DISSERTATION

ADJUSTING ATTITUDES ABOUT ALTITUDE:

NOVEL APPROACHES TO PROMOTE HUMAN PERFORMANCE IN HIGH-ALTITUDE

Submitted by

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ABSTRACT

ADJUSTING ATTITUDES ABOUT ALTITUDE: NOVEL APPROACHES TO PROMOTE HUMAN PERFORMANCE IN HIGH-ALTITUDE

Military personnel frequently operate in environmental extremes, such as high-altitude, without adequate time for acclimatization. Altitude mediated decrements in human physiological function jeopardize mission success and personal safety. The following dissertation describes three experiments directed at the identification of non-traditional, military specific approaches to promote human functional performance in high-altitude. The specific aims of the following experiments were: 1) to compare the difference in time trial performance in normoxia and hypoxia following oral administration of a placebo, a non-specific phosphodiesterase inhibitor/adenosine receptor antagonist (Aminophylline), a carbonic anhydrase inhibitor (Neptazane), or the combination of Aminophylline and Neptazane; 2) to assess endurance exercise performance in hypoxia following an intravenous infusion of glucose with and without prior sympathetic nervous system inhibition (clonidine); and 3) to determine endurance exercise performance in hypoxia following a high-carbohydrate meal with and without prior/concurrent administration of an oral insulin sensitizer (metformin) and to compare hypoxic endurance exercise performance with endurance exercise performance in normoxia following the same meal. When compared with normoxia, hypoxia attenuated endurance exercise performance in these experiments. In experiment 1, we found that concomitant administration of Aminophylline and Neptazane attenuated the hypoxia-mediated deficit in endurance exercise performance compared with placebo. Neither Aminophylline nor Neptazane

alone ameliorated this decrement. In experiment 2, prior clonidine administration attenuated the cardiovascular response to hypoxia assessed by heart rate and blood pressure responses at rest but did not deleteriously impact endurance exercise performance in hypoxia. Finally, the preliminary data from experiment 3 suggest metformin improved the metabolic response to a high-carbohydrate meal in hypoxia, and potentially augmented skeletal muscle glycogen synthesis. Endurance exercise performance was unaffected in hypoxia following metformin administration. Collectively, the data from these experiments suggest these pharmacological treatments, compatible with military specific demands, effectively promote human physiological function in high-altitude.

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CHAPTER I – INTRODUCTION AND EXPERIMENTAL AIMS

Human physiological function is impaired in hypoxic environments, resulting in decreased endurance exercise performance (38). There are many potential contributors to this impairment including limited oxygen delivery to active tissues, and sympathetically mediated reductions in insulin sensitivity and subsequent glucose uptake in skeletal muscle. We have designed three studies to investigate different strategies to improve physiological function and attenuate the hypoxia-mediated decrement in endurance exercise performance in humans.

Study 1

Many pharmacological treatments have been proposed to improve oxygen delivery to active tissues in hypoxia, some with more success than others. These treatments include xanthene derivatives (39), corticosteroids (36), carbonic anhydrase inhibitors (75), beta-adrenergic receptor antagonists (142), and phosphodiesterase inhibitors (56, 72, 102). Recently, administration of these drugs in combination rather than individually has been shown to be a superior strategy for improving hypoxic exercise performance in rodents (108). We hypothesized that Aminophylline, Neptazane, and/or the combination of Aminophylline and Neptazane would attenuate the hypoxia-mediated decrement in endurance exercise performance in humans.

Specific Aim: To compare the difference in time trial performance in normoxia and hypoxia following oral administration of placebo, Aminophylline, Neptazane, or the combination of Aminophylline and Neptazane.

Study 2

We have previously demonstrated that hypoxic activation of the sympathetic nervous system attenuates insulin sensitivity (105); one of the consequences of insulin resistance is reduced uptake of glucose by skeletal muscle. As pre-exercise skeletal muscle glycogen is a primary determinant of endurance exercise performance (5, 6) attenuated insulin sensitivity in hypoxia could contribute to decrements in hypoxic exercise performance following a meal. In our earlier work, pharmacological inhibition of the sympathetic nervous system improved insulin sensitivity in hypoxia (105), however, this same strategy may impair endurance exercise performance on account of the contribution of the sympathetic nervous system to oxygen delivery and cardiovascular regulation (65, 113). Therefore we hypothesized that endurance exercise performance following intravenous glucose feeding in a low oxygen environment will be attenuated when feeding occurs during sympathetic inhibition.

Specific Aim: To assess endurance exercise performance in hypoxia following an intravenous infusion of glucose with and without prior transdermal clonidine administration.

Study 3

In contrast to use of a pharmacological strategy with established cardiovascular effects (that is, a sympathetic inhibitor; Study 2), we have chosen to use an alternative strategy: metformin, a commonly used pharmacological treatment that augments skeletal muscle glucose uptake (96) and skeletal muscle glycogen synthesis (3), without influencing oxygen delivery. Accordingly, we hypothesized that metformin will reduce the hypoxia mediated decrement in endurance exercise performance following a high carbohydrate meal in hypoxia.

Specific Aim 1: To determine endurance exercise performance in hypoxia following a high carbohydrate meal with and without prior/concurrent oral metformin use and to compare it to endurance exercise performance in normoxia following the same meal.

Specific Aim 2: To measure the change in skeletal muscle glycogen content following a high carbohydrate meal in hypoxia with and without prior/concurrent oral metformin use, and to compare that to skeletal muscle glycogen content in normoxia following the same meal.

CHAPTER II – REVIEW OF LITERATURE

Military personnel are frequently deployed to austere environments, including extremes in altitude, where the success of missions and the safety of the soldiers are dependent on sustained physical and mental performance. The challenges of high-altitude can be addressed with several physiological modifications (e.g. acclimatization), however decisions to deploy military personnel are often made swiftly, leaving inadequate time for these adaptations. Therefore, strategies to promote rapid acclimatization to high-altitude would be of obvious benefit to the military. Several previous investigations have identified pharmacological approaches that promote human physiological function in high-altitude. Reviewed here are these strategies, their applicability to the military, and the novel approaches to be addressed in subsequent chapters.

Acute Responses to High-Altitude

High-altitude environments impact human physiological function via the hypobaric mediated decline in the availability of oxygen. As altitude increases the partial pressure of oxygen falls, narrowing the pressure gradient of oxygen from alveoli to capillaries, diminishing arterial oxygen content. The decline in arterial oxygen content is sensed by smooth muscle cells in the pulmonary vasculature, triggering several cellular mechanisms culminating with pulmonary vasoconstriction (138) in an attempt to align pulmonary perfusion and ventilation. The cumulative effect of the diminished oxygen pressure gradient and pulmonary vasoconstriction is a decline in oxyhemoglobin saturation, i.e. systemic hypoxia. To elucidate the physiological impact of the reduction in arterial oxygen content, field experiments have been

conducted in high-altitude as well as artificial manipulations of barometric pressure (hypobaric-hypoxia) and ambient oxygen content (hypoxic-hypoxia) in laboratory settings.

High-altitude exposure evokes several physiological responses intended to satisfy the oxygen requirements of metabolically active tissues. Pulmonary diffusion capacity (47), and tidal volume and breathing frequency (20) increase to improve arterial oxygen content. Augmented ventilation is initiated via peripheral chemoreceptors in response to the decline in arterial oxygen content, but these responses can be detrimental due to excessive unloading of carbon dioxide and subsequent respiratory alkalosis. The drop in arterial carbon dioxide content blunts the initial/immediate ventilatory response to high-altitude and increases arterial pH, shifting the oxyhemoglobin saturation curve to the left, increasing the affinity of hemoglobin for oxygen thereby reducing the amount of oxygen unloaded at active tissues.

Chemoreceptor activation, via the decline in arterial oxygen content, also instigates an increase in sympathetic nervous system activity, which acts through several cardio-pulmonary mechanisms to maintain oxygen availability for active tissues. Signaling via cardiac beta-adrenergic receptors increases heart rate and cardiac output (17, 33, 79, 92), and the baroreflex is adjusted to prevent a reactionary decline in mean arterial pressure (53). In the lungs, beta-adrenergic stimulation augments bronchodilation (35, 73) thereby improving air movement through the lungs. Additionally, in the periphery, beta-adrenergic vasodilation outpaces alpha-adrenergic vasoconstriction augmenting peripheral blood flow (15, 27, 148).

The sympathetically mediated cardio-pulmonary response also has metabolic consequences, namely, disrupted glucose regulation and impaired insulin sensitivity (78, 103, 105). Endogenous glucose production is increased via norepinephrine and epinephrine signaling pathways (103, 144). Epinephrine impairs both skeletal muscle insulin signaling via diminished

interaction of insulin receptor substrate-1 and phosphatidylinositol 3-kinase (59), and glycolysis through inhibition of hexokinase (111) and glycogen synthase (130). Further, sympathetic activation stimulates lipolysis, augmenting the circulating concentration of free fatty acids (67, 101), impairing insulin mediated glucose disposal (145). The ramification of disrupted glucose homeostasis in high-altitude is inhibited glycogen replenishment in skeletal muscle. As pre-exertional glycogen content is a primary determinant of endurance exercise performance (5, 6, 16), and dependence on carbohydrate is increased when arterial oxygen content declines (11, 68, 87, 120), impaired glucose handling is detrimental to exercise performance in high-altitude.

Human Physiological Function in High-Altitude

Despite the activation of several systems to promote the maintenance of oxygen delivery, human physiological function is impaired in high-altitude. A quantifiable assessment of the decrement in physiological function is endurance exercise performance as it is dependent on oxygen delivery (arterial oxygen content and cardiac output) and carbohydrate availability (skeletal muscle glucose uptake and glycogen content). In high-altitude environments, there are marked declines in submaximal and maximal endurance exercise performance (17, 33, 79, 92) that become more apparent as altitude increases (38). Decrements at high-altitude range from 0 to ~30%, depending on the duration of the activity and/or the severity of hypobaria (38). For example, in a recent review of marathon performance and high-altitude, marathon completion times were reported to increase by approximately 10-12% per 1,000 m gain in altitude (77).

One of the limiting factors to exercise performance in high-altitude is cardiac output. At submaximal intensities, cardiac output is increased when arterial oxygen content is compromised, however it is reduced during maximal exercise (54). Additionally, maximal

exercise performance is limited by the expansion of the alveolar-arterial oxygen content difference: the product of mismatched perfusion and ventilation in the pulmonary system (40, 55, 140). Further, oxygen uptake (VO_2) kinetics are slowed when arterial oxygen is reduced, creating an even greater mismatch between oxygen supply and demand during the on-transient of submaximal exercise (31, 57, 74, 132, 152) potentially impairing endurance performance (23). The oxygen cost of a standardized sub-maximal workload is unaffected by the partial pressure of oxygen (37, 38, 70, 121). However, due to the hypobaria-mediated decline in maximal oxygen uptake (VO_{2max}), the same standardized workload may represent a greater relative effort. The increase in relative intensity is especially apparent during discrete tasks requiring completion of a fixed amount of work as quickly as possible (e.g. time trials, marches) (38, 52, 106); routine activities performed by deployed military personnel. The reduction in submaximal and maximal aerobic exercise capacity relates to a decrease in physical performance and translates to a potential for mission failure and the threat of grave injury or death.

In addition to impairments in aerobic exercise performance, traveling to high-altitude reduces physiological function via the development high-altitude related illness. Acute mountain sickness, a condition that manifests in pulmonary and cerebral edema, loss of physiological function, and mental acuity (50), develops most often following rapid gains in altitude. Another health consequence of traveling to high-altitude is sleep disordered breathing characterized by periods of apnea and further, but transient, decreases in arterial oxygen content (2). Intermittent apnea during sleep may increase the risk for the development of acute mountain sickness symptoms and is related to poor physiological function (86). Together, these physiological decrements associated with high-altitude highlight the difficulties military personnel face during deployment in these environments.

