.g., natural Tregs), most are polarized by environmental cues (e.g., cytokines present) when they become activated by an antigen presenting cell (21). These environmental cues upregulate specific combinations of transcription factors that control the transcriptional activity of the cells to endow them with the characteristics of their Th subset. Of interest, Th17 cells have emerged as major players in intestinal immune function (21). Th17 cells are generated in the presence of cytokines including IL-6, interleukin-22 (IL-22), and transforming growth factor-β (TGF-β) (22), which induce the expression of the master transcriptional regulator RORγt, enabling these cells to secrete their signature cytokines such as interleukin-17 (IL-17), TNF-α, and IL-22 (23). Th17 cells serve protective roles secreting various cytokines and chemokines, recruiting phagocytic immune cells, and causing the production of antimicrobial proteins from non-immune cells in the intestinal microenvironment; these cells aid in the appropriate response to intestinal barrier damage, inflammation, repair, and resolution (24).

Disruption to any one of the components of the intestinal barrier can lead to increased intestinal barrier dysfunction. For example, mice lacking Mucin 2, a main component of the intestinal mucosal layer, display increased intestinal permeability (25). Likewise knockdown of the tight junction protein occludin *in vitro* (Caco-2 cells) and *in vivo* (mice) leads to increased macromolecule flux across the intestinal barrier (26). Both hypoxia and oxidative stress are known to disrupt the intestinal barrier (27, 28). Cultured intestinal cells (Caco-2:HT-29 co-culture) exposed to hypoxia (5% O_2) display decreased expression of tight junction proteins ZO-1, claudin-3, and occludin (27) which indicates an impaired intestinal barrier. Hypoxia also downregulates at the mRNA levels of genes related to the intestinal mucosal layers (MUC-2 and MUC-5AC) (27). Hypoxia and ischemia-reperfusion stress

result in the release of reactive oxygen species which damage epithelial cells and disrupt tight junction proteins (28) resulting in increased intestinal permeability. Moreover, while the immune system serves a vital protective role in the intestinal microenvironment, dysregulated immune functions and/or responses can contribute to intestinal barrier dysfunction. For example, Th17 cells which are hyperactive or remain expanded/activated for prolonged periods of time can contribute to intestinal barrier dysfunction and are also associated with harmful autoimmune and inflammatory conditions (22), including conditions marked by intestinal injury and intestinal barrier dysfunction (24).

Methods for Assessing the Intestinal Barrier in Humans

Assessing the intestinal barrier in hypoxic conditions is pertinent to understand the etiology of high-altitude related GI complications as well as the potential contribution of intestinal injury in the development of AMS and HACE. There are a variety of methods which can be used to assess the integrity of the intestinal barrier. In humans, these methods most often include the collection and analysis of biological specimens such as blood or urine, though these specimens may be difficult or impractical to obtain, process, transport, and store in the field. Nonetheless, several researchers have used measures of intestinal injury and permeability to determine the impact of high-altitude exposures on the intestinal barrier. A more comprehensive and thorough description of the methods used to assess the intestinal barrier *in vivo* and *in vitro* has been covered elsewhere (7, 29, 30), however for reference we will review some of the most common methods used in the high-altitude literature.

Intestinal permeability is commonly assessed using the dual sugar absorption test where two non-metabolizable sugars of varying size (e.g., lactulose 342 Da and L-rhamnose 162 Da) are orally ingested and the presence of the two sugar probes is measured in the blood or

urine. The dual sugar absorption test operates under the assumption that smaller sugar probes can move freely from the intestinal tract into circulation while larger sugar probes are unable to pass the intestinal barrier (31). Thus, increases in the ratio of the large to small sugar probes can be used to determine intestinal permeability. The dual sugar absorption test has been used in both lab (13) and field (11) settings during high-altitude exposures. However, the cumbersome nature of the test may limit its application in some high-altitude scenarios.

Accordingly, many researchers opt for measuring biomarkers of the intestinal barrier in the blood or urine. Intestinal cell damage can be assessed by measuring the presence of fatty-acid binding proteins in the blood or urine. There are three types of fatty-acid binding proteins present within the GI tract which include intestinal fatty-acid binding protein (I-FABP) predominately located in the jejunum, liver fatty-acid binding protein (L-FABP) located in the liver, kidney and throughout the intestine, and ileal bile-acid binding protein (I-BABP) found exclusively in the ileum. Among these, I-FABP is the most commonly used to measure intestinal cell damage as it has been shown to have high test, re-test reliability in situations of rest and exercise (32). The measurement of the proteins zonulin and claudin-3 in circulation have been proposed as assessments of intestinal barrier breakdown (30), though more research is needed to confirm the sensitivity and reliability of these measurements. It is also worth noting that there is some controversy regarding the assays used to measure zonulin, thus this marker should be interpreted with caution (33).

Other assessments of intestinal barrier integrity include measurement of bacterial translocation. For example, increased intestinal permeability allows for gram-negative bacteria, and their harmful constituent endotoxins (also known as lipopolysaccharides (LPS)), to leak from the intestinal tract and enter circulation. The most common assessment

of bacterial translocation is measurement of LPS in in the blood. However, due to the challenges of avoiding sample contamination with exogenous LPS during collection or assay, endotoxins are not often measured (34, 35). Other indirect measurements of bacterial translocation include measurements of LPS binding protein (LBP) and soluble CD14 (sCD14) which are involved in the monomerization and transport of LPS to immune cells (36). These methods may provide more feasible options for high-altitude researchers in the field as they avoid common issues of contamination. More recently the measurement of bacterial DNA (i.e., *Bacteroides*) has been used as an indicator of bacterial translocation due to the robust and sensitive assessment of the 16S gene sequencing. However, these measurements in their current form appear to lack test re-test reliability (32).

The Effect of Hypoxia on the Intestinal Barrier

High altitude environments are characterized by low barometric pressures which cause low partial pressures of oxygen (often referred to as hypobaric hypoxia). Experimentally, high altitude can also be simulated by lowering the inspired fraction of oxygen (FiO_2) through addition of nitrogen without altering the barometric pressure, referred to as normobaric hypoxia. Acute hypoxia at high altitude results in low blood oxygen (hypoxemia), and increased sympathetic tone causing vasoconstriction which may ultimately cause intestinal ischemia (37, 38). Under normal physiological conditions the intestinal epithelium operates in a state of physiologic hypoxia, often characterized as a steep oxygen gradient from the submucosa to the intestinal lumen (39). In fact, the low basal oxygen tension in intestinal epithelial cells stabilizes hypoxia inducible factors which elicit several intracellular adaptations that collectively promote intestinal barrier integrity (40). Thus, it may appear that the intestinal barrier is inherently more resilient to hypoxic stress compared

to other organs. However, in scenarios of hypoxemia and/or ischemia this physiologic hypoxia can be exacerbated and become pathological leading to local tissue hypoxia, energy depletion, and tissue acidosis which can damage intestinal epithelial cells and disrupt tight junction protein complexes (41) resulting in intestinal barrier dysfunction (42–48). In addition, oxidative stress (41) and inflammation (49) are other mechanisms that contribute to intestinal barrier dysfunction.

Indeed, hypoxic exposures have been shown to elicit several physiological changes to the intestinal barrier. For example, rodents exposed to hypoxia (both hypobaric and nomobaric) display marked epithelial cell injury and a compromised intestinal barrier which result in increased permeability and bacterial translocation (42–48, 50) (Table 1). In humans, evidence of an impaired intestinal barrier at high altitude was reported by Dinmore et al. (11) who studied a group of males (n=10) and females (n=4) on an expedition from sea level to 6300 m. The authors noted a twofold increase in intestinal permeability during the first few days of travel from 60 m to 1050 m and again at 5570 m (Table 1). The authors did not detect a significant increase in permeability on day 11 (at an altitude of 5730 m) of their 12-day ascent to 6300 m, which might suggest partial acclimatization. However, based on the reported means and standard deviations (estimated Cohen's d of 0.67) it is likely that the study was underpowered with just 11 participants assessed for intestinal permeability. More recently, Karl et al. (13) reported increased intestinal permeability in a group of healthy, physically active, but unacclimatized men (n=17) following a rapid (transported by airplane and car), 22-day exposure to 4300 m. The authors noted increased permeability on days 1 and 18 of their exposure when compared to sea level. These findings confirm a similar effect to Dinmore et al. (11), and the sustained increase in permeability indicate that their

participants were not fully acclimatized to the hypoxic environment. Nonetheless, the findings of Dinmore et al. (11) and Karl et al. (13) should be interpreted with caution given the limitations within each study design. For example, neither study included a control group which makes it difficult to determine the direct effects of hypobaric hypoxia without the influence of diet, physical activity, or other confounding factors (e.g., circadian disruption, psychological stress, etc.).

During longer hypoxic exposures (several days to weeks), mechanisms of increased intestinal permeability may also involve mucosal layer atrophy and changes to the gut microbiota (51, 52). For example, rats exposed to 30-days of hypobaric hypoxia (412 Torr, ~4870 m) displayed a decreased mucosal layer depth and width compared to normoxic controls (51). This is possibly a consequence of decreased goblet cells and/or mucus section (51). Kleessen et al. (52) reported unfavorable alterations in the intestinal microbiota amongst a group of mountaineers ascending to 6677 m. Specifically, they observed increases in pathogenic, gram-negative bacteria, as well as decreases in bifidobacteria, which are known to contribute to the microbial barrier (53). In addition, the authors noted decreases in serum concentrations of anti-LPS, which are natural antibodies against LPS, indirectly indicating endotoxemia. These findings provide some initial evidence that hypoxic exposures can damage the intestinal barrier which may allow for bacterial translocation. However, it is important to note that much of these data were collected in the field and the influence of confounding factors that impact the intestinal barrier including physical activity, diet, dehydration, and psychological stress are not known, thus these results should be interpreted with caution. In addition, the limited evidence from animal models needs to be confirmed in

humans, and given the current state of the literature, preferably in more controlled laboratory studies where the influence of confounding factors can be minimized.

Hypoxic exposure may also disrupt the intestinal barrier via immunological dysregulation. In rodents, Khanna et al. (42, 43) demonstrated that 7- and 14-days of hypobaric hypoxia (282 Torr, ~7620 m) increased IL-17 (tissue mRNA and circulation protein concentration) suggesting a shift toward Th17 polarization. Elsewhere it was shown that hypoxia, through hypoxia-inducible factor-1α (HIF-1α)- mediated stabilization of RORγt and degradation of the Treg master regulator FOXP3, can promote Th17 cell differentiation and suppress Treg differentiation (54). This could indicate that hypoxia has a pro-inflammatory, and possibly damaging, impact on the intestinal barrier. Consistent with this notion, the genetic knockout of forkhead box P3 (FOXP3) renders mice susceptible to experimental colitis (55), suggesting that anti-inflammatory/immunoregulatory Tregs are protective against intestinal injury. However, the same study showed that hypoxia alone did not induce Th17 cells, and instead promoted Tregs in an HIF-1α-dependent manner. These studies provide conflicting evidence, as one suggests that hypoxia induces pro-inflammatory Th17 cells, while the other shows it upregulates anti-inflammatory Treg cells. Despite this, at minimum, these distinct reports show that hypoxia can indeed have a significant impact on the differentiation of immune cells in the intestinal microenvironment. Further, it is possible that a pro-inflammatory shift toward Th17 differentiation can explain the increase in circulating cytokines observed during prolonged hypoxic exposures which has been noted in rodent models (42–46). Indeed, several of these pro-inflammatory cytokines are known to directly damage the intestinal barrier (56). In addition, TNF-α and IL-1β are also known to stimulate pro-inflammatory pathways (e.g., nuclear factor-κ light-chain-enhancer of activated

B cells -NF-κB) which can activate pro-inflammatory genes (57, 58). However, given that clear evidence of this is lacking in humans, the role of immunological dysfunction on hypoxia-mediated intestinal barrier disruption remains unclear.

There is limited and conflicting evidence regarding the effect of hypoxia on S-IgA. In rodents, hypobaric hypoxia (282 Torr, ~7620 m) was shown to increase intestinal levels of S-IgA with 1-day of exposure, while levels returned to baseline at day 3 and remained there at days 7 and 14 (42). These findings were partially confirmed in humans by Meehan et al. (59) who reported increased plasma IgA concentrations following passive exposure to 7620 m of simulated hypobaric hypoxia (282 Torr). However, elsewhere it was shown that 5-days in hypobaric hypoxia (7000 m) reduced intestinal S-IgA in rodents (45). Given this equivocal data, more research is needed to confirm the impact of hypoxia on intestinal S-IgA. Further, the protective role of S-IgA in mounting an adequate defense to hypoxia-induced bacterial translocation raises an important question regarding a potential therapeutic target (60). Future investigations may consider methods to enhance mucosal IgA production and/or delivery in efforts to protect the gut from hypoxia-induced injury.

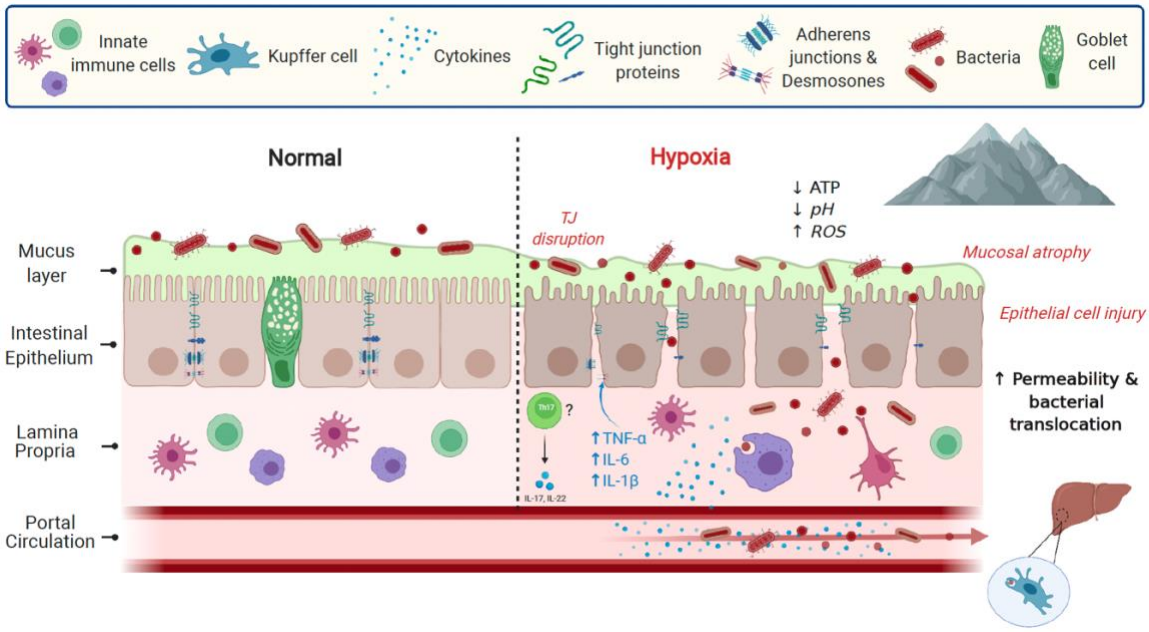

Figure 1: An overview of the effects of hypoxia on the intestinal barrier. Hypoxic exposures decrease the amount of blood oxygen (hypoxemia) and increases sympathetic outflow causing vasoconstriction in the splanchnic region. Consequently, blood is shunted away from the gut resulting in local tissue hypoxia and oxidative stress. This can cause energy (ATP) depletion, decreased pH, and the formation of reactive oxygen species (ROS) which damage intestinal epithelial cells and disrupt tight junction (TJ) protein complexes. In addition, chronic hypoxic exposures may lead to atrophy of the mucosal layers, a result of fewer goblet cells or decreased mucus secretion, further compromising the intestinal barrier. Gram-negative bacteria containing lipopolysaccharides, which are present within the intestinal lumen, cross the compromised intestinal barrier and activate resident macrophages to release pro-inflammatory cytokines. Immunological events in the gut during hypoxia – and even hypoxia itself – might augment pro-inflammatory Th17 cell polarization, however clear evidence of this is currently lacking. Bacteria entering portal circulation are met by Kupffer

cells in the liver furthering the systemic immune and inflammatory cascade. Figure was created using BioRender.com.

Exertion in Hypoxia and the Intestinal Barrier

For people traveling to high altitude, it may be prudent to consider the effects of physical work or exertion that may worsen hypoxia-induced intestinal injury. A consequential effect of exercise on the GI tract is profound redistribution of blood flow. During exercise, splanchnic perfusion is reduced which allows for greater perfusion of the skin and contracting skeletal muscle (61, 62). The reduction in blood flow to the GI system during exercise has largely been attributed to an increase in sympathetic tone (63, 64). This explains why people who lack sympathetic control in the splanchnic region (e.g., high spinal cord injuries) do not decrease portal vein blood flow during exercise (64). The effects of exercise on gut blood flow are especially problematic in hypoxic environments because low oxygen also results in greater sympathetic outflow and subsequent vasoconstriction (37). Further, in hypoxic conditions, such as those observed at high altitude, more blood is redistributed away from the intestinal tract to meet the increased demands for skeletal muscle perfusion (65, 66) which exacerbates intestinal ischemia. Shunting of blood away from the intestinal tract has been shown to cause intestinal cell damage and increased permeability during a bout of exercise (62).

Three studies have examined the effects of exercise in hypoxia on markers of the intestinal barrier in humans (Table 1). Lee and Thake (67) showed that 40-minutes of cycling at 50% of VO_{2max} in normobaric hypoxia (14% FiO_2; simulated altitude of ~3500m) caused a significant increase (143%) in I-FABP. In addition, these authors noted significant increases in IL-6 (425%) following exercise in hypoxia which might suggest a possible immune

response triggered by bacterial translocation. However, the role of IL-6 is complicated in exercising scenarios as it can act as both a pro- and anti-inflammatory cytokine. In addition, the source of release (e.g., muscle or immune cells) is not known, therefore it is unclear if this increase represents a true pro- inflammatory response. Hill et al. (68) later corroborated these findings by showing that running (60-minutes at 65% of VO_2max) in a simulated altitude of ~4000 m (13.5% FiO_2) caused a significant increase in I-FABP (168%), IL-6 (473%), and TNF-α (111%). Importantly, they noted that the same exercise stress, in the absence of altitude exposure, was not sufficient to induce changes in I-FABP or TNF-α in the control trial (~290 m), suggesting that the added stressor of hypoxia was responsible for these responses. Elsewhere it has been shown that 60-minutes of running at 50% VO_2max caused a significant increase in circulating total endotoxins in normobaric hypoxia (13.5% FiO_2, 4000 m) but not normoxia (~760 m) (69). It is important to note that all of these studies simulated altitude using normobaric hypoxic chambers which may elicit different physiological responses than hypobaric hypoxia (70, 71). Of note, hypobaric hypoxia may increase alveolar dead space leading to a greater hypoxemia, hypocapnia, blood alkalosis and a lower SaO_2 compared to nomobaric hypoxia (72) which could exacerbate hypoxia-induced injury to the intestinal barrier. Thus, these effects may be modest estimations of the potential impact of exertion in hypobaric hypoxia on the intestinal barrier, and future investigations are needed to replicate these findings in hypobaric hypoxia. Nonetheless, the observed rise in circulating endotoxins reported by Machado et al. (69) is consistent with the increase in circulating cytokines reported by Lee and Thake (67) and Hill et al. (68). These data demonstrate that exercise performed in hypoxic environments can damage the intestinal barrier allowing for bacterial translocation which triggers an inflammatory response.

Indeed, the translocation of endotoxins (i.e., LPS) across the intestinal barrier can activate innate immune cells via toll like receptor-4 (TLR-4) to produce and release pro-inflammatory cytokines. In addition, TLR-4 activation can lead to the recruitment of other immune cells (e.g., B cells), proliferating this immune response (73). Locally released pro-inflammatory cytokines can directly disrupt tight junction proteins further increasing intestinal permeability (56, 74). For example, several cytokines (e.g., TNF-, IL-1β, interferon- γ (IFN-γ)) have been shown to activate myosin light chain kinase resulting in the contraction of the actin cytoskeleton thereby disrupting tight junction protein complexes and increasing permeability (56, 75). Endotoxins entering portal circulation are met by Kupffer cells in the liver which contribute to the systemic inflammatory response (76). Hypoxia-induced oxidative stress (77) may also promote the activation of pro- and anti-inflammatory cytokine cascades (78). Therefore, it is likely that the combination of intestinal permeability resulting in bacterial translocation and hypoxia-induced oxidative stress are responsible for the release of pro-inflammatory cytokines. Recent insights from RNA sequencing suggest that the inflammatory and immune responses to high-altitude may underlie the development or progression of AMS (79). However, more robust data are needed to determine the influence of these responses on the progression of high-altitude illnesses (e.g., AMS and HACE), especially in exercising scenarios, as these may be mechanisms by which exertion worsens AMS symptoms (80).