Pharmacological Treatments to Augment Arterial Oxygen Content

Acclimatization to hypobaria at high-altitude, and restoration of physiological function, may require days to weeks depending on the altitude (14, 19, 85, 106, 109). Military personnel often do not have sufficient time for these adaptations prior to enemy engagement. Pharmacological strategies aimed at augmenting arterial oxygen content have been thoroughly investigated for civilian applications. Their applicability to the military population, however, is questionable due to rapid nature of military action/deployment and military specific health/safety concerns.

The most pervasive pharmaceutical in high-altitude research is acetazolamide, a carbonic anhydrase inhibitor (119) commercially available as Diamox, that augments renal secretion of bicarbonate, leading to metabolic acidosis. The decline in pH counterbalances respiratory alkalosis and attenuates the leftward shift in the oxyhemoglobin dissociation curve usually observed during ascent in altitude (34, 48, 136, 137). Administration of acetazolamide, over a wide range of durations, is an effective treatment to increase arterial oxygen content during submaximal and maximal exercise (9, 32, 42, 64, 75, 129); however, maximal aerobic exercise performance following acetazolamide administration is variable with reports of improved (129), unchanged (32, 51, 134), or decreased VO_{2max} (42). The effect of acetazolamide on submaximal aerobic exercise is also unclear. In two investigations (9, 134), arterial oxygen content was manipulated by the change in barometric pressure; however, in addition to a small difference in exercise intensity, there is a major discrepancy between the method of acetazolamide administration. In one study (9), acetazolamide was given to all study participants during the three days prior to ascent to 4826 m. Once the participants reached the goal altitude, half were switched to a placebo while the others continued on acetazolamide. In the other study (134),

participants received only acetazolamide or placebo eight hours and again two hours prior to exercise in hypobaria. The absorption, and bioavailability of acetazolamide may dictate that the most effective method of administration is to begin several days prior to departure for high-altitude, and continue its use during high-altitude exposure. Unfortunately, this regimen may not be appropriate for military personnel on account of unanticipated deployment, and also reports of nausea associated with acetazolamide administration at sea-level (150, 151). With respect to the latter, pre-deployment nausea is unlikely to be perceived as ideal preparation for military missions.

Regarding high-altitude health related outcomes, acetazolamide is an effective prophylaxis for acute mountain sickness, reducing the risk of developing symptoms by half (119). In several double-blind studies, administration of acetazolamide for one-to-three days prior to ascent in altitude successfully diminished acute mountain sickness symptoms (9). The results are not always positive; a small number of investigations have found no difference between acetazolamide and placebo in the prevention of acute mountain sickness when examining both hypoxic-hypoxia (129) and hypobaric-hypoxia (134). The difference between these two studies and the successful interventions with acetazolamide is not readily apparent. The dose and timing of administration were similar to other investigations, however 62% of the participants in one of the studies (134) had suffered from acute mountain sickness during prior ascents in altitude. As a previous history of acute mountain sickness is a major predictor of sickness during subsequent high-altitude exposures (128), this study cohort may have been at greater risk for developing symptoms thus decreasing the effectiveness of acetazolamide . In addition to reducing the symptoms of acute mountain sickness, acetazolamide also attenuates high-altitude associated apneic episodes during sleep (34, 48, 136, 137). In summary, while

acetazolamide is an adequate treatment for civilians traveling to high-altitude it is unacceptable for military personnel due to the required timing of administration and an increased risk of paresthesia (sensation of tingling on skin). In addition, of greatest concern to the military, is an amplified threat for bleeding following an injury while taking acetazolamide (123), a potentially catastrophic risk considering the bodily harm military personnel may sustain during high-altitude combat.

An alternative to acetazolamide is methazolamide, a carbonic anhydrase inhibitor that is not known to affect the fibrinolytic system. Methazolamide is as effective as acetazolamide in curtailing the symptoms of acute mountain sickness and elicits a greater rise in arterial oxygen content following acute administration than acetazolamide (150, 151). Additionally, paresthesia occurrence is diminished with methazolamide (150). Together, these advantages of methazolamide compared with acetazolamide identify methazolamide as one potential strategy for the military to address the high-altitude-mediated decline in physiological function.

In addition to carbonic anhydrase inhibitors, the use of anti-inflammatory agents, such as glucocorticoids (dexamethasone), to improve function in high-altitude has been considered. Inflammation is a primary mechanism of high-altitude pulmonary edema development; dexamethasone prevents high-altitude cerebral and pulmonary edema, and acute mountain sickness (29, 30, 88), and is also an effective treatment of acute mountain sickness (49). Additionally, from a function/performance perspective, 24 hours of dexamethasone use attenuated the hypoxia-mediated decline in VO_{2max} in participants susceptible to high-altitude pulmonary edema (36). Dexamethasone, however, does not improve breathing during sleep in hypobaria (82), and pertinent to metabolic health and maintenance of skeletal muscle glycogen at

high-altitude, dexamethasone administration attenuates insulin sensitivity and glucose tolerance (1, 69, 99) making it a less than ideal approach to promote function in the military population.

A third class of pharmaceuticals frequently studied in high-altitude medicine is phosphodiesterase inhibitors. Phosphodiesterases in the lung cause vasoconstriction via cyclic AMP and GMP activation. Clinically, phosphodiesterase inhibitors are utilized to treat pulmonary artery hypertension (41) and to attenuate pulmonary vasoconstriction in environments where arterial oxygen content is challenged (44, 112, 116). Several reports highlight the beneficial effect of phosphodiesterase inhibition on arterial oxygen content during exercise (44, 102, 115, 116) but findings pertaining to the influence on endurance exercise performance are diverse. There are reports of improved submaximal exercise in hypoxic-hypoxia (56) and maximal exercise at high-altitude (44, 116) while others show no improvement in high-altitude at submaximal intensities (72) or in hypoxic-hypoxia at maximal exercise (36). All of these investigations utilized phosphodiesterase 5 inhibitors (sildenafil and tadalafil), therefore the inconsistency between the findings of these studies may be related to the timing of drug administration as it varied between hours to days, and in normoxia prior to hypoxia/high-altitude exposure versus once participants were already in the experimental environment. Although phosphodiesterase inhibitors reduce pulmonary vasoconstriction, their efficacy for treating acute mountain sickness is less clear. Sildenafil administered at high-altitude did not change the occurrence of acute mountain sickness (116), and when administration was initiated three days prior to the ascent, acute mountain sickness symptoms increased (4). Despite the amelioration of pulmonary vasoconstriction, phosphodiesterase 5 inhibition is not an operative strategy to improve physiological function in military personnel deployed in high-altitude environments.

The combination of phosphodiesterase inhibition with a cardio-stimulant may be a better approach for the military.

Theophylline, a combined non-specific phosphodiesterase inhibitor and adenosine receptor antagonist may be more suited for military personnel operating in high-altitude. Theophylline reduces symptoms of acute mountain sickness (73) and is an effective treatment for disruptions in breathing during sleep (73) in high-altitude; however, little is known about the impact of theophylline on endurance exercise performance in humans when arterial oxygen content is challenged. Extrapolating from investigations of phosphodiesterase inhibitors and adenosine receptor antagonists (39), one could speculate that the cardio-stimulant properties in addition to the reduction of pulmonary vasoconstriction would promote the transport of oxygenated blood to working tissues and improve military personnel performance during high-altitude deployment.

Methazolamide and theophylline may be better-suited strategies for military personnel compared with the accepted civilian approaches to promote human physiological function in high-altitude. Concomitant administration of these novel pharmaceutical agents is also worth consideration. Recently, pre-clinical investigations in a rodent model demonstrated the combination of pharmaceuticals targeting multiple points of the cardiopulmonary system to improve oxygen availability/delivery were more effective for improving exercise tolerance in hypobaric-hypoxia compared to the individual treatments alone (107, 108). Voluntary activity, determined by wheel running distance, increased in hypobaria with concomitant administration of theophylline and sitaxsentan or ambrisentan, both endothelin receptor antagonists that are used to treat pulmonary artery hypertension and pulmonary vasoconstriction (108). Wheel running performance was also augmented by the combination of ambrisentan with either of the

anti-hypotensive medications ephedrine and methylphenidate (107). This approach may prove to be a plausible strategy to promote rapid acclimation to high-altitude for military personnel. In adult humans, the combination of dexamethasone and acetazolamide was more effective for preventing acute mountain sickness than either drug alone or placebo (154), and a reduction in severe acute mountain sickness was observed with concomitant administration of a phosphodiesterase inhibitor and acetazolamide compared with acetazolamide alone (81). We are unaware of an investigation in humans examining exercise performance outcomes in response to concomitant pharmacological treatments.

Pharmacological Treatments to Improve Carbohydrate Availability in High-Altitude

In addition to targeting arterial oxygen content, addressing hypoxia-mediated insulin resistance in order to maintain skeletal muscle glycogen content may be a feasible strategy to promote physiological function of military personnel in high-altitude. Clinical presentations of insulin resistance related to reduced arterial oxygen content are often treated with continuous positive applied pressure (for sleep apnea) (7, 22, 24, 28) or by augmenting oxyhemoglobin saturations with supplemental oxygen (obstructive lung diseases) (61). The application of pharmacological inhibition of the sympathetic nervous system with the pharmaceutical clonidine has been examined to attenuate hypoxia associated insulin resistance in healthy males (105). Clonidine, a blood pressure medication, acts via pre-junctional stimulation of alpha-2-adrenergic receptors to reduce central sympathetic activation. Short-term clonidine use (2-to-7 days) results in centrally mediated peripheral sympathetic inhibition, as reflected by attenuated skeletal muscle sympathetic nerve activity (97, 98), decreased plasma norepinephrine concentration and release (95, 97, 98, 105), and increased heart rate variability (80, 153). Transdermal

administration of clonidine for 48 hours attenuated the hypoxia-mediated decline in insulin sensitivity as assessed via the hyperinsulinemic-euglycemic clamp technique (105). For the military population, the implication of attenuating insulin resistance associated with the decline in arterial oxygen content, is improved physical performance as a function of maintained skeletal muscle glycogen content (5) via preserved glucose regulation. A consideration for this proposed strategy is the effect of sympathetic activation on the cardio-pulmonary system during aerobic exercise. The sympathetic nervous system is a powerful regulator of cardio-pulmonary function, and sympathetic inhibition in high-altitude may actually impair endurance exercise performance via decreased cardiac output and oxygen delivery (65, 113). It is unclear if the metabolic benefits of sympathetic inhibition outweigh the cardio-pulmonary encumbrance, however augmented insulin sensitivity and skeletal muscle glycogen maintenance is of obvious advantage to military personnel.

To circumvent the issues pertaining to sympathetic inhibition and endurance exercise performance, an alternative approach would be to administer an insulin sensitizer without direct cardiovascular effects. Metformin is the first line treatment for type 2 diabetes (60) and is also utilized clinically to halt the progression of impaired glucose control to diabetes (26). Metformin regulates both fasting and post-prandial blood glucose by reducing hepatic glucose production (25, 58) and improves glucose uptake and utilization in skeletal muscle (3, 25, 96, 141) by augmenting the translocation of glucose transporter 4 to the muscle cell membrane (18). As with sympathetic inhibition, augmented glucose uptake and glycogen synthesis prior to exercise may promote endurance exercise performance. Therefore, metformin may be a viable non-traditional strategy to promote military performance in high-altitude via maintenance of skeletal muscle glycogen content without any known cardio-pulmonary encumbrance.

Military operations occurring in high-altitude environments are physiologically challenging because of low ambient oxygen partial pressure. The resultant decrease in arterial oxygen content diminishes physical and mental performance and can result in mission failure and unsafe operating conditions for military personnel. Strategies that promote rapid acclimation to high-altitude and improved physiological function are of benefit to the military population, however, accepted approaches developed for civilian travelers are inadequate for several military specific reasons. Accordingly, the purpose of the following investigations is to explore alternative strategies for promoting rapid acclimatization to high-altitude in military personnel.

CHAPTER III – MANUSCRIPT I

Neptazane plus Aminophylline abrogates hypoxia-mediated endurance exercise impairment[1]

Summary

In hypoxia, endurance exercise performance is diminished; pharmacotherapy may abrogate this performance deficit. Based on positive outcomes in pre-clinical trials, we hypothesized oral administration of Neptazane, a carbonic anhydrase inhibitor, Aminophylline, a non-selective adenosine receptor antagonist and phosphodiesterase inhibitor, and/or Neptazane combined with Aminophylline would attenuate hypoxia-mediated decrements in endurance exercise performance in humans. Fifteen healthy males (26±1 years, body mass index: 24.9±0.4 kg/m^2; mean±SE) were randomly assigned to one of four treatments: placebo (n=9), Neptazane (250mg; n=10), Aminophylline (400mg; n=9), or Neptazane (250mg) with Aminophylline (400mg; n=8). On two separate occasions, the first in normoxia (FIO_2=0.21) and the second in hypoxia (FIO_2=0.15), participants sat for 4.5 hours before completing a standardized exercise bout (30 minutes, stationary cycling, 100W) followed by a 12.5km time trial. Blood was sampled before and immediately following each exercise bout. The magnitude of time trial

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performance decrement in hypoxia did not differ between placebo (+3.0±1.0 minutes), Neptazane (+1.4±0.5 minutes) and Aminophylline (+1.8±0.4 minutes), all $P>0.09$, however the performance decrement with Neptazane combined with Aminophylline was less than placebo (+0.6±0.5 minutes; $P=0.01$). This improvement may have been partially mediated by increased SpO_2 in hypoxia with Neptazane combined with Aminophylline, compared with placebo (73±1 vs. 79±2%; $P<0.02$). In conclusion, co-administration of Neptazane and Aminophylline may promote endurance exercise performance during a sojourn to high altitude.

Introduction

Rapid transition from sea level to high-altitude is accompanied by impaired physiological function; impairments that may have important health implications for both professional (such as the military) and recreational travelers. Documented responses to high-altitude and/or simulated high-altitude (hypoxia) include compromised cognitive ability (83), sleep disturbances (133), decreased insulin sensitivity (105), and reduced exercise capacity/performance (17, 33, 79, 92). With regards to the latter, performance decrements at high altitude range from 0 to ~30%, depending on the duration of the activity and/or the severity of hypobaria (38). For example, in a recent review of marathon performance and high-altitude, marathon completion times were reported to increase by approximately 10-12% per 1,000 m gain in altitude (77). Several nutraceutical and pharmacological strategies, including administration of acetazolamide, sildenafil, and caffeine (9, 39, 56, 72, 134), have been explored to prevent the hypoxia/hypobaria-mediated decline in function. Some of these strategies have focused on alleviating nausea and acute mountain sickness (35, 73, 119), while others have targeted improved oxygen delivery (39, 56, 72). Recent published studies (108) and unpublished preliminary data demonstrate the success in a rodent model of the asthma medication,

theophylline, when combined with either a pulmonary hypertension medication, such as Sitaxsentan and Ambrisentan, or the glaucoma medication, Neptazane (122). The purpose of this investigation was to determine, in humans, the efficacy of Aminophylline (active ingredient: theophylline), Neptazane (active ingredient: methazolamide), and Aminophylline combined with Neptazane to attenuate hypoxia-mediated decrements in endurance exercise performance. Identification of an effective pharmacological strategy to abrogate the deleterious effects of short-term exposure to hypoxia would be of benefit to professional and recreational travelers during brief sojourns to high-altitude. We hypothesized that Aminophylline, Neptazane, and/or the combination of Aminophylline and Neptazane would attenuate the hypoxia-mediated decrement in endurance exercise performance in humans.

Methods

Drug Safety

Prior to the initiation of the current investigation, an in-patient study was undertaken by our collaborators at University of Colorado Health (formerly Poudre Valley Health System) to determine the safety of Aminophylline and Neptazane when consumed independently and in combination, and also to provide insight into the pharmacokinetics of the drug combination. This safety study was registered as a Clinical Trial (ClinicalTrials.gov Identifier: NCT01587027). Sixteen healthy adults participated in a 5-day study protocol comprising single dose administration of Aminophylline (500 mg), Neptazane (250mg), and Aminophylline (500 mg) combined with Neptazane (250 mg), with a 24-hour washout period between each dosing. The order of individual administration of Aminophylline and Neptazane was randomized; the combined administration was always the third/final treatment. The studied doses were based on

recommended clinical dosing. Vital signs were monitored and venous blood sampled prior to and during absorption. The primary findings of the study were: 1) when consumed during rest, the drugs, either independently or in combination, were reasonably well tolerated at the administered doses; 2) the time to peak circulating concentrations of the primary active agents of each drug (ie., theophylline for Aminophylline, and methazolamide for Neptazane) were 2-3 hours following administration; co-administration did not affect these time to peak concentration values; and, 3) consistent with previous literature, the half-lives of the drugs were 8 and14 hours, for Aminophylline (149) and Neptazane (90), respectively. Based on these observations, and on the advice of our physician collaborators (D.G.L. and G.J.L.), in the current study the administered dose of Aminophylline was decreased from 500 mg to 400mg.

Participants

Fifteen young, healthy men were recruited for participation in the current investigation. The experimental protocol conformed to the standards set by the Declaration of Helsinki of 1975, as revised in 1983, and was approved by the Institutional Review Board at Colorado State University. The nature, purpose and risks of the study were explained to each research participant before written informed consent was obtained. The study was also registered as a Clinical Trial (ClinicalTrials.gov Identifier: NCT01702025).

Inclusion criteria consisted of age within the range 18-40 years, body mass index within the range 18.5-30 kg/m^2, body mass greater than 68.2 kg, free from overt disease as determined via medical history and assessment of blood pressure and heart rate (via 12-lead electrocardiogram) at rest and during incremental exercise to volitional exhaustion, and approval from a supervising cardiologist (D.G.L or G.J.L). Exclusion criteria included current use of

tobacco products or any prescribed medications, excessive use of caffeinated or theophylline containing food or beverages, history of acute mountain sickness, history of allergic reaction, hypersensitivity or idiosyncratic reaction to methazolamide and/or theophylline or an allergy to any sulfa or sulfonamide derivatives, asthma or any other type of lung/pulmonary dysfunction, and/or contraindications to vigorous exercise. Consequently, research participants demonstrated physiological attributes typical of fit and healthy, recreationally active young men. Noteworthy, all listed cycling as a component of their habitual physical activity.

Following screening procedures research participants completed additional assessment that included measurement of body composition (via dual-energy x-ray absorptiometry: DEXA-IQ; Lunar Radiation Corp., Madison, WI, USA, software v. 4.1) and determination of maximal oxygen uptake (VO_{2max}) during incremental stationary cycle ergometer exercise (via indirect calorimetry) as previously described (117).

Protocol Overview

Research participants were randomly assigned, in a double-blind fashion, to one of four treatments: placebo, Neptazane, Aminophylline, or Neptazane combined with Aminophylline. Participants reported to the laboratory on two separate occasions, separated by 7-28 days, to be studied during normoxia and hypoxia. In light of the half-lives of Aminophylline (149), and Neptazane (90), (8 and 14 hours, respectively) this duration of separation between visits was sufficient for drug clearance. During each visit, following oral administration of the drug(s), participants were studied at rest, during standardized exercise, and during performance of an exercise time-trial. To further minimize potential health risks associated with administering this novel drug combination to exercising adults in a laboratory environment, the normoxic trials

always preceded the hypoxic trials; thus, this design represented a controlled, incremental risk (that is, study of treatments in normoxia at rest, during standardized moderate-intensity exercise, and during vigorous exercise, and then, after a minimum of 7 days, study of treatments in hypoxia during rest, during standardized moderate-intensity exercise, and during vigorous exercise). Neither participants, nor research staff, were naïve as to the environmental condition. In order to familiarize participants with procedures prior to the treatment visits, standardized exercise and habituation time trials were completed in normoxia without drug intervention.

Colorado State University (Fort Collins campus) is situated at an altitude 1,525 m (~5,000 ft) above sea level, thus the normoxic conditions may be considered mildly hypobaric (typical barometric pressure ~ 640 mmHg). All research participants had been residents of Fort Collins for a minimum of 12 months and refrained from travel to sea level destinations prior to and during study participation.

Protocol

Following a 12-hour fast and 24-hour abstention from vigorous physical activity, research participants arrived at the laboratory and were instrumented for measurement of heart rate (3-lead ECG), blood pressure, and peripheral oxygen saturation (SpO_2; pulse oximeter; physiological monitor; Cardiocap 5, GE Datex-Ohmeda, Madison, WI, USA). An intravenous catheter was inserted into an antecubital vein for subsequent and repeated blood sampling. The venous catheter was kept patent via a saline drip. After recording of baseline vitals, participants were transferred to a clinical laboratory or to an environmental chamber within which the inspired oxygen concentration could be manipulated (Colorado Altitude Training, Louisville, CO, USA). During the first visit, in the clinical laboratory, the inspired oxygen concentration

was normoxic ($FIO_2 = 0.21$), and during the second, it was hypoxic ($FIO_2 = 0.15$) for the entire duration of the trial. Participants rested in a seated position for 15 minutes before oral consumption of either placebo (200 mg of white cornmeal packaged in gel capsules), Neptazane (250 mg), Aminophylline (400 mg), or Neptazane (250 mg) combined with Aminophylline (400 mg). Participants remained resting in the seated position for 4.5 hours. Heart rate, blood pressure and SpO_2 were recorded every 15 minutes. Fifteen and 120 minutes post drug administration, standardized nutrition was provided: a liquid meal (250 kcal: 57% carbohydrate, 28% fat, 15% protein; Ensure, Ross Laboratories, Abbott Park, IL, USA) and an energy-dense sports bar (220 kcal, 58% carbohydrate, 25% fat and 17% protein; PowerBar Triple Threat, Fremont, MI, USA). Water was consumed ad libitum.

Four and a half hours after drug administration participants began 30 minutes of standardized exercise on a computer controlled, electrically braked stationary cycle ergometer (Dynafit Velotron; Racermate Inc., Seattle, WA, USA); resistance was set to 100 W. Based on the physiological characteristics and habitual physical activity of the research participants, this workload represented low-to-moderate intensity exercise that could easily be completed in both normoxia and hypoxia. On completion, participants were permitted a brief (up to 5 minutes) break before beginning an exercise time-trial, the goal of which was to perform stationary cycle ergometer exercise equivalent to a distance of 12.5 km (7.75 miles) as quickly as possible. During the time trial all time cues were hidden from the participants but feedback regarding distance cycled was provided continuously. Measurements of heart rate, blood pressure, oxyhemoglobin saturation, and ratings of perceived exertion (8) were made at regular intervals during both the standardized exercise bout and the time trial.

Blood Sampling and Analysis

During rest, blood (~10 ml into a chilled tube and preserved with ethyl-enediamine-tetra-acetic acid) was sampled prior to drug administration and again at time 0, 60, 120, 180, 240 and 270 minutes. During exercise, blood was sampled immediately following completion of the standardized exercise and the time trial. Plasma and red blood cells were separated via chilled (4°C) centrifugation from resting and exercise samples and stored at -80°C for subsequent determination of circulating concentrations of theophylline (the active ingredient of Aminophylline) and methazolamide (the active ingredient of Neptazane). Approximately 1 ml of each sample collected prior to and during exercise was also analyzed immediately for lactate concentration via an automated device (2300 STAT Plus Glucose Lactate Analyzer, YSI Inc., Yellow Springs, OH, USA).