Interestingly, while an increase in pro-inflammatory cytokines is observed with exercise in hypoxia, the overall immune response may be biased towards anti-inflammation (81). Specifically in humans, Hill et al. (81) demonstrated reduced TNF-α:IL-1RA and IL-1β:IL-1RA ratios as well as lower circulating monocyte chemoattractant protein-1 (MCP-1)

following exercise in hypoxia compared to normoxia. In addition, post-exercise phosphorylation of NF-κB in peripheral blood mononucleated cells was lower in hypoxia compared to normoxia. While bacterial translocation was not directly measured, these data point to a diminished immune response, despite greater intestinal injury likely leading to greater bacterial translocation. Thus, while pro-inflammatory cytokines are known to increase with exercise in hypoxia, it appears that anti-inflammatory cytokines may increase to a greater degree. This is potentially concerning given the increased permeability and subsequent bacterial load that needs to be challenged. It is necessary to better understand the direct effect of hypoxia on the intestinal barrier to construct countermeasures to prevent increases in permeability and/or bolster the immune response.

Table 1. *Summary of selected studies examining the effect of hypoxic environments on the intestinal barrier.*

Reference	Sample	Protocol	Outcomes
		Human Studies	
Dinmore et al. (11)	10 males and 4 females (n=11 assessed)	12-day expedition to 6300 m	Increased intestinal permeability (L/R) on day 1 (1050 m) and day 5 (5570 m).
Kleessen et al. (52)	5 males and 2 females	47-day expedition 6677 m	Increased pathogenic bacteria on day 12 (5200 m), day 15 (5600 m), and day 29 (6677 m). Decreased bifidobacteria on day 15 (5600 m) and day 29 (6677 m). Increased CRP at day 12 (6677 m).
Karl et al. (13)	17 males	22-day rapid exposure to 4300 m*	Increased intestinal permeability (L/M) at day 1 (4300 m) and day 18 (4300 m). No change in circulating IL-6 with high-altitude exposure.
Lee and Thake (67)	21 males	Acute exercise (40-miuntes 50% of VO_2peak) in nomobaric hypoxia (14% FiO_2, ~3500 m)	Increased I-FABP and IL-6 pre to post exercise in hypoxia.

Hill et al. (68)	9 males and 1 female	Acute exercise (60-miuntes 65% of VO_2max) in nomobaric hypoxia (13.5% FiO_2, ~4000 m)	Increased I-FABP, TNF-α, and IL-6 pre to post exercise in hypoxia.
Machado et al. (69)	9 males	Acute exercise (60-miuntes 50% of VO_2peak) in nomobaric hypoxia (13.5% FiO_2, ~4000 m)	Increased endotoxins at post and 1-hr post exercise in hypoxia.
		Animal Studies	
Khanna et al. (42)	Sprague-Dawley rats	14-days in 282 Torr (~7620 m) of hypobaric hypoxia	Intestinal barrier damage and decreased goblet cells observed at 1-day, 3-days, and 7-days. Increased circulating zonulin at 7-days, non-significant decrease in jejunum occludin protein expression. Increased mRNA expression and concentration of circulating IL-17 after 7-days. Increased S-IgA at 1-day.
Xu et al. (44)	Sprague-Dawley rats	5-days at 7000 m of hypobaric hypoxia*	Intestinal villi damage observed after 5-days. Decreased ileum occludin protein expression after 5-days. Increased circulating concentrations of IL-6, TNF-α, and IFN-γ.
Li et al. (46)	Sprague-Dawley rats	9-days of exercise training in nomobaric hypoxia (12.7% FiO_2, ~4000 m)	Intestinal barrier damage after 3-days, 6-days, and 9-days. Increased mRNA expression of IL-6 and TNF-α and increased protein expression of NF-κB.
Khanna et al. (43)	Sprague-Dawley rats	7-days in 282 Torr (~7620 m) of hypobaric hypoxia*	Intestinal barrier injury, increased intestinal permeability (FITC dextran), and increased concentration of circulating zonulin observed after 7-days. Increased mRNA expression of IL-17. Increased circulating concentration of IL-6, IFN-γ, TGF-β1, BLC, MCP-1, SDF-1, and IL-17.
Luo et al. (47)	Sprague-Dawley rats	3-days at 4000 m of hypobaric hypoxia	Intestinal barrier damage, increased bacterial translocation, and apoptotic index of epithelial cells after 3-days.
Zhou et al. (48)	Sprague-Dawley rats	3-days at 7000 m of hypobaric hypoxia*	Intestinal barrier damage, and increased bacterial translocation observed after 3-days.

Xu et al. (45)	Sprague-Dawley rats	5-days at 7000 m of hypobaric hypoxia*	Intestinal villi damage observed after 5-days. Decreased ileum occludin protein expression and mRNA after 5-days. Increased IL-2, IFN-γ, IL-4. Increased protein expression of NF-κB and TLR-4, and decreased S-IgA after 5-days.
Adak et al. (51)	Albino rats	30-days, 8-hrs per day, at 412 Torr (~4870 m) of hypobaric hypoxia	Intestinal villi damage, atrophy of the mucosal layers, and fewer goblet cells after 30-days.
Zhang et al. (50)	Wistar rats	3-days at 3842 m and 4767 m of hypobaric hypoxia	Intestinal barrier damage at 3842 m and 4767 after 3-days.

** Indicates that only data from control condition were included*
Abbreviations: BLC – B lymphocyte chemoattractant; CRP – C-reactive protein; FiO_2 – fraction of inspired oxygen; FITC – fluorescein isothiocyanate; I-FABP – intestinal fatty acid binding protein; IFN-γ – interferon-γ; IL-2 – interleukin 2; IL-4 – interleukin 4; IL-6 – interleukin 6; IL-17 – interleukin 17; L/R – lactulose to rhamnose ratio; L/M – lactulose to mannitol ratio; MCP-1 – monocyte chemotactic protein-1; NF-κB – nuclear factor-κ light-chain-enhancer of activated B cells; S-IgA – secretory immunoglobulin A; SDF-1 – stromal differentiation factor-1α; TGF-β – transforming growth factor-β; TNF-α – tumor necrosis factor-alpha; TLR-4 – toll like receptor-4.

Medications and Dietary Supplements

There are a variety of medications commonly used at altitude to prevent and/or treat high-altitude illnesses such as AMS or high-altitude headache. Yet, the influence of these medications on the GI system is not often considered. Studying the impact of various medications on the intestinal barrier is relevant for the wilderness medical community as it may elucidate unknown mechanisms by which they can prevent or treat clinical GI related symptoms. Some medications may have side effects that negatively impact the intestinal barrier. Therefore, the following section will review commonly used medications in high altitude environments including acetazolamide, non-steroidal anti-inflammatory drugs (NSAIDs) (e.g., ibuprofen), and dexamethasone. In addition, we will present some evidence regarding interventions which may strengthen the intestinal barrier in high altitude environments.

Acetazolamide

Acetazolamide is a carbonic anhydrase inhibitor which is commonly prescribed for the prevention and treatment of AMS (82). Indeed, the efficacy of acetazolamide in preventing/treating AMS has been well documented by various groups (83–86). The primary mechanism of action by which acetazolamide ameliorates symptoms of AMS is the inhibition of carbonic anhydrase in the kidneys, red blood cells, and other tissues leading to the induction of urinary bicarbonate diuresis and subsequent propagation of mild metabolic acidosis (87). Central chemoreceptors respond to the slight fall in fluid pH by stimulating a compensatory respiratory response to hypoxic stimuli (88). This compensatory respiratory response is characterized by an increase in minute ventilation at high altitude (89), which may lead to improved maintenance of arterial oxygen content as well as acute elevations in cerebral blood flow (90). In addition, it has been proposed that the diuretic properties of acetazolamide may contribute to the reduction in AMS symptoms (82), as more severe forms of the disease, such as HACE and high-altitude pulmonary edema (HAPE), are often associated with sodium and water retention (91).

GI distress is one of the more common side effects reported with the use of acetazolamide (92), but the cause of these side effects is not known. The effect of acetazolamide on the intestinal barrier is not well characterized in humans, and studies using rodent models have provided conflicting results. For example, some have shown that acetazolamide can damage the GI tract through inhibition of carbonic anhydrase and mucus secretion (93), while elsewhere it has been reported that acetazolamide can prevent gastric ulcerations via prostaglandin biosynthesis in rodents (94, 95). Future studies are needed to

directly determine acetazolamide's impact on the intestinal barrier during high altitude exposures.

Non-steroidal anti-inflammatory drugs (NSAIDs)

NSAIDs are over-the-counter drugs commonly carried and used by travelers to high altitude to prevent high-altitude illnesses (96). Indeed, researchers have shown that ibuprofen, a common NSAID, can prevent some symptoms of AMS and high-altitude headache (97, 98). NSAIDs work by inhibiting enzymes (cyclooxygenases) involved in the formation of thromboxanes, prostacyclins, and prostaglandins thus reducing the inflammatory cascade associated with hypoxic exposures (99). This anti-inflammatory effect is likely beneficial, as recent reports have highlighted the potential role systemic inflammation (increased expression of IL-1β, IL-6, and TNF-α) plays at the onset of AMS (15).

While the benefit of such pharmacological intervention is well reported, it is also necessary to consider its possible side-effects, specifically as it relates to the GI tract. The main mechanism of NSAIDs can be problematic for the intestinal barrier as prostaglandins are known to contribute to mucosal defense by decreasing gastric acid secretion, promoting antiulcer activity, providing cytoprotection, and increasing mucus production (100, 101). Moreover, some have noted that ibuprofen may worsen symptoms of GI distress or even increase the risk of GI bleeding (102). In fact, ibuprofen has been shown to aggravate intestinal injury following exercise (103, 104). Yet, the direct impact of ibuprofen on gastrointestinal permeability during high-altitude exposure has not been properly investigated.

Dexamethasone

Dexamethasone is a glucocorticoid that is commonly used to treat severe symptoms of AMS, HACE, and HAPE, and it has also been suggested for prophylaxis of AMS (105). The mechanisms behind dexamethasone's action in treating and preventing AMS and other high altitude-illnesses (HACE and HAPE) are not fully understood but are believed to be multifaceted and perhaps not mutually exclusive (106). These include immune cell apoptosis (eosinophils and T cells), decreased production of inflammatory cytokines, and suppression of cyclooxygenase (107). In addition, it has been suggested that dexamethasone prevents hypoxia-induced endothelial dysfunction which may explain its efficacy in treating HACE (108). In addition, dexamethasone has been shown to increase the expression of the tight junction protein claudin-4 in cultured intestinal epithelial cells (Caco-2) which conferred decreased permeability (109). Further, corticosteroids, such as dexamethasone, have been proposed as a potential treatment for irritable bowel syndrome (110), perhaps by offering acute gastroprotective effects (111). However, prolonged use of glucocorticoids may make people more susceptible to ulcers due to their inhibition of prostaglandin formation (112). Nonetheless, the effects of dexamethasone on the intestinal barrier during hypoxic exposures has not been studied.

Dietary supplements

Several dietary supplements (e.g., amino acids, select polyphenols, probiotics, etc.) have been shown to strengthen or protect the intestinal barrier from various stressors (reviewed by 76). However, the use of these substances for protecting the intestinal barrier during hypoxic exposures, especially during exertion, is relatively unknown. Importantly, many of these substances are easily accessed over the counter with relatively few known side effects. Thus, their role in protecting the intestinal barrier during hypoxic exposure warrants discussion.

Glutamine is one of the most abundant amino acids in the human body and is well utilized by the intestinal endothelium. In fact, glutamine is the preferred fuel source for enterocytes, thus glutamine supplementation may protect intestinal epithelial cells from stress-induced energy substrate depletion (114). Glutamine has also been shown to fortify tight junctions (115), and play a role in suppressing pro-inflammatory pathways, such as NFκB (116). The underlying mechanism behind glutamine's improved stability of tight junctions is likely the upregulation of heat shock factor 1 (HSF-1), which subsequently enhances heat shock protein (HSP) expression (117–119). It is suggested that glutamine increases the hexosamine biosynthetic pathway activity leading to the transcriptional activation of HSF-1 and specificity protein 1, which are important transcriptional factors of HSP70 (117). An increase in the cell's basal stores of HSP characterizes an enhancement in a cell's ability to endure physiological stress. Further, overexpression of HSF-1 has been shown to play a vital role in tight junction regulation and stabilization under stressful conditions (e.g. heat stress), as demonstrated by Dokladny et al. (120). Glutamine has been shown to increase tissue HSP70 and HSF-1 expression in the cecum following passive hyperthermia in rats (121). Importantly, this study reported a decrease in intestinal permeability and circulating endotoxins and increased survival following exposure to otherwise lethal hyperthermia, thus, demonstrating the potential for glutamine to protect the intestinal barrier from hypoxia-induced injury.

Regarding hypoxia, glutamine administered (5.0 g/kg of body weight) 3 days before and 5 days during a simulated altitude of 7,000 m was effective in reducing hypoxia-induced damage to intestine morphology and structure in rats. (44) The authors also reported that glutamine supplementation reduced serum oxidative stress (higher serum superoxide and

lower malondialdehyde) and pro-inflammatory markers (IL-6, TNF-α and IFN-γ). Finally, Caris et al. (122) has previously reported an anti-inflammatory effect (shift toward Th1 response) after exercise at simulated altitude (~4,500 m) following 6-days of glutamine and carbohydrate supplementation. In summary, glutamine has been shown to protect the intestinal barrier possibly through increases in intracellular HSPs which may protect the intestinal barrier by preventing increases in permeability and suppressing the release of pro-inflammatory cytokines.

Bovine colostrum is the first milk produced after calving and is rich in growth factors, immunoglobulins, and antimicrobial peptides (123). It is suggested that bovine colostrum can prevent stress induced intestinal injury via upregulation of tight junction proteins and increased expression of intracellular HSPs (124–126). Marchbank et al. (126) showed that 14 days of bovine colostrum supplementation (20 g/day) reduced intestinal permeability (assessed via Lactose/Rhamnose ratio) following treadmill running (20 minutes, 80% VO_2max) in normoxia. This same group later confirmed these findings using an indirect marker of intestinal permeability (I-FABP) following treadmill running (60 minutes, 70% VO_2max) in the heat (30° C, 60% RH) (125). However, data are equivocal as we (127), along with others (128), have failed to see a significant effect with bovine colostrum following exercise in the heat. Nevertheless, the effects of bovine colostrum on the intestinal barrier during hypoxic exposures has not been studied.

The polyphenol curcumin derived from the plant Curcuma longa, has been reported to infer some intestinal barrier protection through reduced oxidative stress and apoptosis (129, 130). However, the mechanisms underlying these effects remain to be fully elucidated. In hypoxia induced pulmonary edema, curcumin supplementation has been reported to inhibit

the disruptions of tight junctions in adenocarcinoma human alveolar basal epithelial cells (A549) (131). While there is a lack of studies using intestinal cells, it is reasonable to hypothesize that curcumin may prevent intestinal permeability during hypoxic exposures though future investigations are needed to test this hypothesis.

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (132). Certain probiotics have been shown to alter the gut microbiota, increase mucin secretion, and prevent stress induced increases in intestinal permeability (133, 134). While not currently known, probiotics may help protect the intestinal barrier during hypoxic exposures. The use of probiotics may be even more relevant for prolonged exposures to prevent mucosal atrophy or unfavorable changes in the gut microbiota. However, given that elsewhere probiotics have been shown to have no (135) or even negative (136) effects on the intestinal barrier, more evidence is needed to determine their efficacy in protecting the intestinal barrier from hypoxia-mediated stress.

Perspectives and Significance

In summary, there is a small, but growing body of evidence which suggests that acute exposures to high-altitude damage the intestinal barrier through hypoxic and oxidative stress. Furthermore, prolonged hypoxic exposures likely compromise the intestinal barrier through alterations in immunological function, microbiota, or mucosal layers. Exertion may worsen high-altitude related intestinal injury, increase permeability, and cause greater bacterial translocation. Various medications which are used to alleviate some symptoms associated with altitude exposure might induce greater intestinal injury (e.g., NSAIDs). These responses may result in a pro-inflammatory response which could contribute to other symptoms of AMS including HACE. Other medications commonly used during high altitude ascent (e.g.,

dexamethasone) may confer protection to the intestinal barrier, though more research is needed to determine their impact during hypoxic exposure. In addition, future investigations should aim to determine the relationship between intestinal barrier damage and symptomology as this is relevant for people ascending to high altitude to perform physical work and/or exercise.

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CHAPTER III: Study 1 - Exercise in hypobaric hypoxia increases markers of intestinal injury and symptoms of gastrointestinal distress

This chapter presents a research manuscript entitled “Exercise in Hypobaric Hypoxia Increases Markers of Intestinal Injury and Symptoms of Gastrointestinal Distress” which has been accepted for publication in *Experimental Physiology*. Tables, figures, and references are provided at the end of the manuscript

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Title: Exercise in Hypobaric Hypoxia Increases Markers of Intestinal Injury and Symptoms of Gastrointestinal Distress

Zachary J. McKenna[1], Zachary J. Fennel[1], Quint N. Berkemeier[1], Roberto C. Nava[2,3], Fabiano T. Amorim[1], Michael R. Deyhle[1], Christine M. Mermier[1]

[1] Department of Health, Exercise and Sports Sciences, University of New Mexico Albuquerque, NM, USA

[2] Harvard Medical School, Boston, MA, USA

[3] Research Division, Joslin Diabetes Center, Boston, MA, USA

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Corresponding author: Zachary J. McKenna

Email: zmckenna@unm.edu

Phone: N/A

Mailing address: MSC 063830, 1 University New Mexico, Johnson Center B143 Albuquerque NM 87131

1. What is the central question of this study?

What is the effect of hypobaric hypoxia on markers of exercise-induced intestinal injury and symptoms of GI distress?

2. What is the main finding and its importance?

Exercise performed at 4300 m of simulated altitude increased I-FABP, CLDN-3, and LBP which together suggest that exercise-induced intestinal injury may be aggravated by concurrent hypoxic exposure. Increases in I-FABP, LBP, CLDN-3 were correlated to exercise-induced GI symptoms, providing some evidence of a link between intestinal barrier injury and symptoms of GI distress.

Abstract

We sought to determine the effect of exercise in hypobaric hypoxia on markers of intestinal injury and gastrointestinal (GI) symptoms. Using a randomized and counterbalanced design, 9 males completed two experimental trials: one at local altitude of 1585 m (NORM) and one at 4300 m of simulated hypobaric hypoxia (HYP). Participants performed 60-minutes of cycling at a workload that elicited 65% of their NORM VO_2max. GI symptoms were assessed before and every 15-minutes during exercise. Pre- and post-exercise blood samples were assessed for intestinal fatty acid binding protein (I-FABP), claudin-3 (CLDN-3), and lipopolysaccharide binding protein (LBP). All participants reported at least one GI symptom in HYP compared to just 1 participant in NORM. I-FABP significantly increased from pre- to post-exercise in HYP (708±191 to 1215±518 pg•mL^{-1}; p=0.011, d=1.10) but not NORM (759±224 to 828±288 pg•mL^{-1}; p>0.99, d=0.27). CLDN-3 significantly increased from pre- to post-exercise in HYP (13.8±0.9 to 15.3±1.2 ng•mL^{-1}; p=0.003, d=1.19) but not NORM

(13.7±1.8 to 14.2±1.6 ng•mL^{-1}; p=.435, d=0.45). LBP significantly increased from pre- to post-exercise in HYP (10.8±1.2 to 13.9±2.8 μg•mL^{-1}; p=0.006, d=1.12) but not NORM (11.3±1.1 to 11.7±0.9 μg•mL^{-1}; p>0.99, d=0.32). I-FABP (d=0.85), CLDN-3 (d=0.95), and LBP (d=0.69) were all significantly higher post-exercise in HYP compared to NORM (p≤0.05). Overall GI discomfort was significantly correlated to ΔI-FABP (*r*=0.71), ΔCLDN-3 (*r*=0.70), and ΔLBP (*r*=0.86). These data indicate that cycling exercise performed in hypobaric hypoxia can cause intestinal injury, which might cause some commonly reported GI symptoms.

Introduction

Gastrointestinal (GI) distress is often reported during ascents to high-altitude (> 2500 m) (Anand *et al.*, 2006). Common symptoms include nausea, vomiting, diarrhea, anorexia, and weight loss. More severe symptoms, such as GI bleeding, a potentially fatal condition, have been observed amongst lowland natives performing physical work at high-altitude (Wu *et al.*, 2007). Many of these adverse GI symptoms are attributed to acute mountain sickness (AMS), though their underlying cause is not known. One potential explanation is hypoxia/ischemia-mediated intestinal injury, which has been proposed to underlie some of the GI symptoms that arise during exercise (Zuhl *et al.*, 2014). Indeed, exertion at high altitude may exacerbate GI symptoms, which is especially relevant for groups (mountaineers, military personnel, wildland firefighters, athletes, and tourists) who travel to high altitudes to perform physical work or exercise (Wu *et al.*, 2007).