To quantify theophylline concentrations, 800 µL of methanol and 0.2 mmol/L $ZnSO_4$ (70/30, v/v; protein precipitation solution) were added to 200 µL of plasma. Theophylline-d6 (at 500 ng/mL, Toronto Research Chemicals (TRC), Toronto, Canada) was added as an internal standard (Sigma-Aldrich, St Louis, MO, USA) to the protein precipitation solution. After centrifugation (13,000g, 10 min, 4˚C), 20 µL of the supernatant were injected onto the analytical column of an LC-MS system (Synergi 4u Hydro-RP-80A, 250 x 3.0 mm, 4µm, Phenomenex, Torrance, CA, USA). The mobile phases consisted of A: 0.1% formic acid and B: methanol. The following gradient was run: 0 to 2.7 min with 60% B, 2.7 to 4 min 99% B and finally the column was re-equlibrated until 5 min with 60% B. The flow rate was 0.6 mL/min and the column was kept at 65ºC.

Circulating methazolamide concentration was analyzed using a validated and fully automated inline extraction-tandem mass spectrometry assay. Briefly, 800 µL protein

precipitation solution (vide supra) containing d6-methazolamide (at 100 ng/mL, TRC) was added to 200 µL of red blood cell or plasma sample. After centrifugation (13,000g, 10 min, 4˚C), 50 µL of the supernatant was injected into the HPLC system and loaded onto an extraction column (12.5 x 4.6 mm, Zorbax XDB C8, Agilent Technologies, Palo Alto, CA, USA). Samples were washed with a mobile phase of 70% methanol (B) and 30% 0.1% formic acid (A) with a flow of 3 mL/min. After 1 minute, the switching valve was activated and the analytes were eluted in the back flush mode from the extraction column onto an analytical column kept at 60°C (Zorbax XDB C8, 150x4.6 mm, 5µm, Agilent Technologies, Palo Alto, CA, USA). The mobile phase consisted of B: methanol and A: 0.1% formic acid. The following gradient was run: 0-1 min: 70% B, 1.1-3.5 min: 99% B, 3.6-4 min: 70% B. The flow rate was 1 mL/min throughout the assay.

The HPLC system was interfaced with a triple quadrupole MS (API4000, Applied Biosystems, Foster City, CA, USA). For theophylline, MS was run in positive multiple reaction monitoring (MRM) mode. Peak area ratios obtained from MRM mode of the mass transition for theophylline (181.1→124.1 (quantifier transition; declustering potential (DP): 75V; entrance potential (EP): 10V; collision energy (CE): 29V; collision cell exit potential (CXP): 10V)) and 181.1→96.1 (qualifier transition; DP: 75V; EP: 8V; CE: 24V; CXP: 10V)) and its internal standard d6-theophylline (187.2→127.1; DP: 75V; EP: 10V; CE: 29V; CXP: 10V) were used for quantification.

For methazolamide, peak area ratios obtained from negative MRM mode of the mass transition 235.2→78.1 (quantifier transition; DP: -52V; EP: -12V; CE: -29V; CXP: -5V) and 235.2→129.1 (qualifier transition; DP: -52V; EP: -12V; CE: -19V; CXP: -9V) and its internal

standard d6-methazolamide (241.2→78.1; DP: -58V; EP: -12V; CE: -30V; CXP: -5V) were used for quantification.

Statistical Analysis

As stated, the purpose of this investigation was to determine, the efficacy of Aminophylline, Neptazane, and Aminophylline combined with Neptazane, to attenuate hypoxia-mediated decrements in endurance exercise performance. The overarching goal was to identify an intervention to promote exercise performance in hypoxia, and not to compare the efficacy of three treatments against each other. Accordingly, the influence of the treatments was investigated using a planned comparisons approach where each intervention was compared with placebo. This was accomplished via post-hoc investigation of two-way analysis of variance (placebo *vs*. treatment) with repeated measures on one factor (normoxia *vs*. hypoxia), and also comparison of the magnitude of hypoxia mediated decline in time trial performance (i.e. hypoxia time trial – normoxia time trial). The level of statistical significance was set at $P < 0.05$. Correction for multiple comparisons was made using the modified Bonferroni technique. To address potential drug/FIO_2 interactions and drug combination/FIO_2 interactions, differences in circulating concentrations of theophylline (the active ingredient of Aminophylline) and methazolamide (the active ingredient of Neptazane) over time were examined with three-way analyses of variance (normoxia vs. hypoxia), and treatment (individual drug administration vs. combination), with repeated measures on one factor (time). Throughout the manuscript, data are expressed as mean and standard error.

Results

Participants

Fifteen research participants were randomly assigned to one of four treatments, however several participants completed multiple trials/conditions: four completed all treatments (placebo, Neptazane and/or Aminophylline), four completed three treatments, one completed two, and six completed one. When participants completed more than one treatment, the conclusion of one trial and the start of the next were separated by at least seven days. Selected physiological characteristics of the research participants are presented in Table 1.1. During the trials, one participant reported "facial tingling" after consuming Neptazane in combination with Aminophylline; another reported mild, brief double vision after consuming Neptazane alone. Both participants completed the study. Aside from these two incidents, Neptazane and/or Aminophylline were well tolerated.

Resting Data

Resting heart rate, blood pressure, and oxyhemoglobin saturation during normoxia and hypoxia with and without Neptazane and/or Aminophylline are presented in Table 1.2 (see Supplemental Figures 1.1-1.3 for graphical representation of resting heart rate, blood pressure, and oxyhemoglobin saturation during all trials). In all treatments, hypoxia increased resting heart rate ($P < 0.012$); compared with placebo, Neptazane and/or Aminophylline did not influence the effect of hypoxia on heart rate ($P > 0.60$).

Hypoxia did not influence resting blood pressure (systolic or diastolic; $P > 0.27$). Compared with placebo, systolic blood pressure was lower with the combination of Neptazane and Aminophylline, however this difference did not attain statistical significance ($P = 0.061$).

Hypoxia decreased resting oxyhemoglobin saturation ($P < 0.001$); compared with placebo, Neptazane and/or Aminophylline did not influence the effect of hypoxia on oxyhemoglobin saturation ($P > 0.15$).

Standardized Exercise

Specific to our participants, the absolute standardized exercise bout (100 W) represented low-moderate intensity exercise (~30% of maximal work rate, an estimated metabolic response of ~ 40% of VO_{2max}, and ratings of perceived exertion between 9 and 12). There was no difference in the relative intensity of the standardized exercise between treatment groups ($P = 0.939$). In all treatments, hypoxia increased heart rate during standardized exercise (Table 1.2; Supplemental Figure 1.4; $P < 0.048$); compared with placebo, Neptazane and/or Aminophylline did not influence the effect of hypoxia on exercising heart rate ($P > 0.56$).

Blood pressure during standardized exercise was unaffected by hypoxia for all treatments (Table 1.2; Supplemental Figure 1.5; $P > 0.10$). Hypoxia decreased oxyhemoglobin saturation during standardized exercise in all treatments (Table 1.2; Supplemental Figure 1.6; $P < 0.001$). Compared with placebo, Neptazane, and Neptazane combined with Aminophylline increased oxyhemoglobin saturation ($P < 0.001$). Rating of perceived exertion was increased with hypoxia (Table 1.2; $P < 0.018$), in almost all treatments; Neptazane combined with Aminophylline, compared with placebo, removed the effect of hypoxia ($P = 0.27$).

Time Trial

Normoxic time trial performance was not different from the habituation trials for any treatment group (all $P \geq 0.205$). Neither Neptazane, Aminophylline, nor Neptazane combined

with Aminophylline affected the time to cycle 12.5 km in normoxia ($P > 0.10$). Time-trial performance was slower in hypoxia compared with normoxia with placebo (22.3 ± 0.7 *vs.* 25.2 ± 1.9 minutes, $P < 0.001$), Aminophylline (22.2 ± 0.5 *vs.* 23.9 ± 0.7 minutes, $P = 0.009$), and Neptazane (23.2 ± 0.5 *vs.* 24.6 ± 0.5 minutes, $P = 0.023$), but not with Neptazane combined with Aminophylline (24.0 ± 0.6 minutes *vs.* 24.5 ± 0.6 minutes, $P = 0.376$). There was no difference in hypoxic time trial performances between the treatment groups (all $P > 0.261$). Examination of the delta values (magnitude of hypoxia-mediated performance decrement) revealed neither Neptazane nor Aminophylline was different to placebo (Figure 1.1; Supplemental Figure 1.7; $P > 0.09$); the hypoxia-mediated performance decrement with Neptazane combined with Aminophylline was less than placebo ($P = 0.01$). Noteworthy, inspection of Figure 1.1 reveals two research participants who appear to be outliers (one participant who was considerably slower in hypoxia in the placebo condition, and one participant who appeared to be appreciably faster in hypoxia in the combined Aminophylline/Neptazane condition). Neither participant were statistical outliers, and removal of either or both participants from the final analyses did not change the overall conclusion pertaining to the ergogenic effect of Aminophylline combined with Neptazane in hypoxia.

Hypoxia did not affect heart rate, or systolic and diastolic blood pressures (Table 1.2; Supplemental Figures 1.8-1.9; $P > 0.41$). Hypoxia decreased oxyhemoglobin saturation (Table 1.2; Supplemental Figure 1.10; $P < 0.001$). Compared with placebo, Neptazane, and Neptazane and Aminophylline increased oxyhemoglobin saturation ($P < 0.02$) in hypoxia. Rating of perceived exertion was unaffected by hypoxia ($P > 0.39$; Table 1.2).

Blood Data - Methazolamide and Theophylline Concentrations

The active drug components of Neptazane and Aminophylline are methazolamide and theophylline, respectively; the concentrations of these components in plasma (both methazolamide and theophylline) and red blood cells (methazolamide only) were measured over the course of each condition (Figure 1.2). Hypoxia did not affect plasma theophylline concentrations whether Aminophylline was taken alone or with Neptazane ($P > 0.80$). When Aminophylline was administered alone or in combination with Neptazane, theophylline concentrations peaked at 180 minutes in normoxia and hypoxia. In plasma, hypoxia increased methazolamide concentrations when Neptazane was taken alone ($P = 0.01$) and with Aminophylline ($P = 0.04$). Plasma methazolamide concentrations peaked at 240 minutes in normoxia and hypoxia. Hypoxia also augmented the rate of increase of red blood cell concentration of methazolamide such that it was greater at 60 minutes (normoxia: 22.3 ± 7.9 μg/mL; hypoxia: 30.1 ± 9.5 μg/mL; $P = 0.001$). However, when Neptazane was taken with Aminophylline, the influence of hypoxia on red blood cell concentration of methazolamide was removed ($P = 0.08$). Red blood cell concentrations of methazolamide peaked at 180 minutes when Neptazane was given alone in both normoxia and hypoxia. When Aminophylline was given in combination with Neptazane, the red blood cell concentration of methazolamide peaked at 240 minutes in normoxia and 120 minutes in hypoxia.

Blood Lactate Following Exercise

Blood lactate concentration was unaffected by the standardized exercise bout regardless of hypoxia and treatment (Supplemental Figure 1.11; $P > 0.188$) and was always greater at completion of the time trial compared with any other point for all treatments ($P < 0.001$).

Hypoxia did not affect blood lactate ($P > 0.42$). Blood lactate was lower at the completion of the time trial with both Neptazane alone and in combination with Aminophylline ($P < 0.021$).

Discussion

The novel findings of this study were, compared with placebo, Neptazane combined with Aminophylline abrogated the hypoxia-mediated decrement in endurance exercise performance. This beneficial effect may have been mediated, in part, via a smaller magnitude of decrease in oxyhemoglobin saturation during exposure to the simulated high-altitude environment. When administered alone, neither Neptazane nor Aminophylline influenced endurance exercise performance in hypoxia compared with placebo. These data imply that co-administration of Neptazane and Aminophylline may promote endurance exercise performance during a brief sojourn to high altitude.