The pathophysiology of exercise-induced intestinal injury is complex, but it has been suggested that ischemia and subsequent reperfusion to the intestinal tract damage the intestinal barrier (van Wijck *et al.*, 2012). During exercise, splanchnic blood flow is reduced as more of the cardiac output is diverted to active skeletal muscle in order to support the increased energy demand (Rowell *et al.*, 1964; Qamar & Read, 1987; Rehrer *et al.*, 2001). In hypoxic conditions, such as those observed at high altitudes, sympathetic outflow is increased (Fletcher, 2000) which might exacerbate this redistribution of blood (Loshbaugh *et al.*, 2006). In addition, at increasing altitudes there is a decrease in the partial pressure of oxygen resulting in hypoxemia, a lower-than-normal blood oxygen level. Together hypoperfusion and hypoxemia result in hypoxic and ischemia-reperfusion stress which has the potential to injure intestinal epithelial cells and disrupt tight junction protein complexes,

resulting in increased intestinal permeability (van Wijck *et al.*, 2011; Lian *et al.*, 2021). This is problematic because increased intestinal permeability allows for luminal contents, including Gram-negative bacteria containing lipopolysaccharides (LPS), to leak from the intestinal tract where they interact with local innate immune cells (macrophages and monocytes). This can initiate an inflammatory cascade including the production and release of proinflammatory cytokines (Bouchama & Knochel, 2002). These responses may explain some of the GI complications that are reported by people who ascend to high altitude.

Recently, two groups have shown that moderate intensity running (Hill *et al.*, 2020) and cycling (Lee & Thake, 2017) in normobaric hypoxia increases circulating markers of intestinal injury and proinflammatory cytokines when compared to exercise performed in normoxic conditions. Another study showed that exercise in normobaric hypoxia increases circulating endotoxins (Machado *et al.*, 2017), suggesting increased intestinal permeability and bacterial translocation. However, no study to date has examined exercise-induced intestinal injury using hypobaric hypoxia, which may present a greater physiological stress than normobaric hypoxia (Beidleman *et al.*, 2014). In addition, these previous investigations have not examined the impact of exercise in hypoxia on acute GI symptomology, so it is unclear if the increases in markers of intestinal injury or bacterial translocation relate to GI symptoms. To fill these gaps in the literature, we measured markers of intestinal injury and symptoms of GI distress after 60-minutes of moderate intensity cycling at a simulated altitude of 4300 m (440 Torr) and tested if these markers correlated with GI symptoms during exercise.

Methods

The study was approved by the University Institutional Review Board (protocol no. 1509955) and all experimental procedures conducted conformed to the *Declaration of Helsinki* except for registration in a database. Each participant signed a written informed consent document before beginning the study.

Using a randomized and counterbalanced design, nine male participants (age 28±2 years, weight 75.2±10.7 kg, height 176±8 cm, body fat 12.6±5.6 %, VO_2max 3.73±0.73 L•min^{-1}) completed a total of three visits including baseline testing and two experimental trials: one at the local altitude of 1585 m or 630 Torr (NORM) and one at a simulated altitude of 4300 m or 440 Torr (HYP). Altitude was simulated in a hypobaric chamber at the University of New Mexico (Special Devices Center Office of Naval Research, Guardite Corporation, Chicago, IL). Study enrollment was open to females, however none volunteered for the study. Participants were free from cardiovascular disease and did not have any known GI disease or a regular history of GI distress. In addition, participants self-reported regular physical activity that met the minimum guidelines for exercise participation according to the American College of Sport's Medicine (American College of Sports Medicine, 2014). All participants had resided in Albuquerque, New Mexico for at least 6 months prior to enrollment and had not traveled to high altitude (> 2500 m) at least one week prior to enrollment. Experimental trials were separated by a minimum of fourteen days and completed at the same time of day to avoid diurnal variations.

Preliminary Testing

Preliminary testing included measurements of resting blood pressure, height, weight, body composition, and aerobic capacity. Body density was estimated using three site (chest,

abdomen, thigh) skinfold measurements (Lange, Beta Technology, Santa Cruz, CA). Each site was measured twice in a rotational order and the mean values for each site were summed and incorporated into a standardized regression equation to estimate body density (Jackson & Pollock, 1978), which was then converted to body composition (Brožek *et al.*, 1963). A maximal graded exercise test on a cycle ergometer (Corival, Lode, Groningen, The Netherlands) was used to determine aerobic capacity. During the maximal graded exercise test, expired gases were collected and analyzed using a metabolic cart (TrueOne 2400, Parvo Medics, Sandy, UT) to determine maximum oxygen consumption (VO_2max). In addition, heart rate (Polar H10) and rating of perceived exertion (RPE) (Borg, 1982) were measured and recorded throughout the test. VO_2max was confirmed if three of the following four criteria were met; a plateau in VO_2 despite an increase in workload ($\leq$ 150 ml•min^{-1}), respiratory exchange ratio (RER) $\geq$ 1.1, heart rate within 10 beats of age predicted max, RPE $\geq$ 17. Participants were asked to avoid substances known to influence the intestinal barrier (i.e., glutamine, non-steroid anti-inflammatory drugs, bovine colostrum, curcumin, and probiotics) and were given a food log to track their diets in the 24 hours prior to visit 2. They were then asked to use the food log to mimic their diet on the day prior to visit 3 to limit the influence of diet on intestinal barrier function.

Experimental Trials

Participants arrived at the laboratory in the morning between 08:00 and 09:00 after an overnight fast and abstaining from exercise for 24 hours, alcohol for 24 hours, and caffeine for 4 hours. A urine sample was collected to ensure they were euhydrated (urine specific gravity < 1.020) before beginning exercise. For both experimental trials participants performed 60-minutes of cycle ergometry exercise at a workload that elicited 65% of their

VO_2max in NORM. During each trial the temperature and humidity were maintained between 15-19°C and 30-50%, respectively. Heart rate, oxygen saturation (SaO_2) (Nonin Go2 pulse oximeter), RPE, and metabolic gas variables (VO_2 and RER) were recorded at rest and every 5-minutes during exercise. A modified visual analog scale was administered before, every 15-minutes during, and immediately after exercise to assess symptoms of GI distress and dizziness (Gaskell *et al.*, 2019). The questionnaire included a ratings of overall GI discomfort, specific upper GI symptoms (belching, heartburn, upper abdominal bloating, stomach pain, urge to regurgitate), lower GI symptoms (flatulence, lower abdominal bloating, urge to defecate, abnormal stool), nausea, side stitch, and dizziness. Participants were familiarized with the scale and it was explained that the severity of the ratings were deemed mild (1-3), severe (4-5), or very severe (7-10) as described elsewhere (Gaskell *et al.*, 2019).

Blood Sampling

Blood samples were collected through venipuncture of an arm vein into heparin or EDTA vacutainer tubes immediately pre- and post-exercise. An aliquot of whole blood was set aside for analysis of hematocrit and hemoglobin. Blood samples were centrifuged at 1600 x g for 15 minutes in 4°C to separate plasma. Two 1 mL aliquots of plasma were immediately frozen and stored in a -80°C freezer until later analysis. Hematocrit was measured in triplicate by transferring blood into microcapillary tubes and centrifuging for 5-minutes. The microcapillary tubes were then measured using a micro-capillary. Hemoglobin concentrations were measured in duplicate using a Hemoglobin Reagent Set (Pointe Scientific, Canton, MI) following manufacturer specifications. Absorbance was read in duplicate, and the coefficient of variation was less than 5%. The hemoglobin and hematocrit

measures were used to calculate plasma volume changes from pre to post exercise (Dill & Costill, 1974). Intestinal injury was assessed by measuring the concentration of plasma intestinal fatty acid binding protein (I-FABP) (Hycult Biotech, Uden, The Netherlands) and claudin-3 (CLDN-3) (Cloud-Clone Corp, Katy, TX, USA) pre- and post-exercise using enzyme linked immunosorbent assays (ELISA). I-FABP is a cytosolic protein present within enterocytes located predominantly in the jejunum, which has been shown to correlate with small intestine permeability (van Wijck *et al.*, 2012; March *et al.*, 2017). CLDN-3 is a ubiquitous tight junction protein expressed in a variety of tissues including the intestine, kidney, liver, lung, and endothelia (Rahner *et al.*, 2001; Mitchell *et al.*, 2011; Castro Dias *et al.*, 2019). CLDN-3 is known to play a central role in regulating paracellular permeability (Milatz *et al.*, 2010; Wells *et al.*, 2017) and urinary CLDN-3 has been related to histological tight junction breakdown in the GI tract (Thuijls *et al.*, 2010). In addition, plasma LPS binding protein (LBP) was measured as an indirect marker of LPS translocation (Hycult Biotech, Uden, The Netherlands) via ELISA. ELISA kit procedures were performed according to the manufacturer's instructions and intraassay coefficient of variations were 2.57% for I-FABP, 4.17% for CLDN-3, and 2.64% for LBP.

Data Analysis

An *a priori* power analysis was conducted using the effect size ($\eta_p^2 = 0.3$) of a prior study examining changes in I-FABP following exercise in normobaric hypoxia (Hill *et al.*, 2020). It was estimated with an α-level of $p \leq 0.05$, power of 0.80 (1 - β), and assuming a moderate correlation among repeated measures (0.70) that eight participants would be required to detect differences in the I-FABP response to exercise performed under normoxic versus hypobaric hypoxia conditions.

Statistical analyses were performed in RStudio (version 1.2.5033, R Development Core Team, Vienna, Austria). Data were assessed for normality using the D'Agostino-Pearson method (D'Agostino, 1986) and visual inspection of residual Q-Q plots. One participant terminated exercise at 45-minutes during the HYP trial due to severe GI distress (nausea). However, their data were included in all subsequent analyses as exercise duration was matched in the NORM trial. Thus, physiological data during exercise are reported as an n=9 for 0-45 minutes and n=8 for 45-60 minutes. Accordingly, physiological data collected during the experimental trials (VO_2, RER, HR, workload, SaO_2, and RPE) were analyzed using linear mixed-effects models that are robust to missing data. Comparison of the total work completed between the NORM and HYP trial was made using a paired t-test (two-tailed). I-FABP, CLDN-3, and LBP were each analyzed using a two-way (time x condition) repeated measures analysis of variance. If a significant interaction or main effect was found, pairwise comparisons with Bonferroni corrections were used to determine differences between conditions and time-points. Differences in plasma volume change between NORM and HYP trials were determined using a paired sample t-test (two-tailed). To better understand the magnitude of change for I-FABP, CLDN-3, and LBP, the pre to post changes (Δ) were quantified and comparisons of the Δ between NORM and HYP trials were made using paired-sample t-tests (two-tailed). GI symptom scores were totaled for the exercise trial (15, 30, 45, and 60-minute) and the incidence of GI symptoms (i.e., presence of any one symptom > 1) was calculated and reported as a percentage. Comparisons between the NORM and HYP trials for GI symptoms (overall, upper GI, lower GI, nausea, side stitch, and dizziness) were made using the non-parametric Wilcoxon signed-ranked tests (one-tailed) due to the non-normal distribution of these data. Bivariate repeated measures correlations

were used to determine relationships between GI symptoms (overall, upper GI, lower GI, nausea, side stitch) and ΔI-FABP, ΔCLDN3, and ΔLBP (Bakdash & Marusich, 2017). Statistical significance was set *a priori* to $p \leq 0.05$. Measures of effect sizes were quantified for each dependent variable and are reported as partial eta squared (η_p^2) or Cohen's d (d). Data are reported in text, tables, and figures as mean ± standard deviation (SD) or median and range where specified.

Results

Physiological Variables

Figure 1 displays the physiological responses to exercise during both experimental trials. Three participants had to reduce their workload near the end of exercise in HYP to complete the trial. However, total work completed was similar between NORM (6127 ± 1613 kJ) and HYP (5972 ± 1451 kJ) trials ($p=0.151$, $d=0.10$). Likewise, VO_2 ($p=0.685$, $\eta_p^2<0.01$) and workload in watts ($p=0.825$, $\eta_p^2<0.01$) were similar between NORM and HYP trials. There was a significant main effect of condition for RER ($p<0.001$, $\eta_p^2=0.22$). However, pairwise comparisons showed that RER was not significantly different between NORM and HYP trials at rest (0) or at any time point (5-60) during exercise ($p>0.05$). There was a significant main effect of condition on heart rate ($p=0.027$, $\eta_p^2=0.02$). Pairwise comparisons showed that heart rate was significantly higher at 10-30 minutes during exercise in HYP compared to NORM ($p<0.05$). Likewise, there was a significant main effect of condition on RPE ($p=0.002$, $\eta_p^2=0.04$), and pairwise comparisons showed that RPE was higher in the HYP at 10-60 minutes during exercise compared to NORM ($p<0.05$). SaO_2 was lower throughout the entire HYP trial (0-60 minutes) compared to NORM ($p<0.001$).

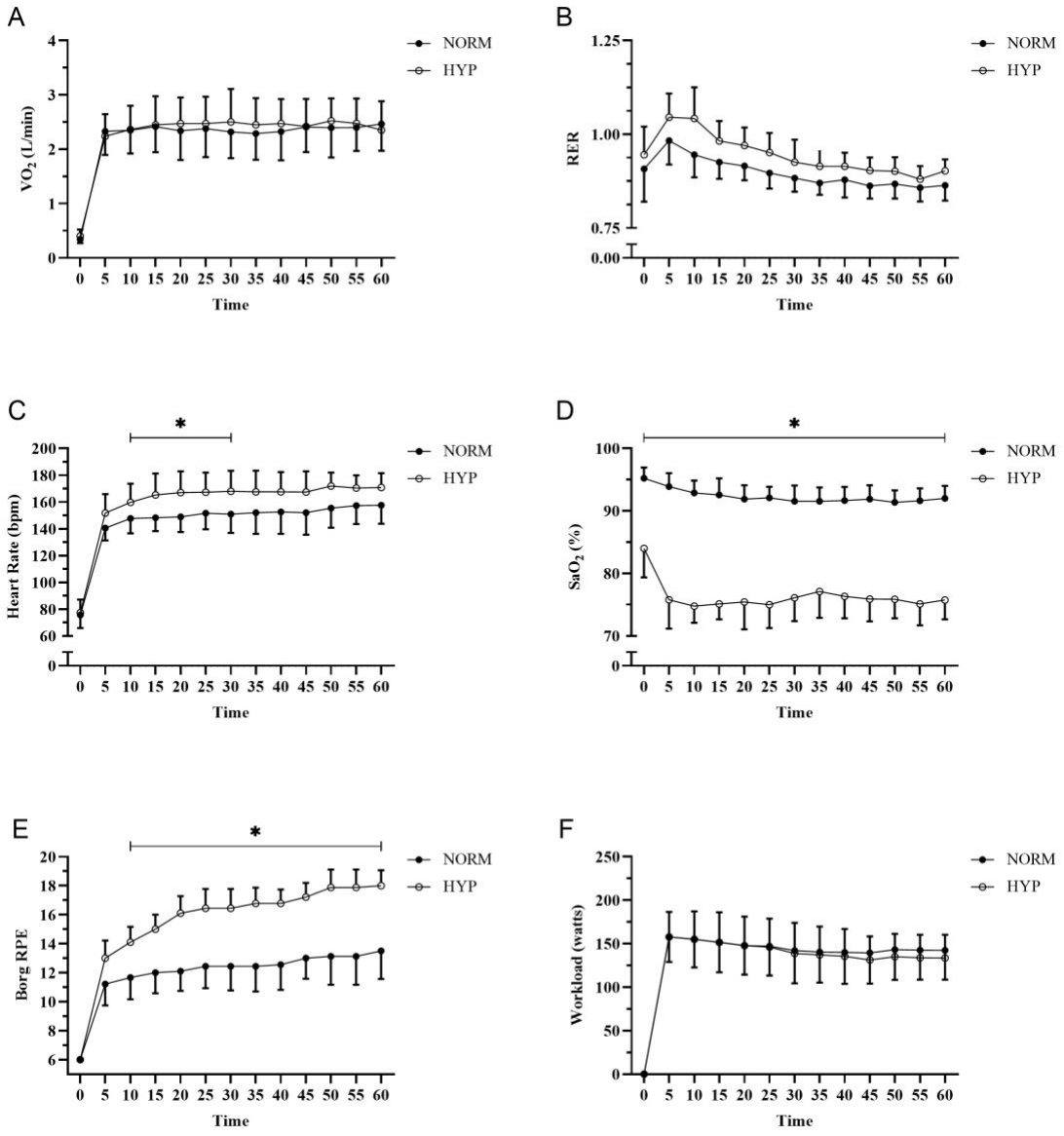

Figure 1. Physiological responses during exercise in NORM (630 Torr) and HYP (440 Torr) trials. A) oxygen consumption, B) respiratory exchange ratio, C) Heart rate, D) oxygen saturation, E) Borg rating of perceived exertion, and F) workload in watts. Data are reported as mean and SD. *n*=9 for 0-45 minutes and *n*=8 for 45-60 minutes. *Denotes significant a difference ($p \leq 0.05$) between NORM and HYP.

Markers of Intestinal Injury

Plasma volume changes were similar between NORM (-0.58 ± 2.49 %) and HYP (-0.77± 2.16 %) trials (p=0.877, d=0.08). A significant interaction between time and condition was detected for I-FABP (p=0.002). In addition, there was a significant main effect of time (p=0.035), but not of condition (p=0.053) for I-FABP. Pre-exercise I-FABP was not significantly different between NORM and HYP trials (p>0.99, d=0.25). I-FABP was significantly elevated from pre- (707.81 ± 190.99 pg•mL^{-1}) to post-exercise (1214.95 ± 518.38 pg•mL^{-1}) in the HYP trial (p=0.011, d=1.10) but was not significantly different between pre- (758.80 ± 224.26 pg•mL^{-1}) and post-exercise (828.33 ± 288.11 pg•mL^{-1}) in the NORM trial (p>0.99, d=0.27) (Figure 2A). Post-exercise I-FABP (p=0.048, d=0.85) and Δ I-FABP (p=0.001, d=1.04) (Figure 2B) were significantly higher in the HYP trial compared to the NORM trial.

There was a significant interaction between time and condition for CLDN-3 (p=0.038). A significant main effect of time was detected for CLDN-3 (p=0.0002), but not of condition (p=0.436). There was no significant difference in pre-exercise CLDN-3 between NORM and HYP trials (p>0.99, d=0.43). CLDN-3 was significantly elevated from pre- (13.82 ± 0.94 ng•mL^{-1}) to post-exercise (15.27 ± 1.22 ng•mL^{-1}) in the HYP trial (p=0.003, d=1.19) but was not significantly different between pre- (13.71 ± 1.77 ng•mL^{-1}) and post-exercise (14.25 ± 1.61 ng•mL^{-1}) in the NORM trial (p=0.435, d=0.45) (Figure 2E). Both post-exercise CLDN-3 (p=0.027, d=0.95) and Δ CLDN-3 (p=0.038, d=1.33) (Figure 2F) were significantly higher in the HYP trial compared to the NORM trial.

A significant interaction between time and condition was detected for LBP (p=0.002). There was a significant main effect of time (p=0.002) but not of condition (p=0.352) for LBP. Pre-exercise LBP was not significantly different between NORM and HYP trials

(p>0.99, d=0.08). LBP was significantly elevated from pre- (10.77 ± 1.18 μg•mL^{-1}) to post-exercise (13.89 ± 2.80 μg•mL^{-1}) in the HYP trial (p=0.002, d=1.12) but was not significantly different between pre- (11.25 ± 1.08 μg•mL^{-1}) and post-exercise (11.71 ± 0.91 μg•mL^{-1}) in the NORM trial (p>0.99, d=0.32) (Figure 2C). Post-exercise LBP (p=0.019, d=0.69) and ΔLBP (p=0.002, d=1.08) (Figure 2D) were significantly higher in the HYP trial compared to the NORM trial.

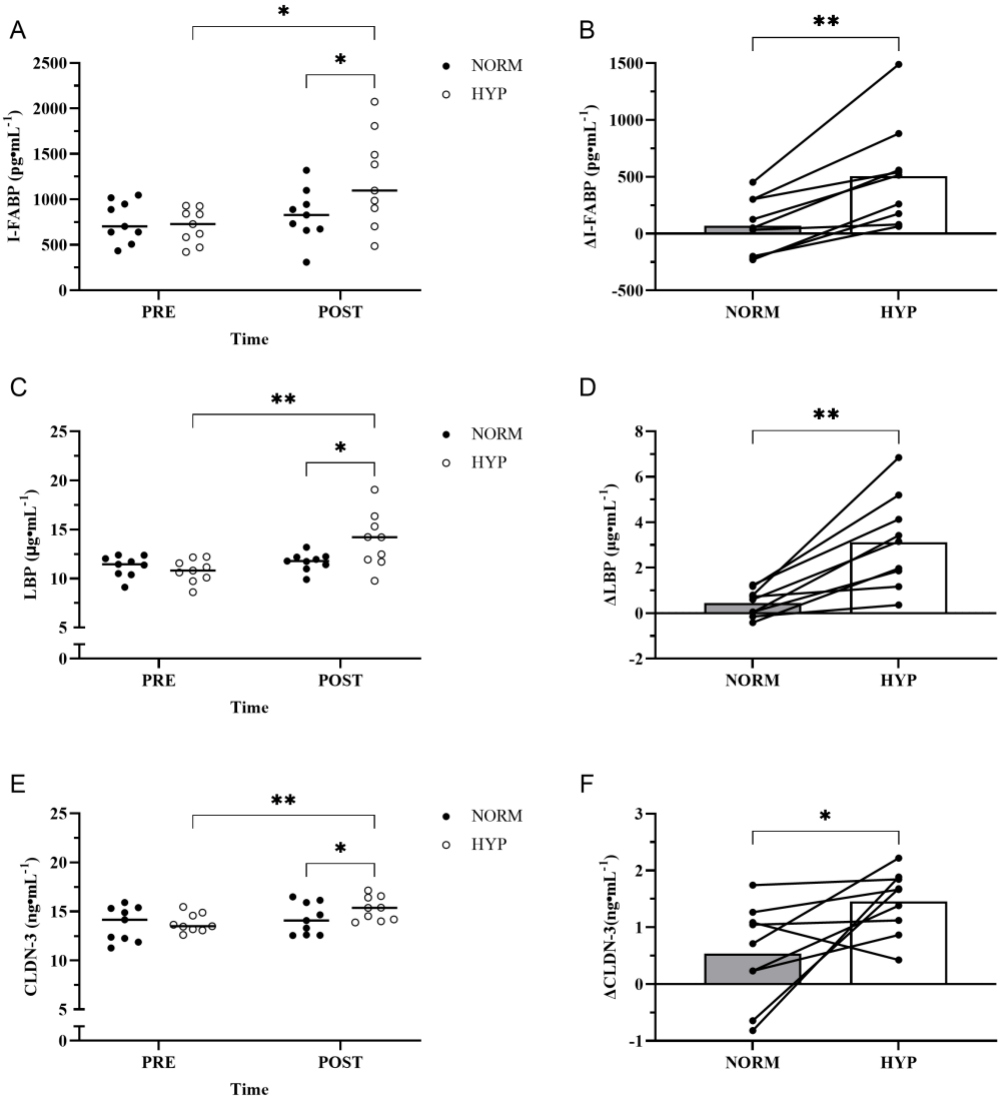

Figure 2. Markers of intestinal injury in NORM (630 Torr) and HYP (440 Torr) trials. A) plasma intestinal fatty acid binding protein before and after exercise in NORM and HYP, B) pre- to post-exercise change in plasma intestinal fatty acid binding protein C) plasma lipopolysaccharide binding protein before and after exercise in NORM and HYP, D) pre- to post-exercise change in plasma lipopolysaccharide binding protein, E) plasma claudin-3 before and after exercise in NORM and HYP, F) pre- to post-exercise change in claudin-3. The horizontal lines or bars mark the mean, and the dots represent individual data points. *n*=9. *Denotes $p \leq 0.05$ and **denotes $p \leq 0.01$.

GI Symptoms

Table 1. Summary of symptoms reported during exercise in NORM (630 Torr) and HYP (440 Torr) trials.

	NORM		HYP		
	Incidence	Median (range)	Incidence	Median (range)	p-value
Overall gut discomfort*	0%	0	66%	3 (0-8)	p=0.016
Upper GI symptoms	11%	0 (0-1)	44%	0 (0-6)	p=0.125
Lower GI Symptoms	0%	0	33%	0 (0-12)	p=0.125
Nausea	0%	0	44%	0 (0-11)	p=0.063
Abdominal Stitch	0%	0	22%	0 (0-7)	p=0.250
Dizziness*	0%	0	89%	8 (0-21)	p=0.004

Note: Symptoms were assessed before and every 15-minutes during exercise using a modified visual analog scale (21). Symptom incidence was calculated as the percentage of participants who reported a symptom at any point during exercise. Symptom scores were then totaled for the entire exercise bout and are reported above as median and (range). Comparisons between the median scores in NORM and HYP trials were made using Wilcoxon sign-ranked tests. *Denotes statistical significance ($p \leq 0.05$). n=9.

A summary of the GI symptoms reported during exercise are presented in Table 1. All nine participants reported at least one symptom from the GI questionnaire in the HYP trial compared to just one participant in the NORM trial. Overall GI discomfort (p=0.016) and dizziness (p=0.004) were higher in the HYP trial compared to NORM. Two participants reported severe GI symptoms in the HYP trial (nausea: n=1 and right intestinal pain: n=1) and one participant had to stop exercise in HYP due to very severe nausea. Figure 3 presents

a correlation matrix for ΔI-FABP, ΔCLDN-3, ΔLBP, overall GI discomfort, upper GI symptoms, lower GI symptoms, nausea, and side stitch.

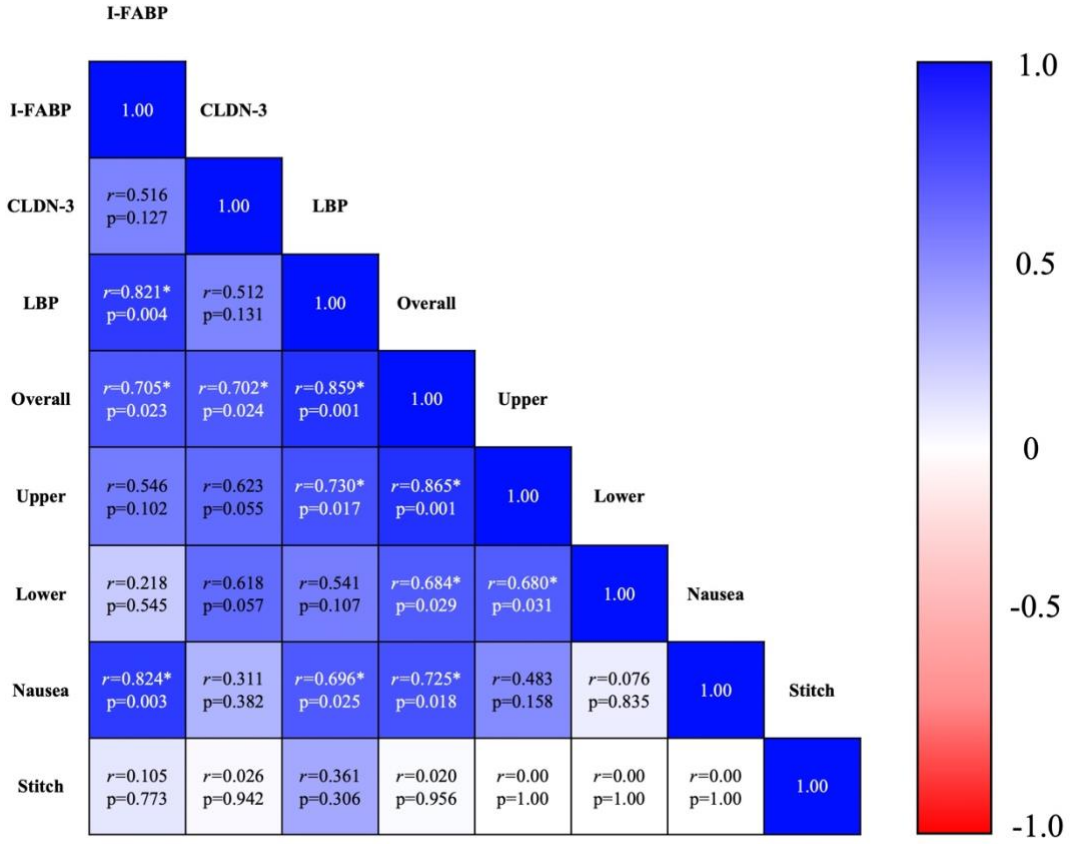

Figure 3. Correlation matrix of the magnitude of change (Δ) for I-FABP, CLDN-3, and LBP, and gastrointestinal symptoms in both experimental trials. Repeated measures correlations were performed to determine the correlation coeffect (*r*) which is presented to indicate the strength and direction of the relationships. I-FABP – intestinal fatty acid binding protein; CLDN-3 – claudin-3; LBP – liposaccharide binding protein; Overall – overall GI discomfort; Upper – upper GI symptoms; Lower – lower GI symptoms; Stitch – side stitch. *n*=9. *Denotes $p \leq 0.05$.

Discussion

The primary findings from this study were as follows: 1) exercise in hypobaric hypoxia (simulated altitude of 4300 m) resulted in significant elevations in markers of intestinal injury (I-FABP, CLDN-3, and LBP), 2) participants reported more frequent and more severe symptoms of GI distress during exercise in HYP compared to NORM, and 3) ΔI-FABP, ΔLBP, and ΔCLDN-3 were significantly correlated with some of the GI symptoms reported during exercise. Collectively these data indicate that intensity matched (absolute VO_2 and workload) cycling exercise performed at a simulated altitude of 4300 m increases plasma biomarkers of injury to the intestinal barrier. Moreover, we show that the magnitude of change for several of these biomarkers correlated with adverse GI symptomology. The significant relationships between these variables are consistent with the theory that loss of intestinal barrier integrity results in bacterial translocation, which may contribute to GI symptoms that are reported during exercise.

Our findings of intestinal injury following exercise in HYP expand upon previous investigations using normobaric hypoxia (Machado *et al.*, 2017; Lee & Thake, 2017; Hill *et al.*, 2020). Previous studies have focused on the effect of exercise in hypoxia on markers of intestinal injury but lacked direct evidence of its consequences. While intestinal permeability was not directly measured, we are the first to show that hypoxia-mediated intestinal injury results in a concomitant increase in the translocation of endotoxins as seen by the elevated concentrations of circulating LBP. These findings further support I-FABP as a robust and reliable marker of small intestinal injury (Ogden *et al.*, 2020) which has been shown elsewhere to correlate well with intestinal permeability (van Wijck *et al.*, 2012; March *et al.*, 2017). Furthermore, previous investigations have not assessed symptoms of GI distress

during exercise in hypoxia, hence the relationship between these biomarkers and acute GI symptomology was not clear. Here we provide novel evidence that these biomarkers (I-FABP, LBP, and CLDN-3) were associated with GI symptoms reported during cycling exercise, suggesting that hypoxia-mediated intestinal injury may have clinical significance. These findings fill several gaps in the literature and build upon the knowledge of the impact of hypoxia on the GI system.

Similar to our findings, Lee & Thake (2017) and Hill *et al.* (2020) reported increases in I-FABP following exercise in hypoxic (13.5% FiO_2) but not normoxic conditions. Pre- to post-exercise changes in I-FABP were higher in the present study than Lee & Thake (2017) which are likely explained by differences in the exercise protocol intensity (65% vs 50% of normoxic VO_2max) and/or duration (60 vs 40 minutes). Indeed, here we report similar pre- to post-exercise changes, and effect size, for I-FABP to Hill *et al.* (2020) who used a similar exercise protocol but different exercise mode (running vs cycling). This is somewhat surprising given that the present study used hypobaric hypoxia which likely presents a greater physiological stress than normobaric hypoxia (Roach *et al.*, 1996; Beidleman *et al.*, 2014). While there is no clear explanation for the different physiological responses to normobaric and hypobaric hypoxia, some of the proposed mechanisms include intravascular bubble formation, increased alveolar deadspace, ventilation/perfusion mismatch, and altered chemosensitivity (Loeppky *et al.*, 1997). However, it is likely that the duration of hypoxic exposure used by the present study and by Hill *et al.* (2020) (60-minutes) was not sufficient to induce these physiological differences (Coppel *et al.*, 2015). Future studies are needed to determine if the differences between normobaric and hypobaric hypoxia exist during longer exposures.

Pre- and post-exercise concentrations of CLDN-3 followed a similar pattern to I-FABP concentrations, though the magnitude of change for these markers were not significantly correlated. We are among the first to measure changes in CLDN-3 with exercise, and we are first group to demonstrate that exercise in HYP increases CLDN-3. CLDN-3 is a tight junction protein which helps regulate paracellular permeability in the intestine (Milatz *et al.*, 2010; Wells *et al.*, 2017). The release of CLDN-3 into circulation is an indirect assessment of tight junction breakdown, indicating intestinal barrier injury. The similar exercise-induced responses in I-FABP and CLDN-3 provide evidence of concurrent validity which further confirms our hypothesis of increased intestinal injury following exercise in HYP but not NORM. Taken together, these data suggest that one-hour of moderate intensity cycling in normoxia is not a sufficient stress to induce intestinal injury. However, when combined with an additional stressor, in this case hypoxia, exercise can result in significant increase in markers of intestinal injury (demonstrated via increases in I-FABP and CLDN-3). This exaggerated effect is similar to reports of increased I-FABP following exercise combined with heat stress or dehydration (Lambert *et al.*, 2008; Yeh *et al.*, 2013).

It has been previously suggested that running causes greater intestinal injury than cycling due to mechanical agitation (de Oliveira *et al.*, 2014). This hypothesis has also been used to explain the why runners report more adverse GI symptoms than other endurance athletes such as cyclists and swimmers. However, the importance of exercise mode on exercise-induced intestinal injury has been recently challenged (Edwards *et al.*, 2021), and it appears that it is the intensity of the exercise may better predict the extent of intestinal injury. In support of this, the changes in I-FABP concentrations from pre- to post-exercise reported here and by Hill *et al.* (2020) suggest that cycling and running in hypoxic environments

(hypobaric or normobaric) induce similar degrees of intestinal injury. Nonetheless, direct comparisons between running, cycling, and other exercise modalities including hypoxic environments as well as mechanistic investigation of intestinal injury are required before definitive conclusions can be made.

LBP is an acute-phase protein which binds to and transports LPS, released from the intestinal tract, to promote an immune and inflammatory response (Schumann & Latz, 2000). Thus, increased circulating LBP has been used as a surrogate marker for endotoxin translocation. We demonstrated that exercise in HYP results in significant elevations in LBP which likely indicate a mild endotoxin response. These data are corroborated by Machado *et al.* (2017) who reported significant elevations in circulating endotoxins following exercise in normobaric hypoxia (14% FiO_2), but not normoxia. Our data also confirm the findings of previous investigations demonstrating increased bacterial translocation following hypoxic exposures in rodent models (Zhou *et al.*, 2011; Luo *et al.*, 2017). The systemic effects of mild increases in markers of endotoxin translocation in exercising scenarios is not entirely clear. However, hypoxia-induced increases in circulating LPS could partially explain the proinflammatory response following exercise in hypoxia which has been reported by Hill *et al.* (2020) and Lee & Thake (2017). Proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukins 1 beta (IL-1β) and 6 (IL-6) are elevated following hypoxic exposures and have been suggested to contribute to the development of high-altitude cerebral edema (Zhou *et al.*, 2017; Wang *et al.*, 2018) which in extreme cases can be fatal (Davis & Hackett, 2017). In addition, the proinflammatory response may contribute to the performance decrement or early onset of fatigue (Vargas & Marino, 2014) which has been observed at

high altitude. These are important considerations for people who travel to altitude to perform physical work, and especially for those who require optimal exercise performance.

Intestinal barrier injury and bacterial translocation may explain some of the GI distress reported during high altitude ascents. Indeed, our data support this hypothesis as we report significant correlations between GI symptoms and markers of intestinal injury (I-FABP and LBP). This might also be an underlying factor for the GI tract related illnesses reported amongst trail runners, who are known to exercise at high-altitudes (Viljoen *et al.*, 2021). Given that LBP was the most strongly correlated (i.e., highest *r* value) with GI symptoms we theorize that hypoxia mediated LPS translocation may be more associated with GI symptoms than intestinal injury alone. Our data contradict previous investigations that did not observe significant correlations between exercise-induced systemic endotoxemia (Jeukendrup *et al.*, 2000) or markers of intestinal injury (Pugh *et al.*, 2017) and GI complaints. These discrepancies are perhaps explained by methodological differences in exercise protocols (i.e., duration, mode, intensity), environmental conditions, measured biomarkers, or GI symptom questionnaires. We acknowledge that the acute exercise protocol used in the present study likely lacks some generalizability. Karl *et al.* (2018) observed no significant relationships between GI symptoms and measures of intestinal permeability (lactulose/mannitol) or LPS translocation (LBP) following passive exposure to 4300 m. Thus, it is possible that the etiology of GI symptoms differs between exercise and non-exercise scenarios, or acute and prolonged exposures. Future investigations are needed to test this hypothesis in more applied field settings, perhaps using several days of high-altitude exposure in combination with physical work or exercise which result in more severe GI distress. Furthermore, more asymptomatic individuals are needed to determine a causal relationship between GI

symptoms and intestinal barrier injury or bacterial translocation as our data only indicate an association. Nonetheless, we do present a model of exercise in hypoxia that can induce mild- to severe GI symptoms which could be used to further study the impact of hypoxia on the GI system.

A potential limitation of the present study was the difference in the relative exercise intensity between the NORM and HYP trials. While we attempted to match for the exercise intensity via workload (watts) and absolute oxygen consumption ($L \bullet min^{-1}$), the lower VO_2max which has been reported at high-altitudes (Calbet *et al.*, 2003) likely increased the relative intensity (% of VO_2max) in the HYP trial. However, the present study sought to determine the effect of exercise performed in hypobaric hypoxia on the GI system, thus we felt that matching for total work performed would provide a more accurate estimation of this effect. This discrepancy in relative intensity provides an explanation for the higher RER observed in the HYP trial. Future studies are needed to determine if the effect of hypoxia on intestinal barrier injury persists when the exercise is matched for relative intensity. The continuous nature of the exercise bout used in the present study likely lacks some ecological validity for certain populations who are more likely to perform intermittent bouts of work at high altitudes. Interestingly, the role of the structure of an exercise bout (continuous or intermittent) on markers of intestinal injury is currently unclear, which is an area that warrants future investigation. In addition, CLDN-3 is also expressed in the vasculature (Castro Dias *et al.*, 2019). Therefore, it is possible that increases in plasma CLDN-3 could reflect broad endothelial stress or damage which has been observed during high-altitude ascent (Swenson *et al.*, 2020). The lack of assessment for proinflammatory cytokines as well as a direct assessment of circulating endotoxins or bacteria limit the interpretation of these

results. Future investigations should include assessments capable of quantifying specific increases bacteria (i.e., blood culture or bacterial DNA) as it appears that hypoxia-mediated bacterial translocation may differ between Gram-negative and Gram-positive species (Keely *et al.*, 2010).

In conclusion, we demonstrate that 60 minutes of moderate intensity cycling exercise performed at 440 Torr (4300 m of simulated altitude) significantly elevates markers of intestinal injury (measured by I-FABP, CLDN-3) and compromised barrier function (LBP). Together these findings suggest that exercise-induced intestinal injury may be aggravated by concurrent hypoxic exposure. Further, increases in I-FABP, LBP, CLDN-3 were correlated to exercise-induced GI symptoms, providing some evidence of a link between intestinal barrier injury and GI complaints. These findings might be considered when determining viable prevention strategies for high-altitude illnesses, including those unrelated to the GI system. For example, medications including non-steroidal anti-inflammatory drugs which are known to damage the intestinal barrier may further complicate high-altitude related GI symptoms (van Wijck *et al.*, 2012). On the contrary, other medications or supplements have potential to protect the intestinal barrier from high-altitude related intestinal injury (Zuhl *et al.*, 2014; King *et al.*, 2021). Future investigations are needed to determine the mechanisms of GI injury including direct measures of intestinal permeability and the role of contributing factors such as pro-inflammatory cytokines. Additionally, the impact of various medications/supplements on the intestinal barrier in efforts to elucidate unknown mechanisms by which they can prevent or worsen GI related symptoms reported during high-altitude ascent are needed.

Data availability: The data that support the findings of this study are available from the corresponding author (ZM) upon reasonable request.

Competing interests: The authors declare no conflict of interest.

Author Contributions: Conception or design of the work: ZM and CM. Acquisition, analysis, or interpretation of data for the work: ZM, ZF, QB, RN, FA, MD, CM. Drafting of the work or revising it critically for important intellectual content: ZM, ZF, QB, RN, FA, MD, CM

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CHAPTER IV: Study 2 - Markers of intestinal barrier dysfunction following exertion in hypobaric hypoxia

This chapter presents a research manuscript entitled “Markers of intestinal barrier dysfunction following exertion in hypobaric hypoxia” which has been submitted for publication in *Experimental Physiology*. Tables, figures, and references are provided at the end of the manuscript

Markers of intestinal barrier dysfunction following exertion in hypobaric hypoxia

Zachary J. McKenna[1], Bryanne N. Bellovary[2], Jeremy B. Ducharme[1], Michael R. Deyhle[1], Andrew D. Wells[3], Zachary J. Fennel[1], Jonathan Specht[1], Jonathan M. Houck[4], Trevor J. Mayschak[1,5], Christine M. Mermier[1]

[1]Department of Health, Exercise and Sports Sciences, University of New Mexico, Albuquerque, NM, USA

[2]Kinesiology Departments, State University of New York at Cortland, Cortland, New York

[3]Department of Health & Exercise, Wake Forest University, Winston-Salem, North Carolina

[4]Health and Human Performance Department, Roanoke College, Salem, Virginia

[5]Department of Emergency Medicine, University of New Mexico, Albuquerque, New Mexico

Corresponding Author: Zachary J. McKenna

Email: zmckenna@unm.edu

Mailing Address: MSC 063830, 1 University New Mexico, Johnson Center B143 Albuquerque NM 87131

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1. What is the central question of this study?