While it was not the purpose of the current study to provide a mechanism of ergogenic action of the drug combination, a brief discussion is warranted. The favorable effects of Neptazane combined with Aminophylline in hypoxia represent a novel, off-target application of two established drugs. Neptazane is indicated for the lowering of intraocular pressure prior to and during treatment for glaucoma. Its primary mechanism of action is via inhibition of carbonic anhydrase. Indications for the use of Aminophylline are usually related to relief from symptoms of asthma and other chronic pulmonary diseases. Its actions are mediated through nonselective inhibition of phosphodiesterase and adenosine receptor antagonism. Given these mechanistic differences between the drugs, it is likely that the benefits of the drug combination in hypoxia represent a synergistic interaction rather than a summation of effects. The carbonic anhydrase inhibition of Neptazane may have increased renal secretion of bicarbonate leading to metabolic

acidosis, thus attenuating the leftward shift in the oxyhemoglobin dissociation curve usually observed during immediate exposure to hypoxia (76). Additionally, carbonic anhydrase inhibition may have augmented ventilation to increase oxyhemoglobin saturation (129). The inhibition of phosphodiesterase, plus the antagonism of adenosine receptors, associated with Aminophylline use may have increased cardiac output, pulmonary blood flow, and systemic circulation (45, 84, 143). Thus in combination, Neptazane and Aminophylline may have promoted oxygen delivery to exercising tissue in hypoxia. Noteworthy, based on observations during habituation, the combination of Neptazane and Aminophylline did not improve time-trial performance in normoxia. While we acknowledge the limitations of comparing habituation trials with the normoxic drug conditions, these comparisons imply that under normal conditions (i.e. normoxia), the drug combination is not ergogenic.

Compared with placebo, Neptazane combined with Aminophylline abrogated the decrement in endurance exercise performance during brief (~ 5 hours) exposure to simulated high-altitude. One important limitation of the current investigation is the data do not provide any insight as to the beneficial effects of this drug combination over longer durations (hours/days). Presumably the ergogenic benefits over longer duration visits would require additional doses of the drug combination, potentially increasing the risk of unfavorable reactions (side-effects) to the drugs. Another important consideration pertains to the gradual acclimation to high altitude as the duration of the visit increases. The influence of Neptazane combined with Aminophylline on the rate of acclimation to high altitude is unknown; it is possible that the short-term benefits of the drug combination may interfere with some of the physiological processes required for high altitude acclimation. Consistent with this, administration of the carbonic anhydrase inhibitor, Acetazolamide, over two weeks attenuates the improvement of oxyhemoglobin saturation during

exercise in hypoxia (75) implying that pharmaceutical strategies to promote rapid acclimation to hypoxia may be better suited for brief exposures rather than longer term visits. It may be that repeated drug dosing negates the need for acclimatization over extended high altitude exposure.

Many nutraceutical and pharmacological strategies have been explored to prevent hypoxia/hypobaria-mediated decline in physiological function. Some of these strategies have focused primarily on alleviating nausea and acute mountain sickness (35, 73, 119). In this regard, Acetazolamide, commonly administered as Diamox, is widely accepted as an effective prophylaxis, reducing nausea symptoms by half (119) and even improving periodic sleep disordered breathing in hypoxia (34, 48). In the current investigation we did not record symptoms of acute mountain sickness and therefore have no direct observations pertinent to how Aminophylline and/or Neptazane might influence this disorder. However, previous studies have reported on the beneficial effects of theophylline administration, including reduced acute mountain sickness and apneic episodes during sleep (34, 35, 73). Additionally, methazolamide, the active component of Neptazane, has been reported to be as effective as acetazolamide in reducing acute mountain sickness symptoms (150). Collectively, these observations imply that in addition to an ergogenic effect, the combination of Aminophylline and Neptazane may also alleviate acute mountain sickness.

Several other considerations pertaining to the current study and outcomes are worthy of mention: 1) A repeated measures, cross-over design in which the same research participants completed the placebo and all of the drug treatments (individual and combination) would have strengthened our conclusions, however the required number and duration of laboratory visits for each participant, in addition to the cost-benefit and safety concerns of repeated exposures to hypoxia and/or drug treatments made such an approach unfeasible. 2) Research participants

completed only one habituation protocol prior to normoxic time trial performance with one of the treatments. It is plausible that one habituation protocol may have been insufficient, thus when combined with the standardized order of normoxic and then hypoxic time trials, the hypoxia mediated performance decrement may have been under exaggerated. We do not believe this to be case given the lack of appreciable difference between the habituation and normoxic time trials, and also based on previous reports of high consistency and reproducibility across self-paced laboratory tests (43, 135, 139). 3) The active ingredient in Aminophylline, theophylline, has a narrow therapeutic window. A small dose may prove to be ineffective, while a larger dose may increase the risk of overdose and/or toxicity. In the current study all research participants were administered the same absolute dose. To decrease the chances of overdose, a body mass greater than 68 kg was adopted as an inclusion criterion. Adults with a body mass less than 68 kg may need to consider a smaller dose. Noteworthy, the data collected during the standardized exercise (100W; Table 1.2) suggest that the drug treatments do not evoke abnormal or exaggerated responses to low-moderate intensity exercise in either normoxia or hypoxia. 4) Finally, all of the research participants in the current investigation were male. While we have no reason to suspect drug tolerance and the beneficial effects in hypoxia would be different in females, we have no direct evidence to support or refute this.

In summary, rapid transition from sea level to high-altitude is accompanied by impaired physiological function and decreased capacity for physical work. Based on experimental animal data, we have examined, in humans, the efficacy of Aminophylline, Neptazane, and Aminophylline combined with Neptazane to promote exercise performance in simulated high-altitude (hypoxia). Compared with placebo, Neptazane combined with Aminophylline abrogated the hypoxia-mediated decrement in endurance exercise performance. This novel, off-target

application of two established drugs may be of ergogenic benefit to professional and recreational travelers during brief sojourns to high-altitude.

Table 1.1. Select participant characteristics

	Placebo	Aminophylline	Neptazane	Combination
n	9	9	10	8
Age (years)	28 ± 2	27 ± 2	27 ± 2	26 ± 2
BMI (kg/m^2)	24.9 ± 0.6	25.2 ± 0.6	25.1 ± 1.5	24.9 ± 0.7
Body Fat %	20.2 ± 0.9	19.3 ± 0.9	20.6 ± 0.7	19.4 ± 0.8
Fat Mass (kg)	15.4 ± 0.7	15.0 ± 0.7	16.1 ± 0.6	15.4 ± 0.8
Fat Free Mass (kg)	58.4 ± 1.9	59.7 ± 2.2	58.9 ± 2.2	60.8 ± 2.3
VO_{2max} (ml/kg/min)	48.8 ± 2.7	51.4 ± 2.8	49.8 ± 2.6	48.3 ± 2.6
Work $Rate_{max}$ (watts)	358 ± 19	378 ± 22	371 ± 20	360 ± 21

Data are mean ± SE. Body mass index: BMI. Maximal oxygen consumption: VO_{2max}.

Table 1.2. Hemodynamic responses in normoxia and hypoxia

	Placebo n = 9		Aminophylline n = 9		Neptazane n = 10		Combination n = 8	
	N	H	N	H	N	H	N	H
HR_{rest}	64 ± 2	72 ± 3*	65 ± 2	74 ± 3*	62 ± 2	68 ± 3*	66 ± 3	72 ± 4*
HR_{100W}	117 ± 4	132 ± 6*	117 ± 4	134 ± 5*	115 ± 5	126 ± 6*	118 ± 9	130 ± 7*
HR_{TT}	156 ± 4	159 ± 4	162 ± 4	162 ±3	150 ± 4	152 ± 4	155 ± 5	162 ± 4
SBP_{rest}	121 ± 2	125 ± 4	128 ± 4	120 ± 2	125 ± 2	119 ± 2	123 ± 5	117 ± 2
SBP_{100W}	139 ± 4	148 ± 5	140 ± 5	142 ± 5	140 ± 3	141 ± 3	138 ± 3	140 ± 4
SBP_{TT}	156 ± 5	157 ± 4	152 ± 5	156 ± 4	148 ± 4	151 ± 5	152 ± 4	150 ± 5
DBP_{rest}	70 ± 2	72 ± 2	71 ± 3	71 ± 2	69 ± 1	71 ± 2	73 ± 3	68 ± 2
DBP_{100W}	70 ± 3	71 ± 3	73 ± 4	73 ± 3	70 ± 3	68 ± 2	73 ± 3	75 ± 2
DBP_{TT}	71 ± 3	69 ± 3	71 ± 3	73 ± 3	72 ± 4	67 ± 3	74 ± 3	72 ± 2
SpO_{2rest}	95 ± 0	84 ± 1*	96 ± 0	83 ± 1*	96 ± 0	85 ± 1*	97 ± 0	83 ± 2*
SpO_{2100w}	94 ± 0	75 ± 1*	94 ± 0	78 ± 2*	95 ± 1	83 ± 1*†	94 ± 1	84 ± 2*†
SpO_{2TT}	91 ± 1	75 ± 1*	92 ± 1	78 ± 1*	93 ± 1	81 ± 1*†	93 ± 1	80 ± 2*†
RPE_{100W}	11 ± 0	11 ± 0	10 ± 0	12 ± 0	10 ± 0	12 ± 0	11 ± 0	12 ± 0
RPE_{TT}	15 ± 0	16 ± 0	16 ± 0	16 ± 0	16 ± 0	16 ± 0	16 ± 0	16 ± 0

Data are mean ± SE. Normoxia: N. Hypoxia: H. Heart rate: HR. 30 minutes of standardized exercise:100W. Systolic blood pressure: SBP. Diastolic blood pressure: DBP. Oxyhemoglobin saturation: SpO_2. Rating of perceived exertion: RPE. Time trial: TT. *Different than normoxia ($P < 0.05$). †Different than placebo in hypoxia ($P < 0.05$).

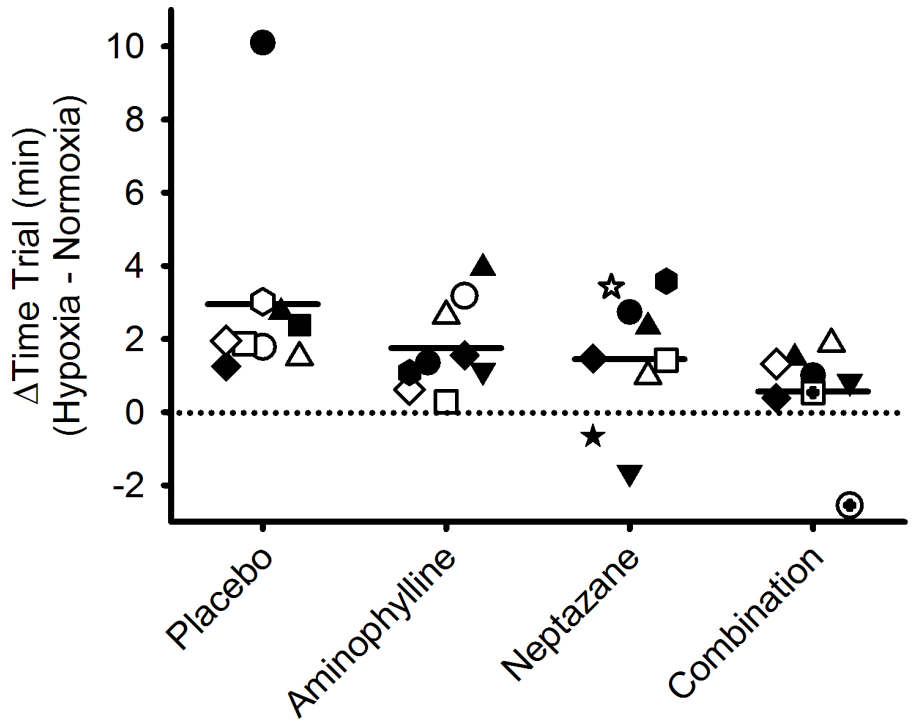


Figure 1.1. Change in 12.5 km time trial performance from normoxia to hypoxia following oral administration of placebo, Neptazane, Aminophylline, or Neptazane with Aminophylline. Symbols represent individual research participants. Heavy line represents group mean.