Do high-altitude exposures lead to intestinal barrier dysfunction, and is that related to the development of AMS?

2. What is the main finding and its importance?

Our findings provide addition evidence that high-altitude exposures can lead to intestinal barrier dysfunction. However, our preliminary evidence that intestinal barrier dysfunction does not contribute to AMS progression.

Abstract

Our primary aim was to determine the effects of hypobaric hypoxic stress on markers of intestinal barrier injury and circulating markers of inflammation. A secondary aim was to determine if intestinal barrier dysfunction or its inflammatory consequence was related to the development of acute mountain sickness (AMS). Thirteen participants were exposed to six hours of hypobaric hypoxia (simulated 4572 m) with two 30-minute bouts of intermittent exercise during the early hours of hypoxic exposure. Pre- and post-exposure blood samples were assessed for intestinal fatty acid binding protein (I-FABP), lipopolysaccharide binding protein (LBP), tumor necrosis factor-α (TNF-α), interleukin-1 receptor agonist (IL-1Ra), and interleukin-1β (IL-1β). I-FABP ($p=0.013$; $d=0.32$), LBP ($p=0.031$; $d=0.48$), TNF-α ($p=0.034$; $d=0.25$), IL-1β ($p=0.042$; $d=0.18$), and IL-1Ra ($p=0.004$; $d=0.23$) significantly increased from pre- to post-hypoxic exposure. Six of the 13 participants developed AMS; however, no differences for any marker were detected between those with and without AMS. These data provide more evidence that high altitude exposures can lead to intestinal barrier dysfunction, which may be an important consideration for mountaineers, military personnel, wildland firefighters, and athletes who travel to high altitudes to perform physical work or exercise.

Introduction

Each year, more than 40 million people visit high altitude areas (> 2500 m), and an estimated 140 million people have a permanent residence above 2500 m (Tremblay & Ainslie, 2021). The decreased barometric pressure at high altitudes and reductions in the partial pressures of inspired oxygen can have a host of physiological consequences including significant impacts on morbidity and mortality (Burtscher, 2014). High altitude illnesses vary from mild to life-threatening and often present in the form of acute mountain sickness (AMS), high-altitude cerebral edema (HACE), and high-altitude pulmonary edema (HAPE). The most common of these is AMS which has an estimated prevalence between 20% and 60% for those traveling to high altitudes (Meier *et al.*, 2017). AMS develops following rapid high-altitude ascent and includes a variety of nonspecific symptoms such as headache, nausea, dizziness, fatigue, and insomnia. In addition, gastrointestinal (GI) distress (e.g., anorexia, nausea, diarrhea, or vomiting) is one of the most commonly reported AMS symptoms with an estimated incidence of 80% amongst people suffering from the illness (Anand *et al.*, 2006). The pathophysiology of AMS is currently unknown, though one prevailing theory is related to dysfunction within the central nervous system (i.e., the brain) (Imray *et al.*, 2010). However, given the high incidence of GI symptoms associated with high altitude exposures, we speculate that the GI system may contribute to the development of AMS as well as other high-altitude associated GI complications (i.e., peptic ulcers (Fruehauf *et al.*, 2020) and GI bleeding (Wu *et al.*, 2007)).

High altitude exposures can reduce splanchnic perfusion (Loshbaugh *et al.*, 2006) and lower blood oxygen levels causing hypoxic and oxidative stress (Dosek *et al.*, 2007). These stressors can injure the intestinal barrier leading to increased intestinal permeability and

bacterial translocation (McKenna *et al.*, 2022*b*). The translocation of gram-negative bacteria which harbor lipopolysaccharides (LPS) on their outer membrane can activate innate immune cells via Toll like receptor-4 (TLR-4) to initiate local and systemic inflammatory responses (Ducharme *et al.*, 2022). Indeed, residence at high altitude has been shown to damage the intestinal barrier and increase intestinal permeability (Karl *et al.*, 2018). Subsequent LPS-mediated increases in pro-inflammatory cytokines could cross the blood brain barrier and contribute to central nervous system dysfunction which may be a contributing factor to development of AMS (Banks *et al.*, 1995). For people traveling to high altitude, it is important to consider the compounding effects of exertion or exercise which could worsen hypoxia-induced intestinal injury. Exercise in normobaric hypoxia was shown to increases circulating markers of intestinal injury and proinflammatory cytokines when compared to exercise performed in normoxic conditions (Lee & Thake, 2017; Hill *et al.*, 2020). For example, increases in intestinal permeability have been observed in soldiers during a four-day cross-country ski-march near sea level (Karl *et al.*, 2017). Recent data from our lab (McKenna *et al.*, 2022*a*) as well as others have shown that an acute bout of moderate intensity exercise in hypoxia increases markers of intestinal injury and inflammation However, less is known about the impact of longer hypoxic exposures in combination with low-moderate intensity exercise on the intestinal barrier.

In the present study, participants were exposed to six hours of hypobaric hypoxia (simulated 4572 m) with brief periods of intermittent exercise during the early hours of hypoxic exposure. This model was used for two reasons: 1) to mimic some of the activity required by those ascending or working at high altitude, and 2) to increase the likelihood of developing AMS. Accordingly, our primary aim was to determine the effects of this hypoxic

exposure on markers of intestinal barrier injury and circulating markers of inflammation. A secondary exploratory aim was to determine if these markers were related to the development of AMS.

Methods

The study was approved by the University Institutional Review Board (protocol no. 18419) and all experimental procedures conducted conformed to the Declaration of Helsinki except for registration in a publicly accessible database. Each participant provided a written informed consent document before beginning the study.

This study is an analysis of secondary objectives from a larger study where the primary aim was to investigate if orthostatic stress responses could predict AMS susceptibility. Thirteen (9 males, 4 females) participants volunteered to participate in this study, their demographics are provided in Table 1. All participants completed two visits including baseline testing and an experimental trial. Participants were apparently healthy and had no known diseases or symptoms of disease and satisfied pre-participation screening guidelines for exercise (Liguori *et al.*, 2021). All participants were residents in the Albuquerque, NM area (~1600 m) for the past year, were not smokers, and not pregnant.

Baseline Testing

Baseline testing consisted of height, weight, body composition, and aerobic capacity. Participants' height and body weight were measured and body composition estimated by the Jackson-Pollock three-site, sex-specific skinfold method (Jackson & Pollock, 1978; Jackson *et al.*, 1980). A maximal graded exercise test on a cycle ergometer (Excalibur Sport Lode, Groningen, Netherlands) was used to determine aerobic capacity. Graded exercise tests were developed using individualized ramp protocols which were based on sex, body weight, and

self-reported fitness level, and were designed to last between 8 and 12 minutes in duration (Yoon *et al.*, 2007). During the maximal graded exercise test, expired gases were collected and analyzed using a metabolic cart (TrueOne 2400, Parvo Medics, Sandy, UT) to determine maximum oxygen consumption (VO_2max). In addition, heart rate (Polar H10) and rating of perceived exertion (RPE) (Borg, 1982) were measured and recorded throughout the test. The test was terminated when the pedal cadence could no longer be maintained above 60 rpm (considered volitional fatigue). VO_2max was confirmed if three of the following four criteria were met; a plateau in VO_2 despite an increase in workload ($\leq$ 150 ml•min^{-1}), respiratory exchange ratio (RER) $\geq$ 1.1, heart rate within 10 beats of age predicted max, RPE $\geq$ 17. VO_2max was determined using 11-breath averaging from breath-by-breath data (Robergs, 2001).

Experimental Trial

Participants were instructed to refrain from alcohol for 24 hours, strenuous exercise for 12 hours, and caffeine for four hours before the experimental trial. During the experimental trial, participants underwent a six-hour exposure to hypobaric hypoxia simulating an altitude of 4572 m. Altitude was simulated using a customized hypobaric chamber at the University of New Mexico. Simulated ascent increased by $\leq$ 305 m per minute to prevent confounding symptoms related to a rapid simulated ascent (e.g., ear pain, dizziness, lightheadedness). During the first three hours of exposure participants completed two 30-minute bouts of cycling at workload equal to 50% of their normobaric VO_2max (Roach *et al.*, 2000). The cycling bouts were separated by at least one hour. Heart rate (Polar H10) and oxygen saturation (SaO_2) (Nonin Go2 pulse oximeter) were measured at the end of each bout of exercise and again at the end of the six-hour exposure just prior to descending. Participants

were free to read or interact with video screens while seated during the resting periods and were permitted to eat a standardized light snack (380 calories) and drink water *ad libitum*. Participants were not permitted to sleep during the hypoxic exposure. AMS was assessed using the modified Lake Louise Score (LLS) (Roach *et al.*, 2018). LLS scores were recorded after six hours of hypobaric hypoxic exposure. Classifications of AMS was AMS+ for LLS scores greater than or equal to 3 with headache and AMS- for LLS scores less than 3 (Roach *et al.*, 2018).

Blood samples were collected through venipuncture of an arm vein into EDTA vacutainer tubes immediately pre- and post-hypoxic exposure. Blood samples were centrifuged at 1600 x g for 15 minutes to separate plasma. 1 mL aliquots of plasma were immediately frozen and stored in a -80°C freezer. Intestinal injury was assessed by measuring the concentration of circulating intestinal fatty acid binding protein (I-FABP) which is a robust and reliable marker of intestinal injury (Ogden *et al.*, 2020) that has been shown to correlate well with intestinal permeability (van Wijck *et al.*, 2011, 2012; March *et al.*, 2017). Circulating LPS binding protein (LBP), a protein involved in the transport of LPS to immune cells, was also measured in circulation as an indicator of intestinal barrier injury. LBP is an indirect measurement of LPS. We chose to measure this surrogate marker of LPS, instead of LPS itself, to avoid the risk that contamination with exogenous endotoxins during sample collection or processing could confound the results. I-FABP and LBP were measured via ELISA kits (Hycult Biotech, Uden, The Netherlands) which were performed according to the manufacturer's instructions with intraassay coefficient of variations of 3.78%, and 3.50%, respectively. Tumor necrosis factor-α (TNF-α), interleukin-1 receptor agonist (IL-1Ra), and interleukin-1β (IL-1β) were analyzed via MAGPIX multiplexing according to the

manufacturer's instructions (Luminex xMAP Technology, San Diego, CA). IL-1β concentrations were undetectable (below the detection limit of the assay) in 9 of the 26 (~35%) assayed sample and these data are presented as zero. Intraassay coefficient of variations for TNF-α, IL-1Ra, and IL-1β were 7.57%, 8.60%, and 9.4%, respectively.

Data Analysis and Interpretation

An *a priori* power analysis was conducted (G*power version 3.1.0) using the effect size (Cohen's d of 1) from our prior study examining the effect of exercise in hypobaric hypoxia on I-FABP and LBP (McKenna *et al.*, 2022*a*). It was estimated with an α-level of 0.05, a power of 0.80 (1 - β), and assuming a moderate correlation among repeated measures (0.70) that eight participants would be required to detect differences in markers from pre- to post-hypoxic exposure. Statistical analyses were performed in RStudio (version 1.2.5033, R Development Core Team, Vienna, Austria). Prior to analysis, data were assessed for model assumptions (i.e., normality, equality of variance). Non-normally distributed data were log transformed prior to analyses. Data were also inspected to detect the presence of outliers, and the influence of suspected outliers were further investigated to determine if their removal or inclusion would significantly impact the results. Paired-sample t-tests (one-tailed) were used to compare pre- and post-hypoxia changes for all dependent variables. Next, linear mixed-effects models were fit to determine main effects of time (pre-and post-exposure), group (AMS+ and AMS-), as well as the interaction effect (time x group) on all dependent variables. Comparisons between AMS+ and AMS- groups for baseline data and LLS scores were made using independent sample t-tests (two-tailed). Statistical significance was set *a priori* to $p \leq 0.05$. Measures of effect sizes were quantified for each dependent variable and

are reported as Cohen's d (d) or partial eta squared (η_p^2). Data are reported in text, tables, and figures as mean ± standard deviation (SD) unless stated otherwise.

Results

Table 1. Participant demographics

	Sex M (F)	Age (yr.)	Height (cm)	Weight (kg)	VO_2max ($ml \cdot kg^{-1} \cdot min^{-1}$)	%BF
Grouped (*n*=13)	9 (4)	23±2	171±12	70.1±15.1	42.4±7.9	15.5±8.4
AMS+ (*n*=6)	3 (3)	23±3	164±7	63.4±11.1	39.1±8.0	18.8±8.4
AMS- (*n*=7)	6 (1)	23±2	176±13	75.8±16.5	45.2±7.18	12.6±7.9
p value		0.941	0.056	0.149	0.177	0.192

Note: M = males, F = females, VO_2max = maximal oxygen consumption, %BF = percent body fat.

Baseline characteristics were similar between AMS+ and AMS- groups (Table 1), however the AMS+ group had had a significantly higher LLS AMS scores (Table 2). Mean heart rate measured at the end of the first and second bouts of exercise was 158 ± 16 bpm and 164 ± 17 bpm, respectively. Average SaO_2 was 74 ± 5 % and 75 ± 2 % at the end of the first and second bouts of exercise, respectively and SaO_2 measured at the end of the sixth hour of exposure but before descent was 78 ± 3 %.

Table 2. Lake Louise Scale scores following 6-hours at simulated 4572 m.

	Headache	GI Symptoms	Fatigue	Dizziness	LLS score
Grouped (*n*=13)	1 (0-3)	0 (0-2)	1 (0-2)	1 (0-2)	2 (0-8)
AMS+ (*n*=6)	2 (1-3)	1 (0-2)	1 (0-2)	1.5 (1-2)	5.5 (4-8)
AMS- (*n*=7)	1 (0-2)	0 (0-0)	0 (0-1)	0 (0-1)	2 (0-2)
p value	0.004*	0.006*	0.032*	0.0001*	<0.0001*

Note: AMS = acute mountain sickness, GI = gastrointestinal, LLS = Lake Louise Scale. Symptoms were assessed at the end of the 6-hour exposure using the LLS AMS score (Roach et al., 2018). Scores reported above as median and (range). Comparisons between AMS+ and AMS- groups were made using independent t-tests. *Denotes significant difference.

Diagnostic testing revealed two suspected outliers: one due to abnormally high I-FABP and LBP and another due to high TNF-α, IL-1β, and IL-1Ra. Both outliers were determined to not be influential and were retained for analyses as their removal or inclusion did not significantly impact the results (i.e., p-value) (See Supplementary Table 1). Figure 1 shows pre- and post-hypoxic exposure changes in I-FABP and LBP, while Figure 2 displays results for TNF-α, IL-1β, and IL-1Ra. Paired-sample t-tests were used to compare pre- and post-hypoxic exposure for I-FABP, LBP, TNF-α, IL-1β, IL-1Ra. I-FABP was significantly increased from pre- (log_{10} 2.68 ± 0.34 pg•mL^{-1}) to post- (log_{10} 2.86 ± 0.28 pg•mL^{-1}) hypoxic exposure (p=0.001; d=0.32). Similarly, there was a significant increase in LBP from pre- (10.29 ± 3.74 μg•mL^{-1}) to post- (12.20 ± 4.22 μg•mL^{-1}) hypoxic exposure (p=0.011; d=0.48). TNF-α (p=0.009; d=0.25), IL-1β (p=0.042; d=0.18), and IL-1Ra (p=0.002; d=0.23) significantly increased from pre- to post- hypoxic exposure.

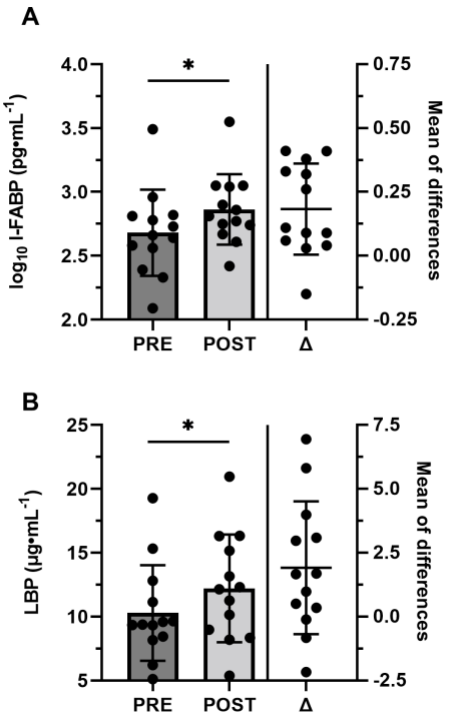

Figure 1. Pre- to post-hypoxic exposure changes in markers of intestinal barrier dysfunction. A) I-FABP and B) LBP. Δ shows mean of differences (post – pre). Data are shown as mean ± SD with dots representing individual data points. n=13. Non-normally distributed data were log transformed prior to analysis. Data were analyzed using paired sample t-tests (one-tailed). *Denotes $p \leq 0.05$. I-FABP- intestinal fatty acid binding protein; LBP- lipopolysaccharide binding protein.

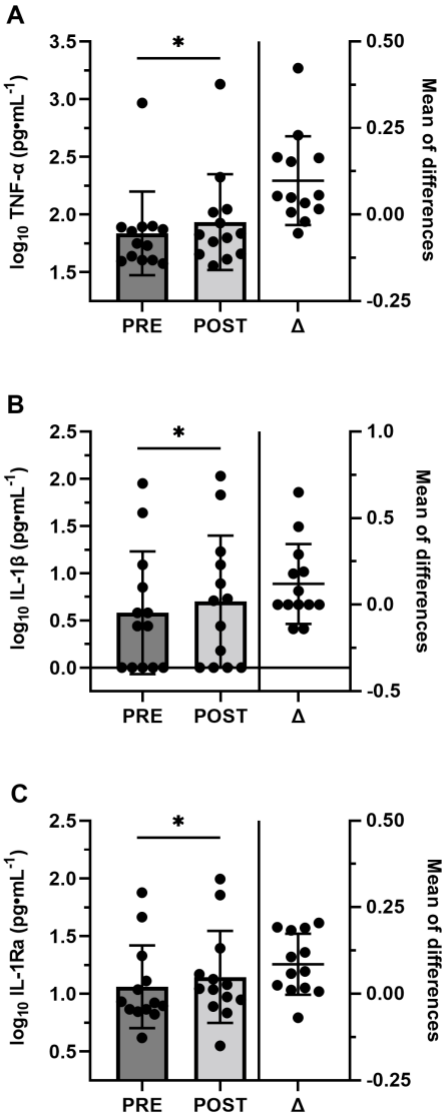

Figure 2. Pre- to post-hypoxic exposure changes in circulating cytokines. A) TNF-α, B) IL-1β, and C) IL-1Ra. Δ shows mean of differences (post – pre). Data are shown as mean ± SD with dots representing individual data points. n=13. Non-normally distributed data were log transformed prior to analysis. Data were analyzed using paired sample t-tests (one-tailed). *Denotes $p \leq 0.05$. IL-1β - interleukin 1-β, IL-1Ra- interleukin 1-receptor agonist, TNF-α- tumor necrosis factor-α.

Linear mixed-effects models were fit to determine main effects of time (pre and post), group (AMS+ and AMS-), as well as the interaction effect (time x group) on I-FABP, LBP, TNF-α, IL-1β, and IL-1Ra (Figure 3 and Figure 4). A significant main effect of time ($p=0.005$, $\eta_p^2=0.512$), but not of group ($p=0.408$, $\eta_p^2=0.058$) was detected for I-FABP. There was not a significant interaction between time and group for I-FABP ($p=0.587$, $\eta_p^2=0.025$). Likewise, there was a significant main effect of time ($p=0.023$, $\eta_p^2=0.364$), but not of group ($p=0.714$, $\eta_p^2=0.011$) for LBP. No significant interaction between time and group for LBP was detected ($p=0.486$, $\eta_p^2=0.041$). There was a significant main effect of time ($p=0.021$, $\eta_p^2=0.391$), but not of group ($p=0.717$, $\eta_p^2=0.012$) for TNF-α. No significant interaction was observed between time and group for TNF-α ($p=0.279$, $\eta_p^2=0.103$). There was not a significant main effect of time ($p=0.102$, $\eta_p^2=0.220$) or group ($p=0.586$, $\eta_p^2=0.027$) for IL-1β. No significant interaction between time and group for IL-1β was detected ($p=0.453$, $\eta_p^2=0.051$). A significant main effect of time ($p=0.007$, $\eta_p^2=0.499$), but not of group ($p=0.906$, $\eta_p^2=0.001$) was detected for IL-1Ra. No significant interaction between time and group for IL-1Ra was detected ($p=0.964$, $\eta_p^2<0.001$).