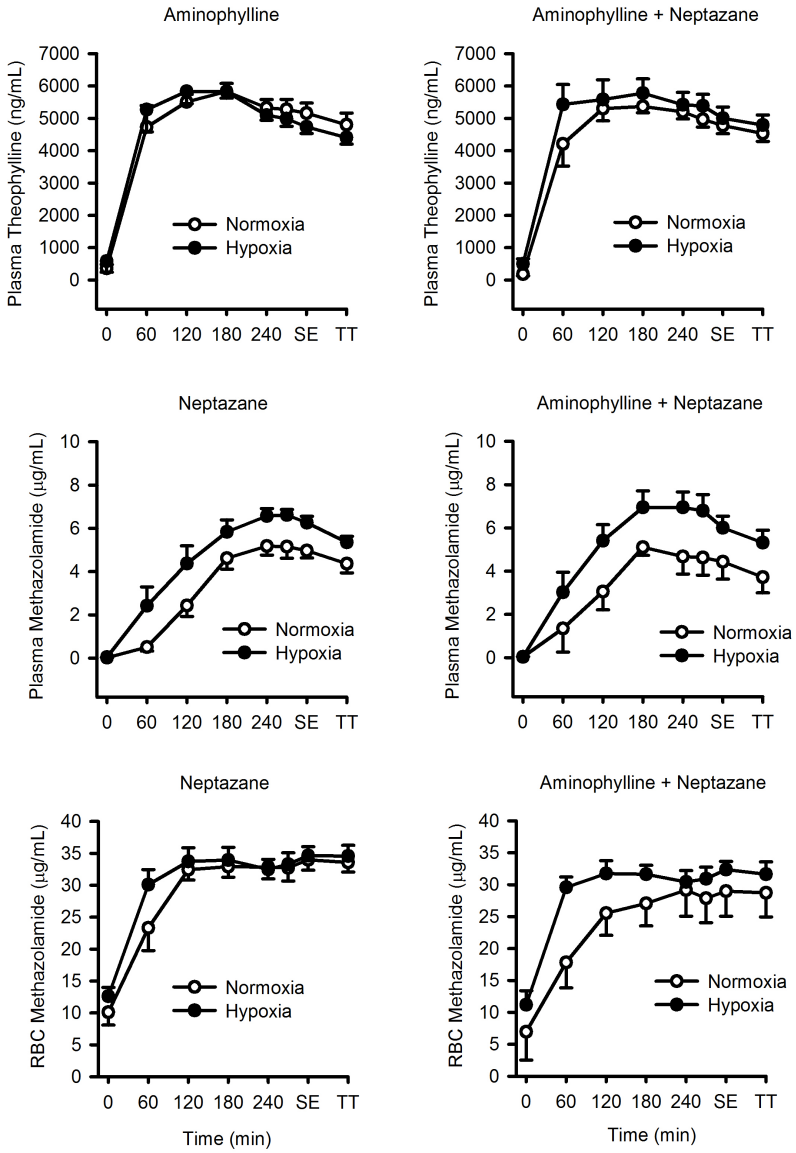
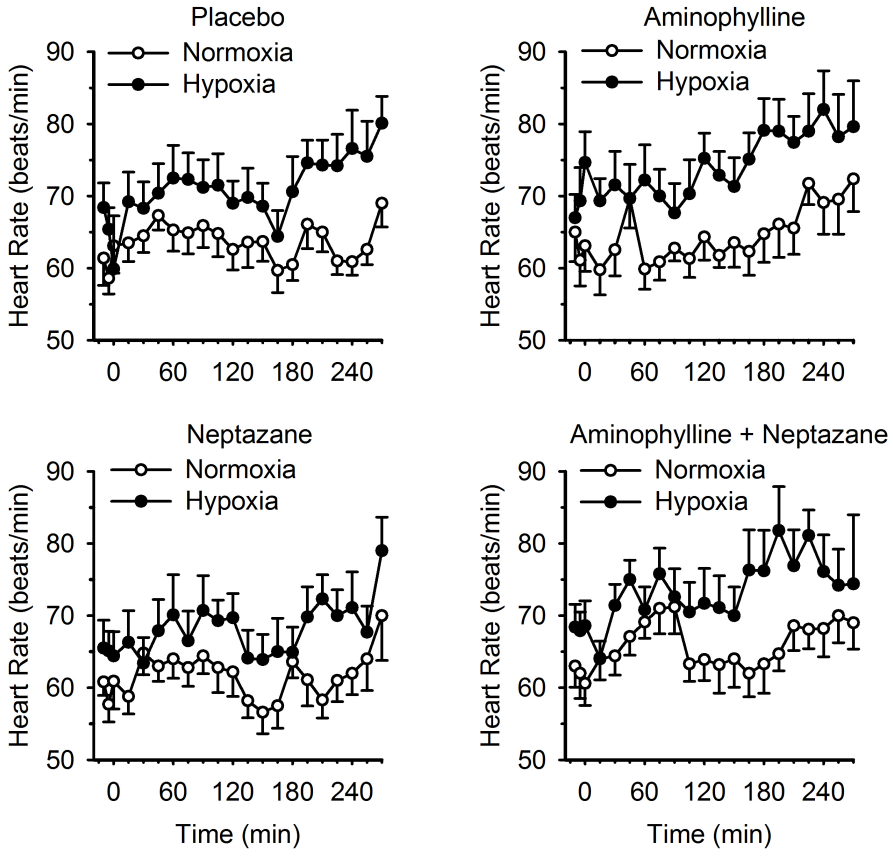
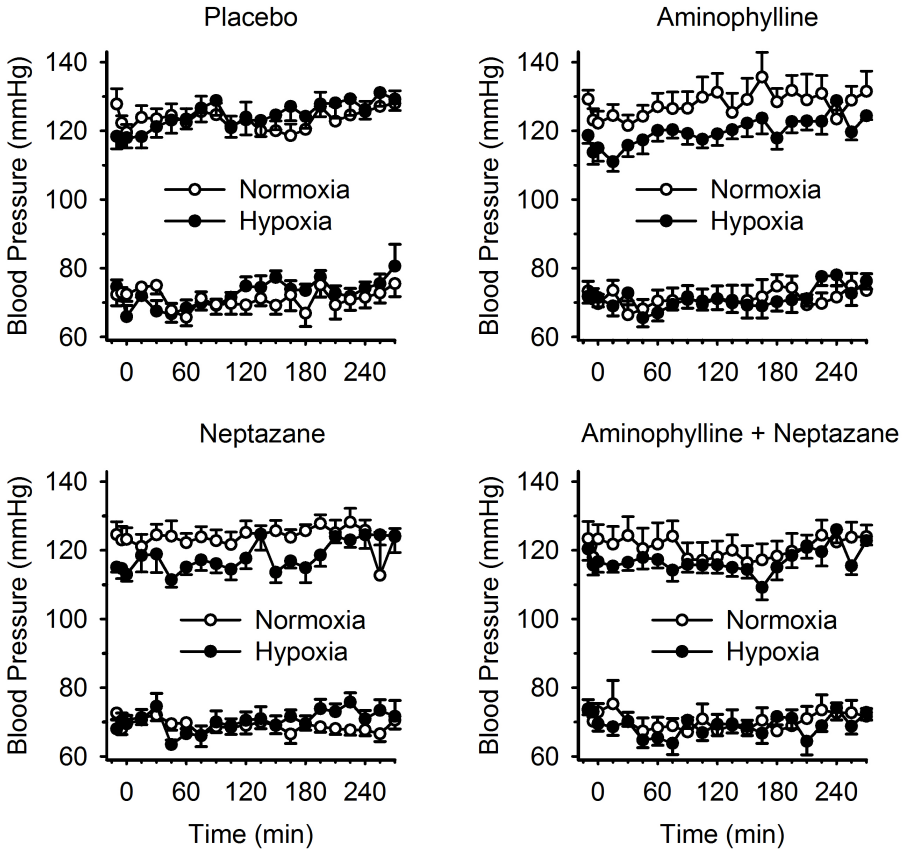


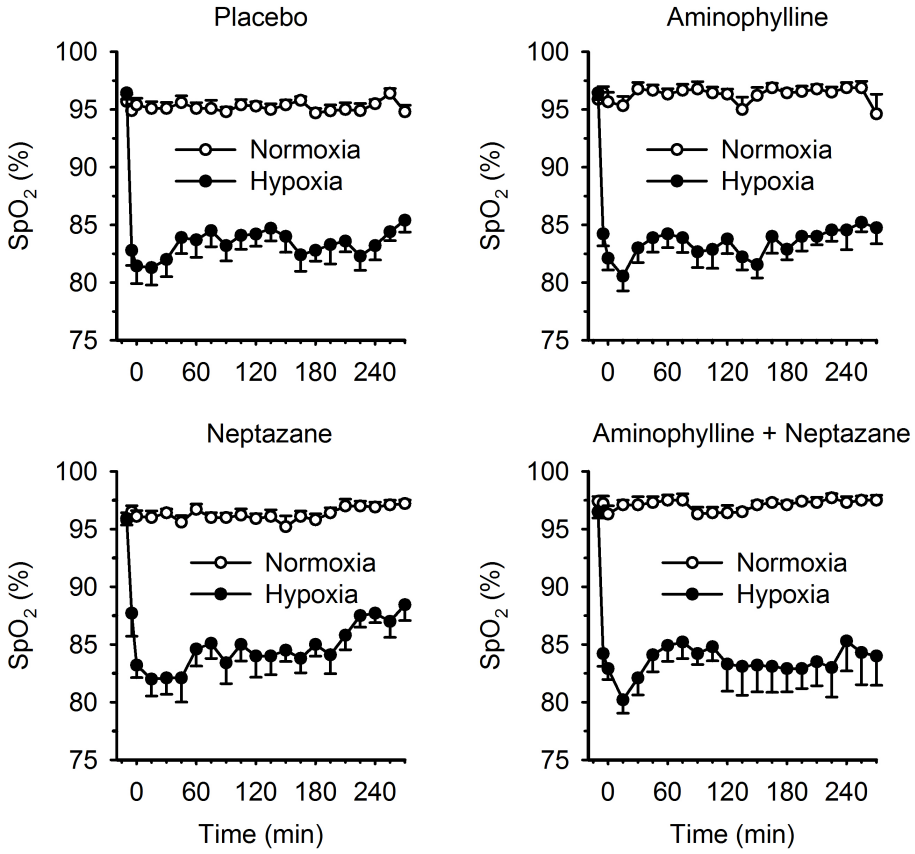
Figure 1.2. Plasma concentrations of theophylline and methazolamide and red blood cell concentrations of methazolamide in normoxia and hypoxia following oral administration of Neptazane (methazolamide) and/or Aminophylline (theophylline). Data are mean ± SE. Hypoxia did not affect plasma theophylline concentrations whether Aminophylline was taken alone or with Neptazane ($P > 0.80$) but did increase plasma methazolamide concentrations when Neptazane was taken alone ($P = 0.01$) or with Aminophylline ($P = 0.04$). Red blood cell concentrations of methazolamide were greater at 60 minutes in hypoxia when Neptazane was taken alone ($P = 0.001$), but not when Neptazane was taken with Aminophylline ($P = 0.08$). Standardized exercise: SE. Time trial: TT