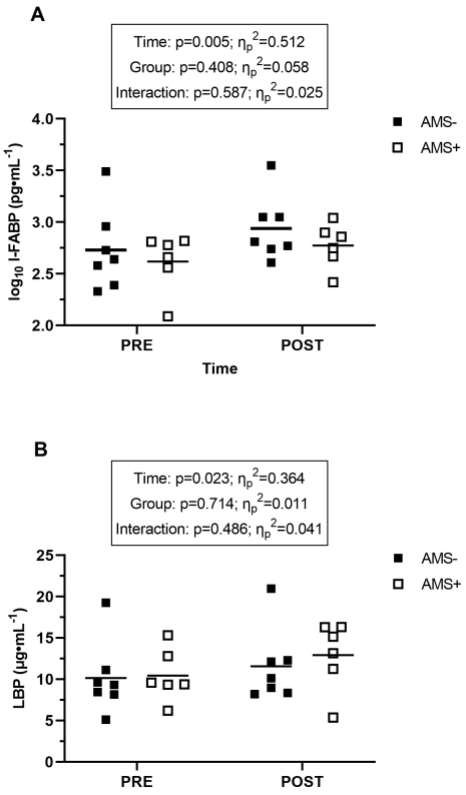

Figure 3. Pre- and post-hypoxia markers of intestinal barrier dysfunction in AMS+ and AMS- groups. A) I-FABP and B) LBP. Data are shown as mean with dots representing individual data points. n=13. Non-normally distributed data were log transformed prior to analysis. Data were analyzed using linear mixed effect models. *Denotes $p \leq 0.05$. I-FABP- intestinal fatty acid binding protein, LBP- lipopolysaccharide binding protein, η_p^2-partial eta squared.

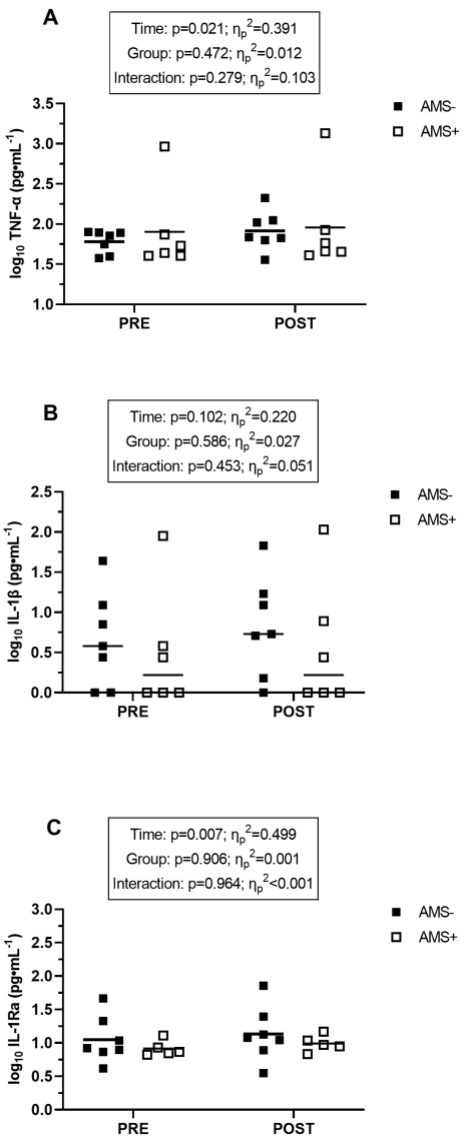

Figure 4. Pre- and post-hypoxia measures of circulating cytokines in AMS+ and AMS- groups. A) I-FABP and B) LBP. Data are shown as mean with dots representing individual data points. n=13. Non-normally distributed data were log transformed prior to analysis. Data were analyzed using linear mixed effect models. *Denotes $p \leq 0.05$. IL-1β - interleukin 1-β, IL-1Ra- interleukin 1-receptor agonist, TNF-α- tumor necrosis factor-α, η_p^2-partial eta squared.

Discussion

The primary aim of this study was to determine the effects of hypoxic stress on markers of intestinal injury and inflammation. Participants in the present study were exposed to six hours of hypobaric hypoxia simulating an altitude of 4572 m with two 30-minute periods of intermittent cycling exercise at 50% of their normobaric VO_2max during the first 3 hours of exposure. This model was used to mimic some of the normal activity which is typically required by those ascending or working at high altitude, and to provoke greater symptoms of AMS within a relatively short period of time. The key finding from this study was that our model induced mild disturbances to the intestinal barrier as evidenced by the increases in I-FABP and LBP. In addition, the pre- to post-hypoxia increases in TNF-α, IL-1β, and IL-1Ra are suggestive of an inflammatory response. These data provide more evidence that high altitude exposures can lead to intestinal barrier dysfunction, which may be an important consideration for groups (mountaineers, military personnel, wildland firefighters, athletes) who travel to high altitudes to perform physical work or exercise.

Our findings of increased I-FABP and LBP build upon previous studies which have shown that high altitude exposures can damage the intestinal barrier (Dinmore *et al.*, 1994; Karl *et al.,* 2018). For example, using a well-controlled but free-living laboratory design, Karl *et al.,* (2018) reported increased intestinal permeability at days 1 and 14 in a group living at 4300 m. However, it seems the primary aim of that study was to determine the influence of high-altitude residence on the intestinal barrier, which may be less relevant for acute exposure to high altitude such as those attempting high altitude ascent. In contrast, Dinmore *et al.,* (1994) studied a group of mountaineers ascending to 6300 m and reported increased intestinal permeability on day 5 after reaching an altitude of 5570 m. Given that

those data were collected in the field during multi-day expedition it is difficult to isolate the effects of exertion and/or hypoxia amid various other confounding variables (e.g., diet, weight loss, circadian disruption, psychological stress). One key advantage of the present study was the use of a tightly controlled methodological design in a laboratory setting which minimized the potential influence of such confounders. The pre- to post-hypoxic exposure increases in I-FABP and LBP confirm the results of those previous investigations and further demonstrate that high altitude ascent can perturb the intestinal barrier.

I-FABP is a robust marker of enterocyte damage which has been shown to correlate well with small intestinal permeability and exercise-induced splanchnic hypoperfusion (van Wijck *et al.*, 2011, 2012; March *et al.*, 2017). Interestingly, there is limited and conflicting evidence regarding the effect of hypoxia on splanchnic perfusion (Loshbaugh *et al.*, 2006; Kalson *et al.*, 2010). However, given the relationship between I-FABP and intestinal ischemia (van Wijck *et al.*, 2011) our data support Loshbaugh *et al.*, (2006) who reported reductions in resting and post-prandial superior mesenteric artery blood flow at simulated 4800 m. Nonetheless, the present study lacked a direct assessment of splanchnic perfusion, and it is possible the increased I-FABP were a result of hypoxemia mediated tissue hypoxia or by hypoxia-induced oxidative stress. The effect of hypoxic environments on splanchnic perfusion is an area that warrants future research as more data are needed to definitively determine if hypoxia does in fact reduce gut blood flow at rest or during exercise. Interestingly, while we observed a significant pre- to post-hypoxia increase in I-FABP, the effect size was relatively small (d=0.32) and lower than what we and others have previously reported using a brief hypoxic exposure with a bout of higher intensity exercise (Lee & Thake, 2017; Hill *et al.*, 2020; McKenna *et al.*, 2022*a*). This is somewhat surprising given

that the present study employed a longer hypoxic exposure, but the higher exercise intensity used previously may have caused greater hypoxemia or hypoperfusion resulting in greater intestinal injury (McKenna *et al.*, 2022*a*). Given that I-FABP has a relatively short half-life (~11 minutes) in the blood it is possible that there were larger increases during the early parts of exposure that were unobserved due to a lack of sampling (van de Poll *et al.*, 2007).

One consequence of intestinal barrier dysfunction is increased translocation of bacteria which may promote local and/or systemic inflammation. After leaving the intestinal lumen, LPS activates innate immune cells (i.e., locally within the lamina propria or Kupfer cells in the liver) via TLR-4 to produce and release pro-inflammatory cytokines. LBP is an acute-phase protein involved in the transport of LPS which has been used as a surrogate marker for circulating LPS (Schumann & Latz, 2000). Our data demonstrate increases in LBP pre- to post-hypoxia which support our previous findings following an acute bout of exercise at simulated 4300 m (McKenna *et al.*, 2022*a*). This also supports Machado *et al.*, (2017) who noted increases in circulating endotoxins following one-hour of moderate intensity exercise in hypoxia. An increase in circulating LPS results in greater activation of TLR-4 which leads to an increased transcription and translation of pro-inflammatory cytokines (Ducharme *et al.*, 2022). Importantly, TLR-4 is widely expressed on various cell types such as those of the intestinal epithelium (Zhao *et al.*, 2021) and cells of the innate immune system, therefore, it's activation by LPS has both local and systemic consequences. For example, TLR-4 activation and subsequent release of pro-inflammatory cytokines (including TNF-α and IL-1β) from intestinal epithelial cells in the jejunum can directly damage tight junction protein complexes thereby causing further increases in intestinal permeability (Zhao *et al.*, 2021). In addition, local release of TNF-α in the intestinal tract has been shown to reduce Na^+/K^+-ATPase

activity which can result in increased fluid accumulation in the intestinal lumen ultimately leading to diarrhea (Musch *et al.*, 2002). Systemically, this increased pro-inflammatory response could result in cytokines crossing the blood brain barrier which may have detrimental effects to the central nervous system (Banks *et al.*, 1995) and although speculative, potentially contribute to the progression of AMS to HACE. In the present study, the observed increase in circulating pro-inflammatory cytokines (TNF-α and IL-1β) coincided with an increase in the anti-inflammatory cytokine, IL-1Ra. Given the current study design, it is unknown whether the exercise bouts or hypoxia increased IL-1Ra, but this anti-inflammatory cytokine inhibits IL-1β signaling and therefore likely mitigates downstream consequences of IL-1β production. Future studies with more robust and frequent measures of the pro- and anti- inflammatory effects of acute hypoxic exposures are warranted. Further, participants in the present study were already acclimatized to ~1600 m, which may have influenced our results. It is possible that those living at or near sea level, may experience greater changes in markers of intestinal barrier injury and/or inflammation.

Our secondary aim was to determine if injury to the intestinal barrier or its inflammatory consequence was related to the development AMS. Using this hypoxic exposure model, nearly half of our participants (n=6) met the criteria for AMS according to the LLS score. These findings corroborate a previous investigation which showed that exercise exacerbates AMS during simulated high altitude ascent (Roach *et al.*, 2000). However, contrary to our initial hypothesis, the responses in intestinal barrier injury or inflammation did not appear to be different between AMS+ and AMS- groups. While we are among the first to explore the relationship between markers intestinal barrier dysfunction and AMS, our findings confirm those of Karl *et al.* (2017) who also noted no differences in

intestinal barrier function between individuals with and without AMS. These data contradict previous studies which have shown elevations in circulating markers of inflammation (e.g., TNF-α, IL-6, IL-1β) in AMS susceptible individuals (Wang *et al.*, 2018). Given that we used exercise in combination with hypoxia to induce AMS it is possible that these responses may differ in those passively exposed to hypoxic stress. Further, given the larger effect size of the interaction between time and group noted for TNF-α, our study may have been underpowered to adequately address this aim. Future studies are warranted with larger sample sizes using more direct markers of intestinal barrier function.

Conclusion

In conclusion, we show that six hours at a simulated altitude of 4572 m with two 30-minute periods of intermittent exercise during the early hours of exposure induced mild increases in markers of intestinal barrier injury and increased circulating markers of inflammation. These data provide additional evidence that even relatively brief periods of high-altitude exposures can lead to intestinal barrier dysfunction. These findings are an important consideration for groups attempting high altitude ascent or those traveling to high altitudes to perform physical work or exercise. However, these initial data suggest that intestinal barrier dysfunction may not be involved in the progression of AMS. Future studies are needed to replicate these findings using more direct assessments of intestinal barrier function.

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CHAPTER V: Study 3 - Ibuprofen increases markers of intestinal barrier injury but suppresses inflammation at rest and after exercise in hypoxia

This chapter presents a research manuscript entitled “Ibuprofen increases markers of intestinal barrier injury but suppresses inflammation at rest and after exercise in hypoxia” which has been submitted for publication in *Medicine & Science in Sports and Exercise*. Tables, figures, and references are provided at the end of the manuscript.

Ibuprofen increases markers of intestinal barrier injury but suppresses inflammation at rest and after exercise in hypoxia

Zachary J. McKenna[1], Jeremy B. Ducharme[1], Quint N. Berkemeier[1], Jonathan Specht[1], Zachary J. Fennel[1], Trevor L. Gillum[2], Michael R. Deyhle[1], Fabiano T. Amorim[1], Christine M. Mermier[1]

[1] Department of Health, Exercise and Sports Sciences, University of New Mexico Albuquerque, NM, USA

[2] Department of Kinesiology, California Baptist University, Riverside, CA, USA

Corresponding author: Zachary J. McKenna

Email: zmckenna@unm.edu

Phone: N/A

Mailing address: MSC 063830, 1 University New Mexico, Johnson Center B143 Albuquerque NM 87131

Abstract

Purpose: The aim of this study was to evaluate the effects of ibuprofen consumption on markers of enterocyte injury, intestinal barrier dysfunction, inflammation, and symptoms of gastrointestinal (GI) distress at rest and during exercise in hypobaric hypoxia. **Methods**: Using a randomized double-blind placebo-controlled crossover design, nine males (age: 28±3 years, weight: 75.4±10.5 kg, height: 175±7 cm, body fat: 12.9±5 %, VO_2peak at 440 Torr: 3.11±0.65 L•min^{-1}) completed a total of three visits including baseline testing and two experimental trials (placebo and ibuprofen) in a hypobaric chamber simulating an altitude of 4300 m. Pre- and post-exercise blood samples were assessed for intestinal fatty acid binding protein (I-FABP), ileal bile acid binding protein (I-BABP), soluble cluster of differentiation 14 (sCD14), lipopolysaccharide binding protein (LBP), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-10. Intestinal permeability was assessed using a dual sugar absorption test (L/R ratio). **Results**: I-FABP ($p=0.012$, $\eta_p^2=0.509$), I-BABP ($p=0.0003$, $\eta_p^2=0.822$), LBP ($p=0.005$, $\eta_p^2=0.651$), and sCD14 ($p<0.001$, $\eta_p^2=0.878$) were all significantly increased in the ibuprofen trial (main effect of condition). In addition, L/R was greater in the ibuprofen trial ($p=0.047$, $d=0.464$). Participants reported greater upper GI symptoms in the ibuprofen trial ($p=0.031$). However, MCP-1 ($p=0.007$, $\eta_p^2=0.613$) and TNF-α ($p=0.047$, $\eta_p^2=0.409$) were lower in the ibuprofen trial. **Conclusion:** These data demonstrate that two 600 mg doses of ibuprofen can worsen markers of enterocyte damage and intestinal barrier dysfunction at rest and following exercise in hypoxia. However, these changes were not accompanied by increased markers of systemic inflammation; in fact, ibuprofen suppresses circulating markers of inflammation.

Key words: permeability, hypobaric, acute mountain sickness, NSAIDs

Introduction

Acute Mountain Sickness (AMS) is a condition used to describe a collection of symptoms (i.e., headache, difficulty sleeping, nausea, anorexia, weight loss, and diarrhea) that commonly develop during rapid ascent to high-altitude (> 2500 m) (1). Ibuprofen is an over-the-counter non-steroidal anti-inflammatory drug (NSAID) which is often used to treat pain, inflammation, and fever (2). Recently, researchers have shown that ibuprofen can prevent symptoms of AMS such as high-altitude headache (3, 4). However, it has been speculated that ibuprofen may worsen symptoms of gastrointestinal (GI) distress or even increase the risk of GI bleeding (5). This is of particular concern for those traveling to high-altitude because GI distress and GI bleeding have been observed during hypoxic exposures in absence of ibuprofen (6–9). Furthermore, ibuprofen is commonly used by athletes and military personnel to treat musculoskeletal injuries and reduce acute pain and thus may be problematic for individuals exercising or performing physical work at altitude (10, 11).

Ibuprofen is a nonspecific NSAID which inhibits both cyclooxygenase 1 (COX-1) and COX-2 to prevent the formation of prostaglandins (12). In the gut, inhibition of COX enzymes and formation of prostaglandins can reduce microvascular blood flow and damage the intestinal barrier (13). In addition to this COX-mediated GI damage, ibuprofen may have COX-independent effects including direct interaction with the phospholipid bilayer and mitochondrial damage which can impair intestinal barrier function (12). Indeed, ibuprofen is known to increase intestinal permeability and elevate markers of intestinal injury at rest and following exercise near sea level (14, 15), which may be an underlying factor of GI distress (8, 16). Increases in intestinal permeability can allow for the translocation of bacteria from the intestinal lumen which can initiate local and systemic inflammatory responses.

Inflammation in the intestinal tract can directly damage the intestinal barrier, increase intestinal permeability, and reduce Na^+/K^+-ATPase activity causing fluid accumulation in the intestinal lumen which may contribute to diarrhea (17, 18). Systemic inflammatory responses could result in cytokines crossing the blood brain barrier which may have detrimental effects on the central nervous system and potentially contribute to symptoms of AMS (dizziness, headache, fatigue) or the progression to high altitude cerebral edema (19).

Recent data from our lab have shown that exercise in hypoxia can elevate circulating markers of enterocyte and intestinal barrier dysfunction (8). Given the known effects of NSAIDs on the gut, we speculate that ibuprofen may worsen these effects and further contribute to high-altitude associated intestinal barrier injury. Therefore, the primary aim of this study was to evaluate the acute effects of ibuprofen consumption on markers of enterocyte injury, intestinal barrier dysfunction, circulating markers of inflammation, and symptoms of GI distress at rest and during exercise in hypobaric hypoxia.

Methods

Using a randomized double-blind placebo-controlled crossover design, nine male participants (age: 28 ± 3 years, weight: 75.4 ± 10.5 kg, height: 175 ± 7 cm, body fat: 12.9 ± 5 %, VO_2peak at 440 Torr: 3.11 ± 0.65 $L \bullet min^{-1}$) completed a total of three visits including baseline testing and two experimental trials (placebo and ibuprofen) at a simulated altitude of 4300 m (440 Torr). Altitude was simulated using a customized hypobaric chamber at the University of New Mexico. For experimental trials participants were given two doses of ibuprofen (2 x 300 mg capsules) or placebo (2 x 5 mg capsules containing microcrystalline cellulose) to ingest 12-hours and 60-minutes prior to each experimental trial. Capsules were opaque and identical in appearance. Two 600 mg doses of ibuprofen were selected as this

dosing strategy is similar to the recommended dosing for individuals traveling to altitude (20). Participant and researcher blinding was monitored by a member of the research team who was not involved with data collection or analysis, and randomization was done using a random number generator. Successful blinding was confirmed by a verbal exit questionnaire following the conclusion of the second trial. Participants were free from cardiovascular disease and did not have any known GI disease or a regular history of GI distress. In addition, they did not use NSAIDs for at least one week prior to enrollment or report regular/ongoing use of supplements known to influence the intestinal barrier (i.e., glutamine, bovine colostrum, curcumin, etc.). Participants self-reported regular physical activity (> 150 minutes per week). All participants had resided in Albuquerque, New Mexico for at least 6 months prior to enrollment and had not traveled above 2500 m at least one week prior to enrollment. Experimental trials were separated by a minimum of seven days and completed at the same time of day to avoid diurnal variations. The study was approved by the University Institutional Review Board (protocol no. 1818088) and all experimental procedures conducted conformed to the Declaration of Helsinki except for registration in a publicly accessible database. Each participant provided written informed consent before beginning the study.

Baseline Testing

Once enrolled, participants underwent preliminary testing consisting of an estimation of body composition via 3-site skinfolds (21, 22), and a maximal graded exercise test on a cycle ergometer at 440 Torr in a hypobaric chamber. The maximal graded exercise test was a 20 watt•minute^{-1} ramp protocol which began at 40 watts. During the maximal graded exercise test, expired gases were collected and analyzed using a metabolic cart (TrueOne 2400,

Parvomedics, Sandy, UT) to determine maximum oxygen consumption (VO_2peak). The highest VO_2 using a 15-second average was considered the VO_2peak. Participants were given a food log to track their diet in the 24 hours prior to the first experimental trial and were asked to mimic their diet on the day prior to their second experimental trial. Participant compliance was confirmed verbally prior to the second experimental trial.

Experimental Trials

Participants arrived at the laboratory in the morning between 06:00 and 08:00 after an overnight fast and abstaining from vigorous or unaccustomed exercise for 24 hours, alcohol for 24 hours, and caffeine for 8 hours. A urine sample was collected to ensure euhydrated status (urine specific gravity ≤ 1.020) prior to exercise. Experimental trials began with 30-minutes of seated rest in the hypobaric chamber maintained at 440 Torr. Participants then exercised on a cycle ergometer for 60 minutes at a workload that elicited 65% of their VO_2peak at 440 Torr. 30-minutes into the exercise bout participants ingested the dual sugar drink for assessment of intestinal permeability. Heart rate (Polar H10, USA), oxygen saturation (SpO_2) (Nonin Go2 pulse oximeter, Plymouth, MN), and rating of perceived exertion (RPE) (23) were measured and recorded every 5-minutes during exercise. A modified visual analog scale was administered before, every 15-minutes during, and immediately after the experimental trials to assess symptoms of GI distress (24). This scale was chosen as it has been shown to have high test-retest reliability during exercise (24). The scale included ratings of overall GI discomfort, specific upper GI symptoms (belching, heartburn, upper abdominal bloating, stomach pain, urge to regurgitate), lower GI symptoms (flatulence, lower abdominal bloating, urge to defecate, abnormal stool), nausea, side stitch, and dizziness. Participants were familiarized with the scale and it was explained that the

severity of the ratings were deemed mild (1-3), severe (4-5), or very severe (7-10) as described elsewhere (24). Participants were free to drink water throughout the experimental trials but remained fasted for the entire duration of the trial.