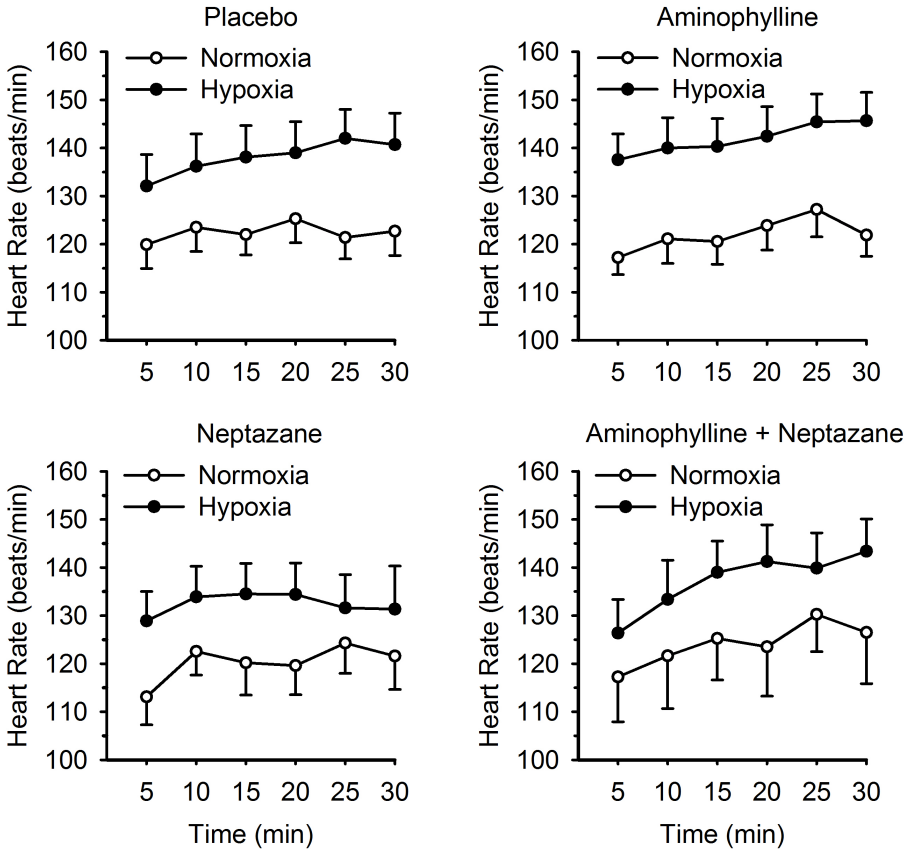
Supplemental Figure 1.1. Heart rate measured during rest in normoxia and hypoxia following oral administration of placebo, Neptazane, Aminophylline, or Neptazane with Aminophylline. Data are mean ± SE. Hypoxia increased resting heart rate ($P < 0.012$); compared with placebo, Neptazane and/or Aminophylline did not influence the effect of hypoxia on heart rate ($P > 0.60$). There was also a main effect of time for all conditions; resting heart rate increased throughout the trials ($P < 0.01$).



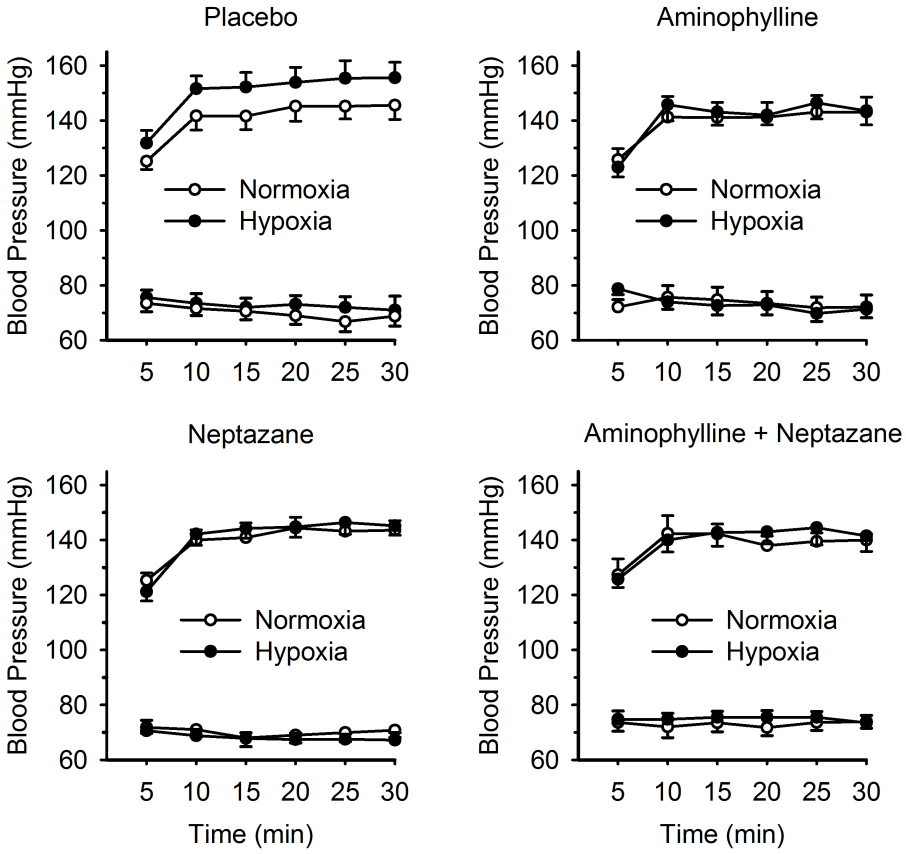
Supplemental Figure 1.2. Blood pressure measured during rest in normoxia and hypoxia following oral administration of placebo, Neptazane, Aminophylline, or Neptazane with Aminophylline. Data are mean ± SE. Hypoxia did not influence resting blood pressure (systolic or diastolic; $P > 0.27$), nor was there any interaction between treatments and hypoxia ($P > 0.18$). Blood pressure increased with time (systolic and diastolic; $P < 0.01$); there were no time-treatment interactions, although compared with placebo, the combination of Neptazane and Aminophylline almost attained statistical significance ($P = 0.061$; all others $P > 0.21$).



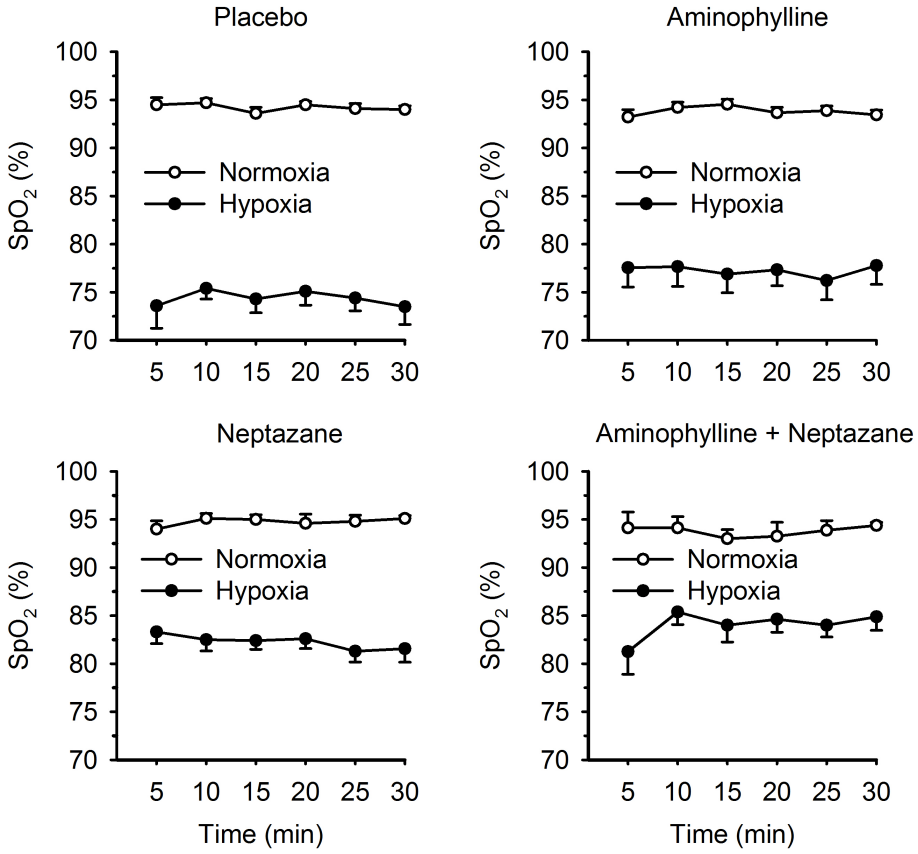
Supplemental Figure 1.3. Oxyhemoglobin saturation (SpO_2) measured during rest in normoxia and hypoxia following oral administration of placebo, Neptazane, Aminophylline, or Neptazane with Aminophylline. Data are mean ± SE. Hypoxia decreased resting oxyhemoglobin saturation ($P < 0.001$); compared with placebo, Neptazane and/or Aminophylline did not influence the effect of hypoxia on oxyhemoglobin saturation ($P > 0.15$). However, compared with placebo, Neptazane alone increased the oxyhemoglobin saturation in hypoxia over time ($P = 0.024$).



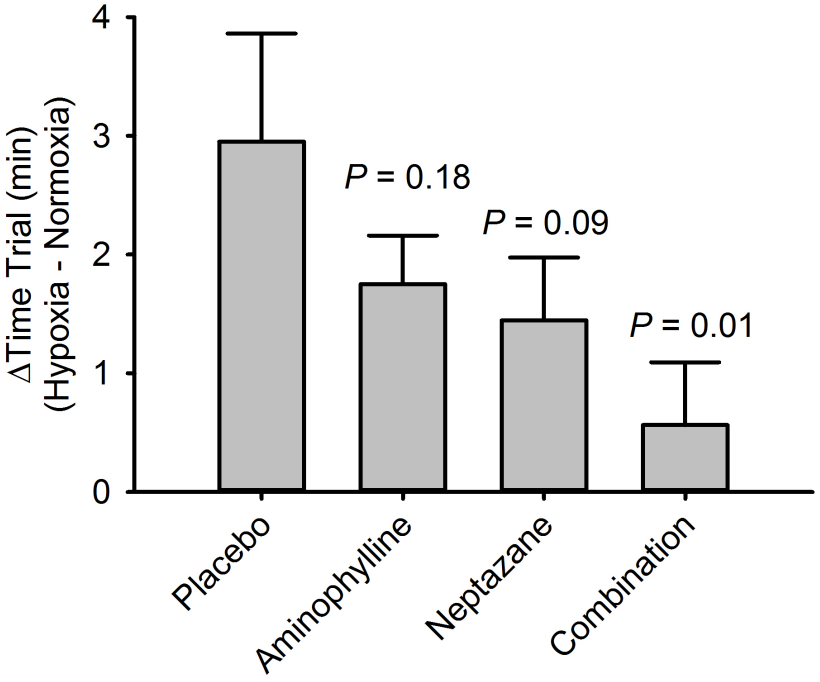
Supplemental Figure 1.4. Heart rate measured during standardized exercise in normoxia and hypoxia following oral administration of placebo, Neptazane, Aminophylline, or Neptazane with Aminophylline. Data are mean ± SE. In all treatments, hypoxia increased heart rate during standardized exercise ($P < 0.048$); compared with placebo, Neptazane and/or Aminophylline did not influence the effect of hypoxia on exercising heart rate ($P > 0.56$). Heart rate during exercise was also increased with time ($P < 0.001$); compared with placebo, Neptazane combined with Aminophylline attenuated the influence of time ($P = 0.02$). None of the treatments influenced the interaction between hypoxia and time ($P > 0.14$).