Blood Sampling

Blood samples were collected through venipuncture of an arm vein into heparin, EDTA, or serum separator Vacutainers® before and immediately after exercise. Blood samples were centrifuged at 1600 x g for 15 minutes in 4°C to separate plasma or serum. Samples were stored in 1 mL aliquots in a -80°C freezer until later analysis. Pre- and post-exercise blood samples (plasma heparin or EDTA) were assayed for intestinal fatty acid binding protein (I-FABP), LPS binding protein (LBP), soluble cluster of differentiation 14 (sCD14) (Hycult Biotech, Uden, The Netherlands), and ileal bile acid binding protein (I-BABP) (RayBiotech, Norcross, GA) using enzyme-linked immunosorbent assay kits with intraassay coefficient of variations of 4.02%, 3.00%, 1.95%, and 3.96%, respectively. In addition, serum samples were assayed for cytokines (TNF-α, interleukin-1β (IL-1β), IL-10, and monocyte chemoattractant protein-1 (MCP-1)) via MAGPIX multiplexing (Luminex xMAP Technology, San Diego, CA). Intraassay coefficient of variations for TNF-α, IL-1β, IL-10, and MCP-1 were 4.1%, 3.0%, 4.4%, and 8.5%, respectively.

Intestinal Permeability

Intestinal permeability was assessed in both experimental trials using a dual sugar drink test as described previously (25). The drink was modified to only include 1 g of lactulose and 0.5 g of L-rhamnose, accordingly only small intestinal permeability was assessed. Stored urine samples were thawed, and 1.5 mL of urine was added to 100 mg of AmberLite® MB (Sigma-Aldrich, Saint Louis, MO). Samples were briefly vortexed and then centrifuged at

10,000 x g for 5 minutes. The supernatant was removed and passed through a 0.22-micron filter. Filtered samples were mixed with an internal standard (melibiose at a concentration of 20 mg•L^{-1}), concentrated under a nitrogen stream, and reconstituted in 100 µl of 50:50 acetonitrile:H_2O for analysis at the Brigham Young University College of Life Sciences Chromatography Center. High-performance liquid chromatography (HPLC) was carried out on an Agilent 1200 system with an evaporative light scattering detector by HILIC gradient using a Shodex HILICpak VG-50 4E 4.6 x 250mm column. Using H_2O as mobile phase A and acetonitrile as mobile phase B, HPLC was performed at 1.0 mL•min^{-1} as follows: 0-17 min, 88% B; 17-26 min, 88% B to 40% B; and 26-30 min, 40% B to 88% B. Quantification of lactulose and rhamnose were based on comparisons against the calibration curves established for each analyte. The urinary excretion of each ingested probe (lactulose and rhamnose) was determined by multiplying the measured concentration of each sugar by the total volume of urine collected and dividing by the dose administered. The ratio of these two values (L/R ratio) was used to determine small intestinal permeability. If lactulose was not detected in the sample the L/R ratio was assumed to be 0. One participant was excluded from the statistical analysis due to a failure to detect any sugar in the placebo trial, thus L/R ratio data are presented for n=8.

Data Analysis

An *a priori* power analysis was conducted (G*power version 3.1.0) using a conservative estimate of effect size (partial eta squared 0.3) from a prior study examining the effect of ibuprofen on I-FABP during exercise near sea level (14). It was estimated with an α-level of 0.05, a power of 0.80 (1 - β), and assuming a moderate correlation among repeated measures (0.70) that eight participants would be required to detect differences in the I-FABP responses

to exercise performed with ibuprofen versus placebo. Statistical analyses were performed in GraphPad Prism (GraphPad Software, San Diego, CA). Prior to analysis, data were assessed for model assumptions (i.e., normality, equality of variance). Non-normally distributed data were log transformed prior to analysis, except for GI symptoms which were analyzed using nonparametric tests. Dependent variables measured over time and across condition were analyzed using two-way (time x condition) repeated measures analysis of variance (ANOVA). Significant main or interaction effects were further explored using pairwise comparisons with Bonferroni corrections. To better understand the magnitude of change for markers of intestinal barrier dysfunction and inflammation (I-FABP, I-BABP, LBP, sCD14, and chemo/cytokines) the pre- to post-exercise changes (Δ) were quantified and comparisons of the Δ between trials were made using paired-sample t-tests (two-tailed). A paired sample t-test (two-tailed) was used to compare the L/R between placebo, and ibuprofen trials. GI symptom scores were totaled for the experimental trials and the incidence of GI symptoms (i.e., presence of any one symptom > 1) was calculated and reported as a percentage. Comparisons between the placebo and ibuprofen trials for GI symptoms (overall, upper GI, lower GI, nausea, side stitch, and dizziness) were made using the non-parametric Wilcoxon signed-ranked tests (two-tailed). Statistical significance was set *a priori* to $p \leq 0.05$. Measures of effect sizes were quantified and are reported as partial eta squared (η_p^2) or Cohen's d (d). Data are reported in text, tables, and figures as mean ± standard deviation or median and range where specified.

Results

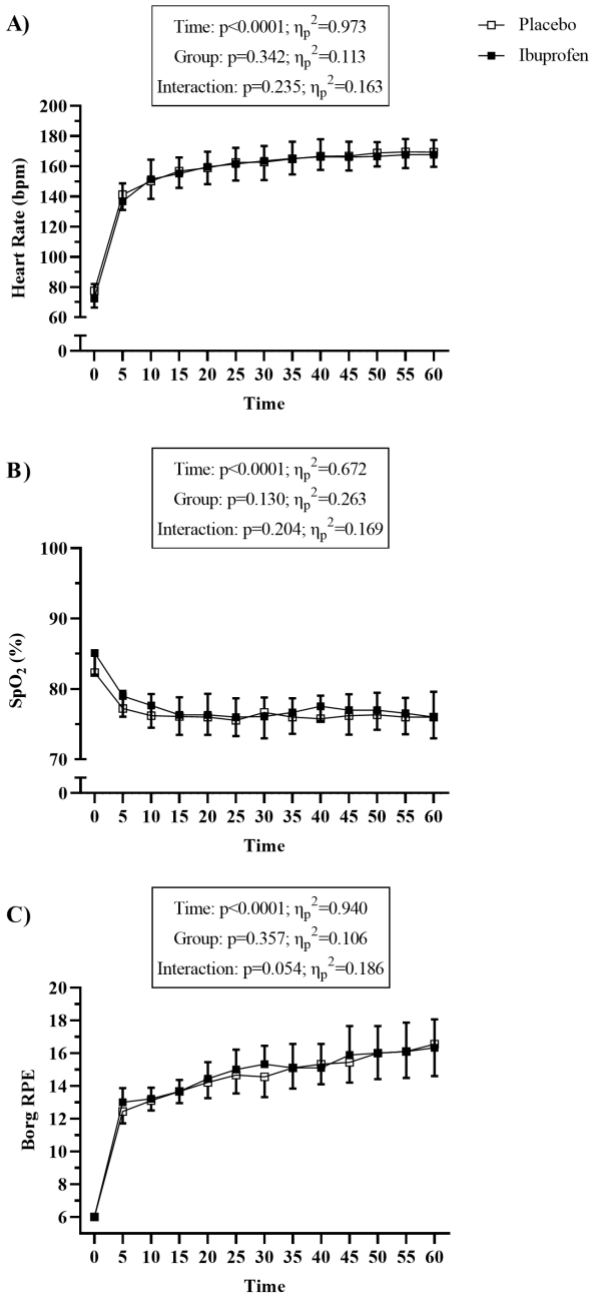

Figure 1. Physiological responses during exercise in placebo and ibuprofen trials. A) Heart rate, B) oxygen saturation, and C) Borg rating of perceived exertion. Data are reported as mean and standard deviation. *n*=9.

Physiological Variables

The physiological responses to exercise during both experimental trials are displayed in Figure 1. Average workload (133 ± 14 watts) and total work were identical between placebo and ibuprofen trials. Likewise, HR ($p=0.342$, $\eta_p^2=0.113$), SpO_2 ($p=0.130$, $\eta_p^2=0.263$), and RPE ($p=0.357$, $\eta_p^2=0.106$) were similar between placebo and ibuprofen trials.

Markers of Enterocyte Injury

Figure 2 shows markers of enterocyte injury during both experimental trials. There was a significant interaction between time and condition was for I-FABP ($p=0.021$, $\eta_p^2=0.509$). In addition, significant main effects of time ($p=0.017$, $\eta_p^2=0.563$) and condition ($p=0.012$, $\eta_p^2=0.509$) were detected for I-FABP. I-FABP was significantly increased from pre- to post-exercise in both the placebo (906.2 ± 394.7 to 1183.3 ± 618.1 pg•mL^{-1}; $p=0.006$, $d=0.530$) and ibuprofen trials (1168.3 ± 394.7 to 1666.2 ± 618.1 pg•mL^{-1}; $p<0.0001$, $d=0.615$). However, pre- ($p=0.008$, $d=0.524$) and post-exercise ($p=0.0001$, $d=0.589$) I-FABP were significantly higher in the ibuprofen trial compared to placebo. In addition, ΔI-FABP was significantly higher in the ibuprofen trial ($p=0.021$, $d=0.543$). Significant main effects of time ($p=0.0003$, $\eta_p^2=0.822$) and condition ($p=0.004$, $\eta_p^2=0.653$) were detected for I-BABP, however no significant interaction was observed ($p=0.128$, $\eta_p^2=0.265$). There were significant pre- to post-exercise increases in I-BABP in both the placebo ($\log_{10}$ 1.57 ± 0.72 to 1.64 ± 0.72 ng•mL^{-1}; $p<0.0001$, $d=0.095$) and ibuprofen trials ($\log_{10}$ 1.60 ± 0.71 to 1.69 ± 0.71 ng•mL^{-1}; $p<0.0001$, $d=0.137$). Pre- ($p=0.006$, $d=0.042$) and post-exercise ($p=0.006$, $d=0.149$) I-BABP were higher in the ibuprofen trial compared to placebo. However, the ΔI-BABP was similar between placebo and ibuprofen ($p=0.106$, $d=0.590$).

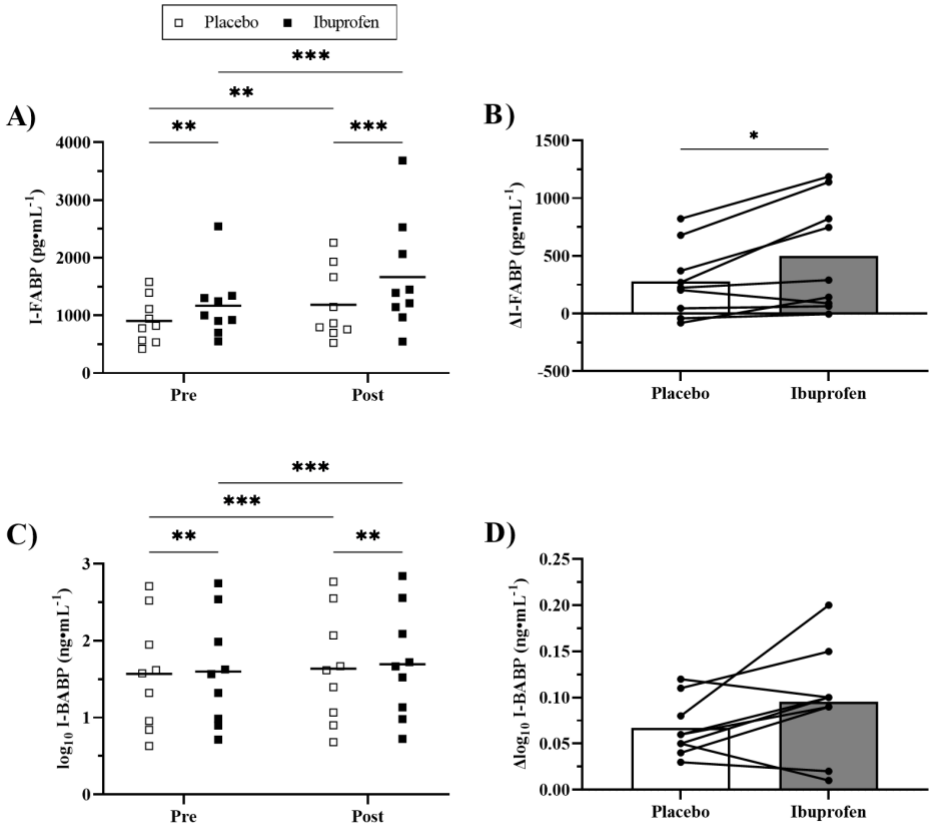

Figure 2. Markers of enterocyte injury in placebo and ibuprofen trials. A) plasma I-FABP, B) pre- to post-exercise change (Δ) in plasma I-FABP C) plasma I-BABP, and pre- to post-exercise change (Δ) in plasma I-BABP. The horizontal lines or bars mark the mean, and the squares/dots represent individual data points. Non-normally distributed data were log transformed prior to analysis. *n*=9. *Denotes $p \leq 0.05$, **denotes $p \leq 0.01$, and ***denotes $p \leq 0.001$. I-FABP – intestinal fatty acid binding protein, I-BABP – ileal bile acid binding protein.

Markers of Intestinal Barrier Dysfunction

Figure 3 displays markers of bacterial translocation during both experimental trials. Significant main effects of time ($p=0.005$, $\eta_p^2=0.654$) and condition ($p=0.005$, $\eta_p^2=0.651$) were detected for LBP, however no significant interaction was observed ($p=0.687$,

η_p^2=0.021). LBP was significantly elevated from pre- to post-exercise in the ibuprofen trial (16.05 ± 3.77 to 17.94 ± 3.87 μg•mL^{-1}; p=0.032, d=0.494) but not the placebo (14.33 ± 4.77 to 15.93 ± 4.34 μg•mL^{-1}; p=0.076, d=0.355). Pre-exercise LBP was similar in placebo and ibuprofen trials (p=0.052, d=0.404), however post-exercise LBP was significantly higher in the ibuprofen trial compared to the placebo trial (p=0.022, d=0.490). There was not a significant difference in ΔLBP between placebo and ibuprofen trials (p=0.686, d=0.001). Significant main effects of time (p<0.001, η_p^2=0.878) and condition (p=0.001, η_p^2=0.755) were detected for sCD14, however no significant interaction was observed (p=0.706, η_p^2=0.018). sCD14 was significantly increased from pre- to post-exercise in both the placebo (1512 ± 297.38 to 1626.11 ± 296.07 ng•mL^{-1}; p=0.030, d=0.388) and the ibuprofen trials (1642.44 ± 313.44 to 1733 ± 308.09 ng•mL^{-1}; p=0.014, d=0.423). Pre- (p=0.014, d=0.429) and post-exercise (p=0.007, d=0.485) sCD14 were higher in the ibuprofen trial compared to placebo. There was not a significant difference in ΔsCD14 between placebo and ibuprofen trials (p=0.655, d=0.212). Figure 4 shows the L/R ratio following the placebo and ibuprofen trials. The L/R ratio was significantly higher (p=0.047, d=0.464) following the ibuprofen trial (0.295 ± 0.152) compared to placebo (0.217 ± 0.187).

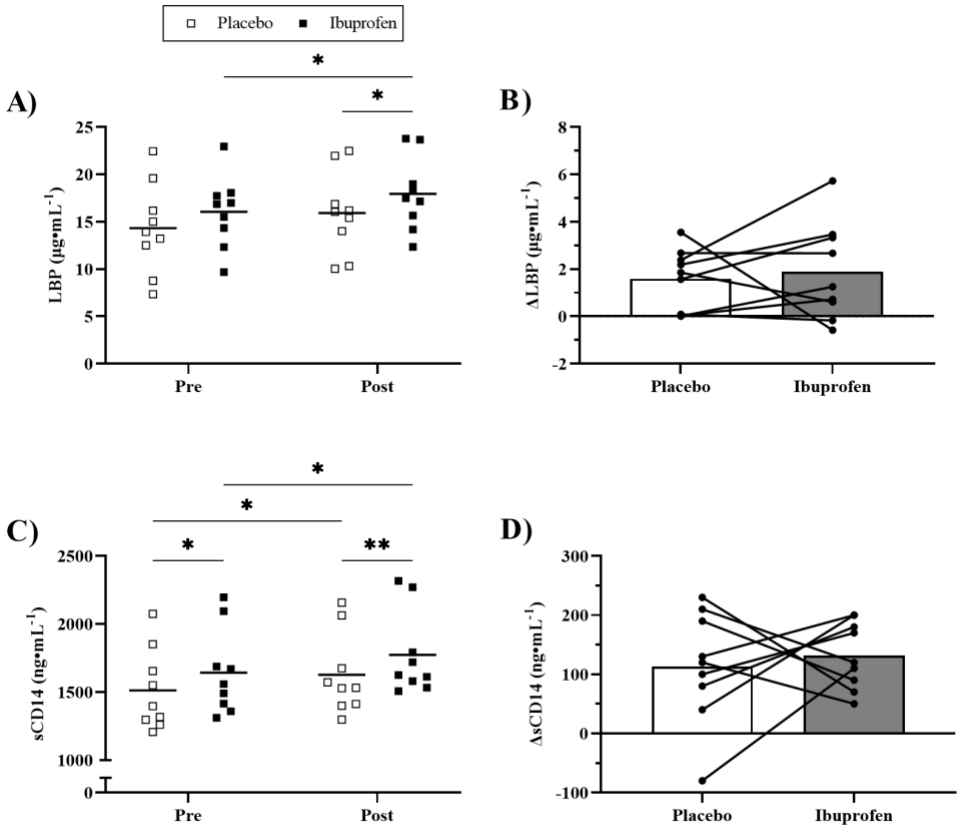

Figure 3. Markers of LPS translocation in placebo and ibuprofen trials. A) plasma LBP, B) pre- to post-exercise change (Δ) in plasma LBP, C) plasma sCD14, and D) pre- to post-exercise change (Δ) in plasma sCD14. The horizontal lines or bars mark the mean, and the squares/dots represent individual data points. *n*=9. *Denotes $p \leq 0.05$. LBP – lipopolysaccharide binding protein, sCD14 – soluble cluster of differentiation 14.

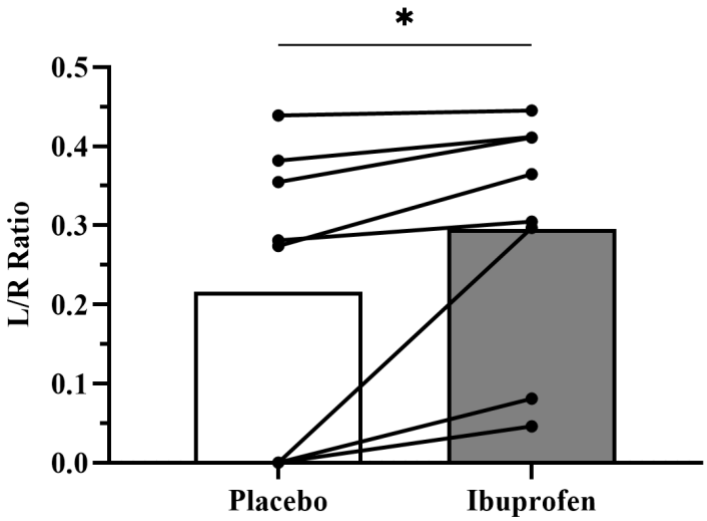

Figure 4. Intestinal permeability (L/R ratio = urine lactulose to rhamnose ratio) assessed following placebo and ibuprofen trials. The bars mark the mean, and the dots represent individual data points. *n*=8. *Denotes $p \leq 0.05$.

Circulating Markers of Inflammation

Circulating concentrations of inflammatory chemo/cytokines during both experimental trials are shown in Figure 5. There were significant main effects of time (p=0.002, η_p^2=0.727) and condition (p=0.007, η_p^2=0.613) for MCP-1, but a significant interaction between time and condition was not detected (p=0.693, η_p^2=0.020). MCP-1 was significantly elevated from pre- to post-exercise in both placebo (149.56 ± 65.40 to 226.46 ± 79.54 pg•mL^{-1}; p=0.004, d=0.950) and ibuprofen trials (104.12 ± 30.63 to 169.20 ± 70.55 pg•mL^{-1}; p=0.004, d=1.041). However, pre- (p=0.030, d=0.829) and post-exercise MCP-1 (p=0.030, d=0.728) were significantly lower in the ibuprofen trial. There was no difference in ΔMCP-1 between placebo and ibuprofen trials (p=0.696, d=0.191). Likewise, significant main effects of time (p=0.034, η_p^2=0.449) and condition (p=0.047, η_p^2=0.409) were detected for TNF-α, but no interaction was observed (p=0.306, η_p^2=0.130). TNF-α was elevated from pre- to post-exercise in both the placebo (30.52 ± 8.79 to 32.70 ± 9.97 pg•mL^{-1}; p<0.001, d=0.238) and

ibuprofen trials (27.91 ± 6.27 to 29.52 ± 8.07 pg•mL^{-1}; p<0.001, d=0.229). Pre- (p<0.001, d=0.347) and post-exercise (p<0.001, d=0.355) TNF-α was lower in the ibuprofen trial compared to placebo. Post-exercise TNF-α was significantly lower in the ibuprofen trial compared to the placebo. ΔTNF-α was similar between the two conditions (p=0.306, d=0.245). A significant main effect of time (p=0.030, η_p^2=0.463) but not condition (p=0.384, η_p^2=0.096) was detected for IL-1β. IL-1β was increased pre- to post-exercise in the placebo ($\log_{10}$ 0.975 ± 0.111 to $\log_{10}$ 0.984 ± 0.113 pg•mL^{-1}; p<0.001, d=0.083) and the ibuprofen ($\log_{10}$ 0.966 ± 0.107 to 0.977 ± 0.112 pg•mL^{-1}; p<0.001, d=0.105) trial. There was no difference in ΔIL-1β between placebo and ibuprofen (p=0.681, d=0.188). A significant main effect of time (p=0.0001, η_p^2=0.860) but not condition (p=0.495, η_p^2=0.060) was noted for IL-10. Further, there was not a significant interaction between time and condition for IL-10 (p=0.971, η_p^2<0.001). IL-10 was significantly elevated from pre- to post-exercise in both the placebo ($\log_{10}$ 0.868 ± 0.130 to $\log_{10}$ 0.902 ± 0.143 pg•mL^{-1}; p<0.001, d=0.253) and ibuprofen trial ($\log_{10}$ 0.861 ± 0.146 to 0.895 ± 0.147 pg•mL^{-1}; p<0.001, d=0.234) trial. There was no difference in ΔIL-10 between placebo and ibuprofen (p=0.919, d=0.019).

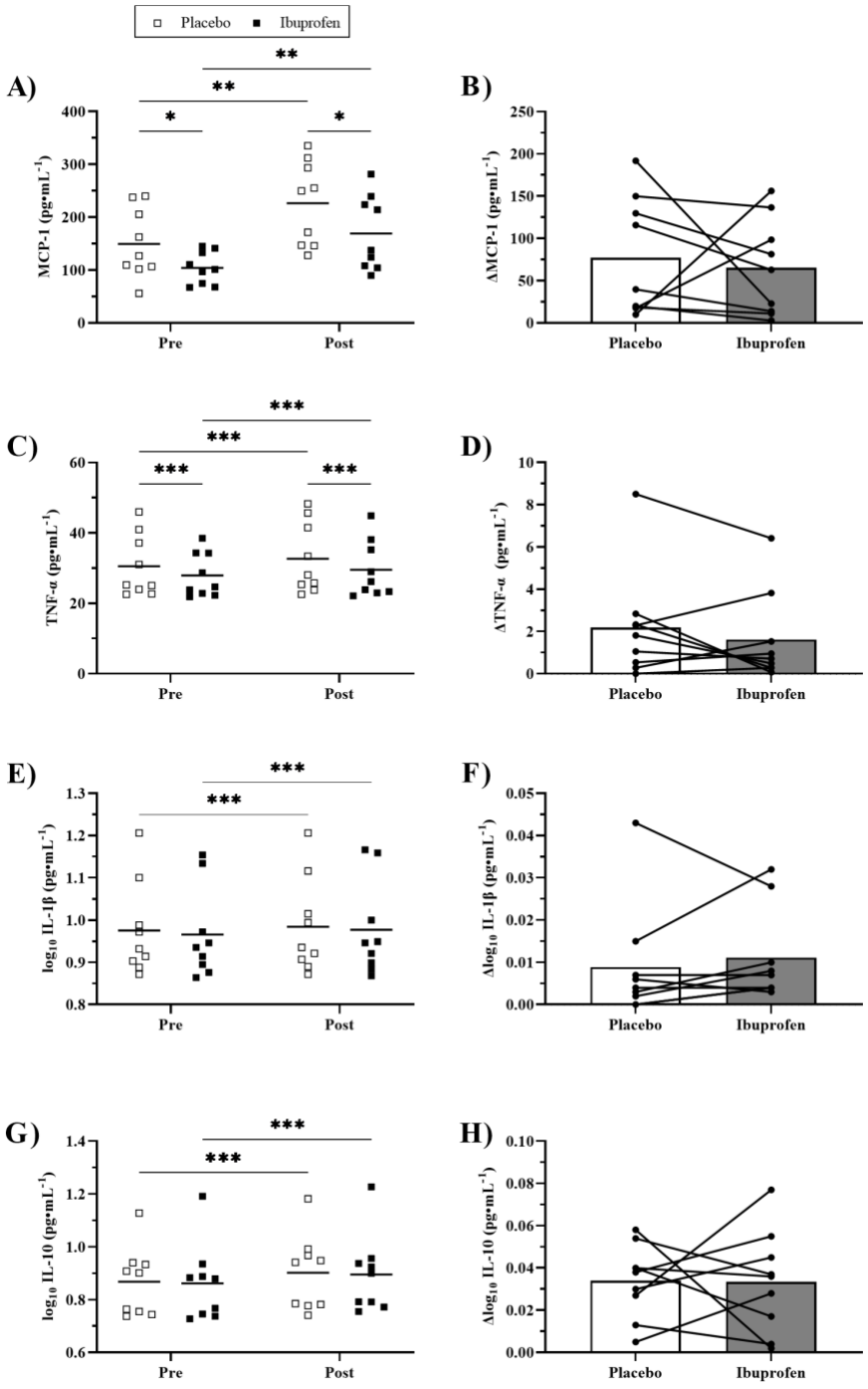

Figure 5. Circulating concentrations of inflammatory cytokines in placebo and ibuprofen trials. A) serum MCP-1, B) pre- to post-exercise change (Δ) in serum MCP-1, C) serum TNF-α, D) pre- to post-exercise change (Δ) in serum TNF-α, E) serum IL-1β, F) pre- to post-

exercise change (Δ) in serum IL-1β, G) serum IL-10, H) pre- to post-exercise change (Δ) in serum IL-10. The horizontal lines or bars mark the mean, and the squares/dots represent individual data points. Non-normally distributed data were log transformed prior to analysis. *Denotes $p \leq 0.05$, **denotes $p \leq 0.01$, and ***denotes $p \leq 0.001$. IL-1β – interleukin 1-β, IL-10 – interleukin 10, TNF-α – tumor necrosis factor-α, MCP-1 – monocyte chemoattractant protein-1.

Gastrointestinal Symptoms

A summary of the symptoms reported during both experimental trials are presented in Table 1. Overall gut discomfort, lower GI symptoms, nausea, side stitch, and dizziness were similar between placebo and ibuprofen trials. However, upper GI symptoms were greater in the ibuprofen trial (p=0.031). Two participants reported severe GI symptoms in the ibuprofen trial (belching: n=1 and lower abdominal bloating: n=1), while one participant reported severe symptoms in the placebo trial (lower abdominal bloating: n=1).

Table 1. Summary of symptoms reported during both experimental trials

	Placebo		**Ibuprofen**		
	Incidence	Median (range)	Incidence	Median (range)	p-value
Overall gut discomfort	78%	2 (0-18)	66%	2 (0-16)	p=0.879
Upper GI symptoms*	44%	0 (0-2)	66%	6 (0-12)	p=0.031
Lower GI Symptoms	78%	5 (0-29)	56%	2 (0-21)	p=0.094
Nausea	11%	0 (0-2)	11%	0 (0-1)	p>0.999
Dizziness	66%	6 (0-12)	66%	4 (0-13)	p=0.250
Abdominal Stitch	11%	0 (0-3)	0%	0	p>0.999

Note: Symptoms were assessed before and every 15-minutes during experimental trials using a modified visual analog scale (22). Symptom incidence was calculated as the percentage of participants who reported a symptom at any point during exercise. Symptom scores were then totaled for the entire experimental trial (maximum score of 70) and are reported above as median and (range). Comparisons between the median scores in placebo and ibuprofen trials were made using Wilcoxon sign-ranked tests (two-tailed). *Denotes statistical significance ($p \leq 0.05$). n=9.

Discussion

The purpose of this study was to evaluate the acute effects of ibuprofen on markers of enterocyte injury, intestinal barrier dysfunction, circulating markers of inflammation, and symptoms of GI distress at rest and during exercise in hypobaric hypoxia. The key findings from this study were as follows: 1) two 600 mg doses of ibuprofen increased markers of enterocyte injury (I-FABP and I-BABP) and intestinal barrier dysfunction (sCD14 at rest, 2) ibuprofen consumption worsened the intestinal barrier dysfunction induced by exercise in hypobaric hypoxia (as seen by ΔI-FABP and L/R), 3) participants reported greater upper GI symptoms during exercise with ibuprofen, and 4) circulating markers of inflammation were lower in the ibuprofen trial. Collectively these data demonstrate that ibuprofen causes enterocyte injury and aggravates intestinal barrier dysfunction at rest and during exercise in hypoxia. However, this compromised intestinal barrier function did not contribute to increased inflammation, and in fact it appears that ibuprofen suppresses circulating markers of inflammation. These findings highlight several mechanisms by which ibuprofen may impact the physiological responses to exercise in hypoxia.

NSAIDs are known to increase the risk for several GI complications including ulceration, perforation, and bleeding (26, 27). Despite this, ibuprofen is widely used by athletes and military personnel to treat musculoskeletal injuries and reduce pain (10, 11). Here, we report an increase in resting I-FABP and I-BABP in the ibuprofen trial which is suggestive of enterocyte injury. I-FABP and I-BABP are cytosolic proteins found within enterocytes located predominately in the jejunum and ileum, respectively. Thus, these markers are often used to detect enterocyte injury as their presence in circulation suggest loss of epithelial cell

membrane potential (28). The fact that both I-FABP and I-BABP were elevated at rest demonstrate that two 600 mg oral doses of over-the-counter ibuprofen was sufficient to induce broad injury to the small intestine. These findings corroborate van Wijck et al. (14) who reported elevated resting I-FABP and small intestinal permeability (L/R) following acute ibuprofen consumption (2 x 400 mg). Small intestinal injury may explain some of the GI complications which have been reported with acute ibuprofen consumption (26, 27). While the risk of GI complications such as GI bleeding with ibuprofen use has been reported to be relatively low (29), this risk may be exacerbated by other contributing factors. For example, in the context of high-altitude, rodent models have demonstrated that hypoxia alone can damage intestinal cells and increase intestinal permeability (30, 31). While not assessed in the present study, the compounding effects of hypoxia and ibuprofen alone on intestinal barrier injury is an interesting area of study that may be relevant for high-altitude travel.

In the present study, sCD14 and LBP were assessed as indirect markers of bacterial translocation (indicating intestinal barrier function) as these proteins are involved in the trafficking of LPS to immune cells (28). Here we report that resting sCD14 was elevated in the ibuprofen trial. In contrast, resting LBP was not significantly different ($p=0.052$) between the two trials, however the large effect size ($d=0.404$) combined with the sCD14 data suggest that ibuprofen alone was likely sufficient to induce a mild endotoxin response. These findings support Nieman et al. (33) who reported higher plasma LPS concentrations in ultramarathoners following ibuprofen consumption (1 x 600 mg dose consumed before and 6 x 200 mg doses taken during a race). While intestinal barrier function or enterocyte injury were not assessed in that study it is likely that ibuprofen caused increases in resting intestinal permeability (14) which allowed for the translocation of LPS. An advantage of the present

study was the use of a double-blind and placebo controlled methodological design which was not employed in previous studies examining the impact of ibuprofen on markers of intestinal barrier dysfunction (14, 33). Thus, our findings provide more convincing evidence that ibuprofen alone induces intestinal barrier dysfunction.

As expected, exercise in hypoxia increased markers of enterocyte injury, intestinal barrier dysfunction, and inflammation which partly confirm and build upon our previous findings (8). Indeed, here we report that exercise at simulated 4300 m induced enterocyte injury, as noted by the pre- to post-exercise increases in I-FABP and I-BABP. In addition, exercise caused an increase in sCD14 suggesting mild LPS translocation. Further, our findings of increased MCP-1, TNF-α, IL-1β, and IL-10 expand upon our previous work and confirm the work of Hill et al. (34) who demonstrated that exercise in hypoxia increases the circulation of both pro- and anti-inflammatory cytokines. However, in contrast to our previous study (8) the present findings show that exercise in hypoxia was not sufficient to increase LBP. This was somewhat surprising given the large effect size (d=1.12) that we previously reported, though this discrepancy is likely explained by differences in exercise intensity (8). For example, in our previous investigation participants exercised at a workload that elicited 65% of their normoxic VO_2max, while the present study used a workload that elicited 65% of their VO_2max at 440 Torr. Thus, the absolute workload in the present study was lower than our previous investigation, likely resulting in less hypoperfusion thereby inducing less damage to the intestinal barrier (35). Together these findings highlight the role of relative exercise intensity in inducing intestinal barrier dysfunction which is an important consideration for groups who are travel to altitude to perform physical work or exercise.

The greater ΔI-FABP and L/R ratio in the ibuprofen trial suggests that ibuprofen aggravated enterocyte injury and intestinal barrier dysfunction induced by exercise in hypoxia. Previous studies have shown that ibuprofen increases markers of enterocyte injury and intestinal permeability (14, 15), however we are the first to show that ibuprofen worsens hypoxia-mediated intestinal barrier dysfunction. Indeed, this finding is further supported by LBP which was significantly elevated from pre- to post-exercise in the ibuprofen trial but not the placebo. The mechanisms underlying this aggravated intestinal barrier dysfunction with ibuprofen are likely multifactorial. It has been speculated that the inhibition of COX induces vascular dysfunction and limits the production of nitric oxide in the intestinal microvasculature which ultimately reduce blood flow (36). This reduction in the splanchnic perfusion may put individuals at greater risk for developing GI complications during exertion in hypoxia. Indeed, this theory is supported by the greater upper GI symptoms reported by participants in the ibuprofen trial. Further, while the pre- to post-exercise changes in I-BABP, LBP, and sCD14 were similar between ibuprofen and placebo trials, these markers were all significantly higher at post-exercise in the ibuprofen trial. Thus, ibuprofen caused greater absolute increases in these markers which further demonstrates that a standard dose of ibuprofen can induce intestinal barrier dysfunction.

Exercise in hypoxia induced significant increases in the markers of inflammation (MCP-1, TNF-α, and IL-1β) in both trials. MCP-1 is a chemokine involved in the recruitment of various leukocytes (primarily monocytes) to sites of inflammation or injury, whereas TNF-α and IL-1β are pro-inflammatory cytokines which play important roles in the immune/inflammatory response to LPS through the interaction with Toll like receptor-4 (TLR-4) (37). Thus, the increase in TNF-α and IL-1β may be explained by exercise-induced

translocation of LPS, an enhanced TLR-4 activation enabled by elevated sCD14 presenting LPS to TLR-4, or a combination of the two (37). Interestingly, while markers of enterocyte injury and bacterial translocation were higher, we report lower circulating markers of inflammation in the ibuprofen trial. Specifically, it seems that the systemic inhibition of COX by ibuprofen caused a decrease in the circulating concentrations of MCP-1 and TNF-α. The fact that pre- and post-exercise MCP-1 and TNF-α were lower in the ibuprofen trial compared to placebo confirm that this dose of ibuprofen can lower systemic inflammation. However, it is important to note that LPS-mediated inflammation likely has both local and systemic consequences, and while these markers of inflammation were measured systemically in circulation, we speculate that local inflammation within the intestinal tract is more relevant in predicting GI complications. In support of this is the findings that NSAID induced intestinal mucosal damage is associated with excessive activation of the TLR-4 pathway in the small intestinal mucosa (38). Therefore, while NSAID supplementation may decrease systemic pro-inflammatory cytokine production, harmful effects local to the gut are likely to occur. In the present study, markers of inflammation were only assessed in circulation thus we are unable to identify the consequences of local inflammation specific to the site of activation. It is possible that the deleterious effects of ibuprofen are GI specific and intestinal inflammation may be increased with ibuprofen consumption. Future studies are needed to further test this hypothesis using animal models or specific markers of intestinal inflammation (e.g., fecal calprotectin) in humans. Furthermore, these results call to question the relevance to circulating inflammatory cytokines in exertional intestinal damage and disfunction.

Conclusions

The aim of this study was to determine the acute effects of ibuprofen consumption on markers of enterocyte injury, intestinal barrier dysfunction, circulating markers of inflammation, and symptoms of GI distress at rest and during exercise in hypobaric hypoxia. Our findings demonstrate that two 600 mg doses of ibuprofen cause enterocyte injury and intestinal barrier dysfunction at rest and following exercise at simulated 4300 m. In addition, the increases in these markers coincided with greater upper GI symptoms during exercise with ibuprofen. However, our data show that this compromised intestinal barrier function was not associated with increased levels of circulating inflammatory cytokines, and instead we show that ibuprofen suppresses circulating markers of inflammation. These findings highlight several mechanisms by which ibuprofen may impact those traveling to altitude. Specifically, ibuprofen may increase the risk of GI complications for those performing physical work or exercise in high-altitude environments.

Data availability: The data that support the findings of this study are available from the corresponding author (ZM) upon reasonable request.

Competing interests: The authors declare no conflict of interest. The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation. The results of the present study do not constitute endorsement by ACSM.

Author Contributions: Conception or design of the work: ZM, TG, MD, FA and CM. Acquisition, analysis, or interpretation of data for the work: ZM, JD, QB, JS, ZF. Drafting of the work or revising it critically for important intellectual content: ZM, JD, QB, JS, ZF, TG, MD, FA, CM.

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CHAPTER VI: Summary, Conclusions, and Recommendations

Summary & Conclusions

In chapter II we reviewed the small but growing body of literature examining the influence of high-altitude exposures on the intestinal barrier. Initial evidence suggests that acute exposures to hypoxia can directly damage intestinal epithelial cells and tight junction protein complexes, whereas prolonged hypoxic exposures may compromise the intestinal barrier through alterations in immunological function, microbiota, or mucosal layers. Further, it appears that exertion may worsen high-altitude related intestinal injury via additional reductions in splanchnic circulation and greater hypoxemia. In chapter III we examined the effects of hypobaric hypoxia on markers of exercise-induced intestinal injury and symptoms of GI distress. Our findings revealed that exercise performed at 4300 m of simulated altitude increased markers of intestinal barrier dysfunction. Further, we showed that increases in these markers were correlated to exercise-induced GI symptoms, providing some evidence of a link between intestinal barrier injury and symptoms of GI distress. In chapter IV we provide more evidence of high-altitude induced intestinal barrier dysfunction as we show that longer hypoxic exposures with low-moderate intensity exercise can increase markers of intestinal barrier injury and inflammation. However, our data indicate that intestinal barrier dysfunction was not related to AMS progression. In chapter V we determined the acute effects of ibuprofen consumption on markers of intestinal barrier dysfunction, circulating markers of inflammation, and symptoms of GI distress at rest and during exercise in hypobaric hypoxia. We revealed that a standard dose (2x 600 mg) of ibuprofen can induce intestinal barrier dysfunction at rest and following exercise at simulated 4300 m. In addition, we showed that increases in markers of intestinal barrier dysfunction coincided with greater

upper GI symptoms during exercise with ibuprofen. However, our data indicate that this compromised intestinal barrier did not contribute to increased inflammation, and instead we show that ibuprofen suppresses circulating markers of inflammation.

Recommendations

While the studies described above provide sound evidence of intestinal barrier dysfunction with exercise and ibuprofen at high altitude, their results leave a few questions unanswered. First, in chapter II we purpose that splanchnic hypoperfusion is a main mechanism by which hypoxia induces intestinal barrier injury. However, splanchnic perfusion was not assessed in any of the studies presented above. Thus, it is unclear if it is a decrease in gut blood flow, hypoxemia, or a combination of the two which induce intestinal barrier injury. In fact, the effects of hypoxia on gut blood flow are not well characterized, and future studies are needed to confirm if hypoxia does in fact reduce splanchnic perfusion. Next, given that hypoxia induced intestinal barrier dysfunction has been observed by us and others (1–4) a search for viable prevention strategies is warranted. These strategies are likely to include dietary or nutritional interventions which improve perfusion, strengthen the intestinal barrier, or target the gut microbiota. Finally, the results provided in chapter V suggest that ibuprofen has two distinct modes of action which include local GI and systemic effects. Due to methodological constraints of obtaining intestinal biopsies the study presented in chapter V did not reveal the direct effects of ibuprofen on the intestinal barrier. Future *in vitro* or *ex vivo* experiments are needed to directly observe these effects.

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