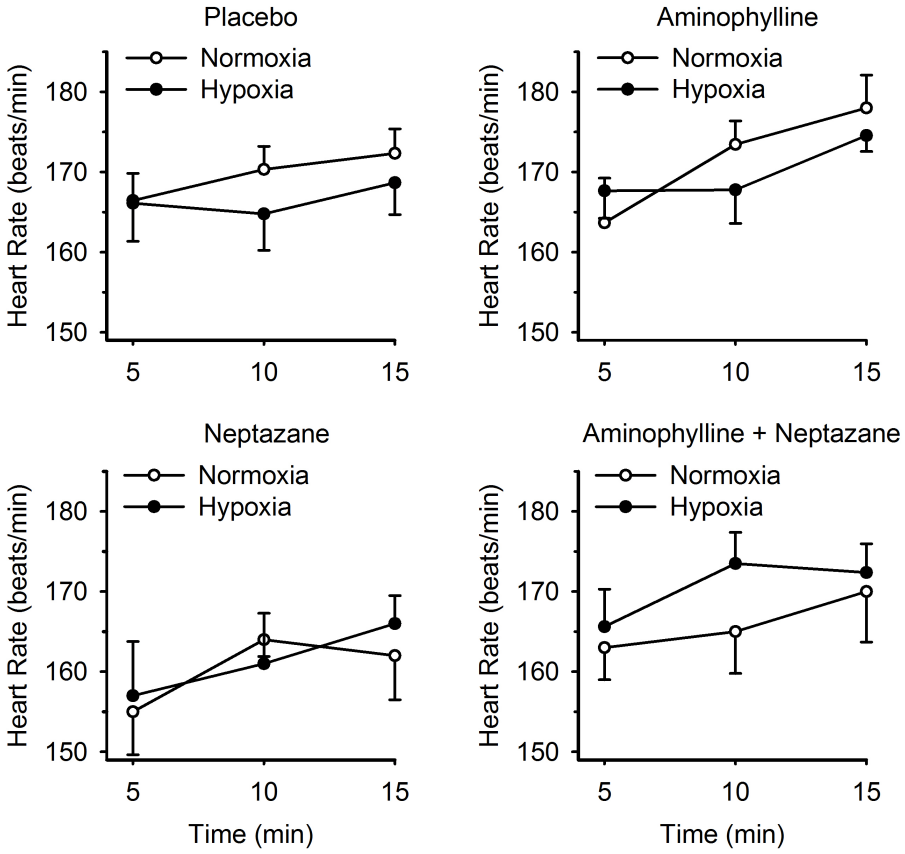
Supplemental Figure 1.5. Blood pressure measured during standardized exercise in normoxia and hypoxia following oral administration of placebo, Neptazane, Aminophylline, or Neptazane with Aminophylline. Data are mean ± SE. Time and/or hypoxia did not influence blood pressure for all treatments ($P > 0.10$) with the exception of Aminophylline; when placebo and Aminophylline were compared, diastolic pressure was slightly decreased with time ($P = 0.004$).



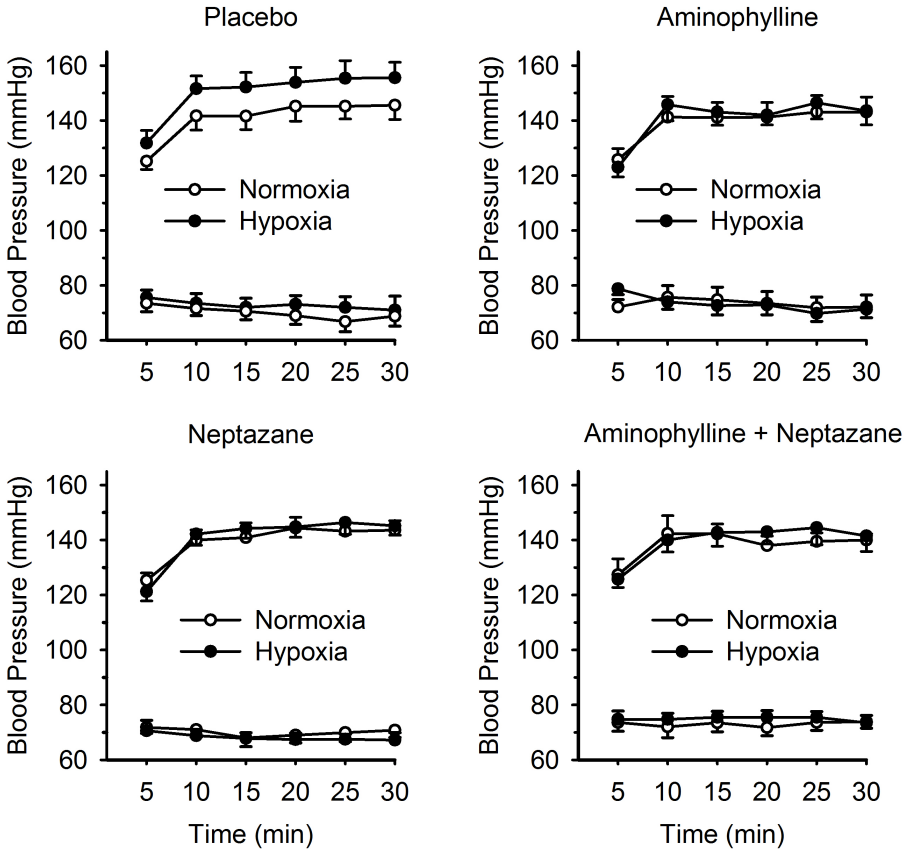
Supplemental Figure 1.6. Oxyhemoglobin saturation (SpO_2) measured during standardized exercise in normoxia and hypoxia following oral administration of placebo, Neptazane, Aminophylline, or Neptazane with Aminophylline. Data are mean ± SE. Hypoxia decreased oxyhemoglobin saturation during standardized exercise in all treatments ($P < 0.001$). Compared with placebo, Neptazane, and Neptazane combined with Aminophylline increased oxyhemoglobin saturation ($P < 0.001$).



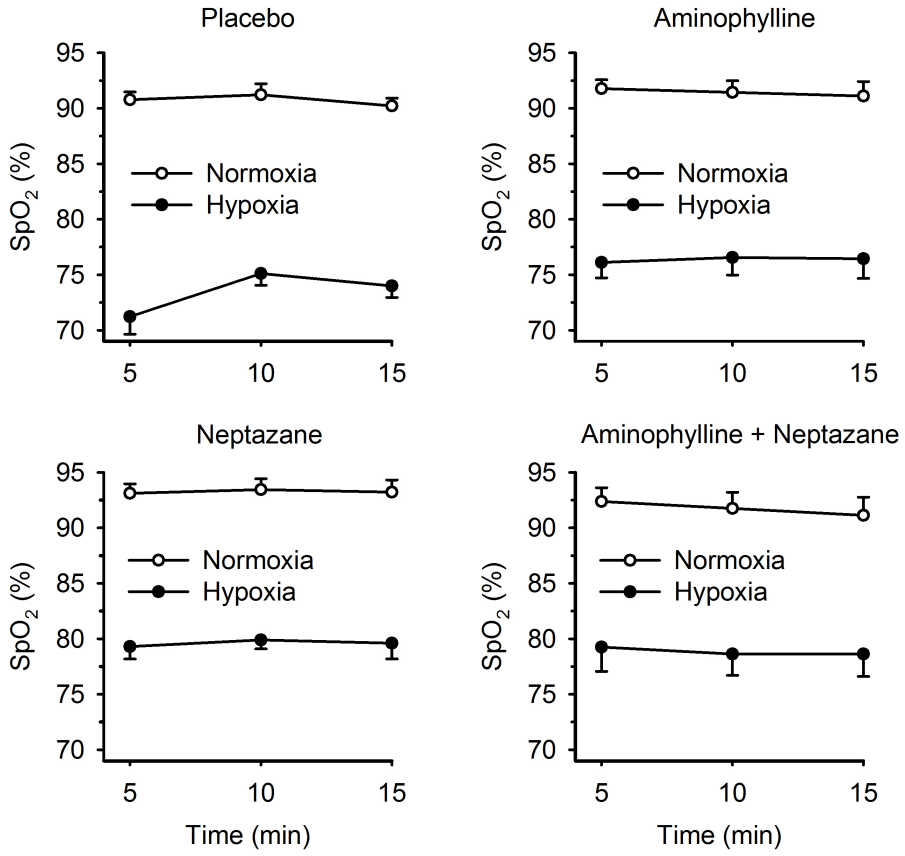
Supplemental Figure 1.7. Change in 12.5 km time trial performance from normoxia to hypoxia following oral administration of placebo, Neptazane, Aminophylline, or Neptazane with Aminophyillne. Each individual participant has been assigned a unique symbol, thus it is clear which participant completed each trial.



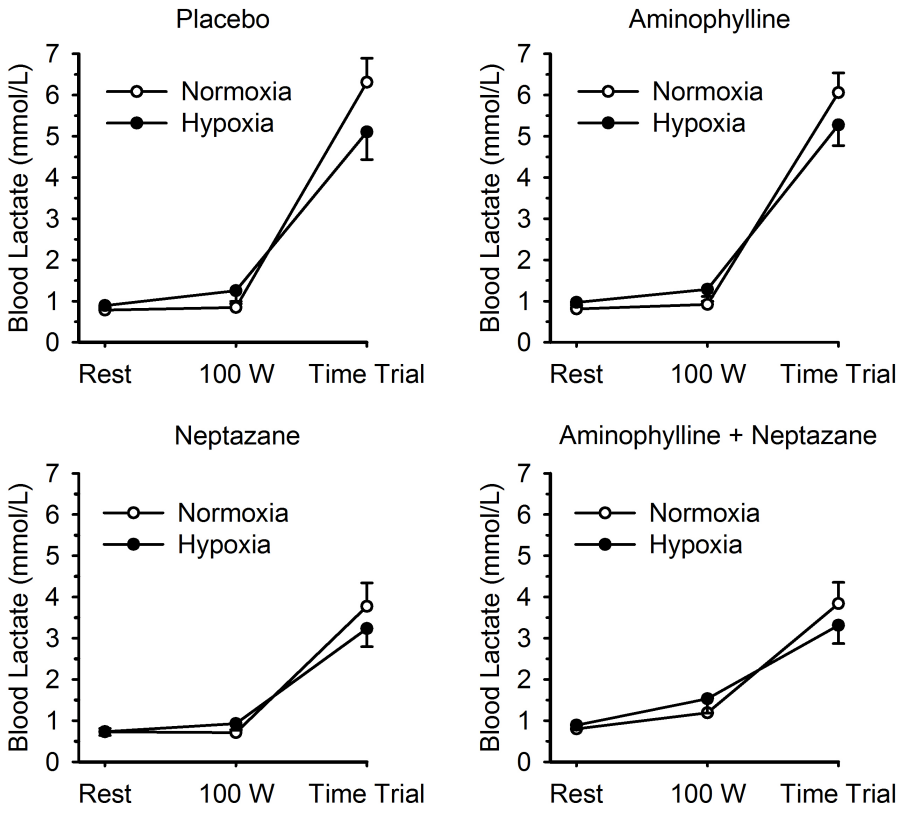
Supplemental Figure 1.8. Heart rate measured during 12.5km time trial in normoxia and hypoxia following oral administration of placebo, Neptazane, Aminophylline, or Neptazane with Aminophylline. Data are mean ± SE. There was a main effect of time for all treatments heart rate ($P < 0.049$); hypoxia did not affect heart rate ($P > 0.41$).



Supplemental Figure 1.9. Blood pressure measured during 12.5km time trial in normoxia and hypoxia following oral administration of placebo, Neptazane, Aminophylline, or Neptazane with Aminophylline. Data are mean ± SE. There was a main effect of time for all treatments for systolic blood pressure ($P < 0.049$); hypoxia did not affect systolic blood pressure ($P > 0.41$). Diastolic pressure was unaffected by time and/or hypoxia in all treatments ($P > 0.18$).



Supplemental Figure 1.10. Oxyhemoglobin saturations (SpO_2) measured during a 12.5 km time trial in normoxia and hypoxia following oral administration of placebo, Neptazane, Aminophylline, or Neptazane with Aminophylline. Data are mean ± SE. Hypoxia decreased SpO_2 ($P < 0.001$). Compared with placebo, Neptazane, and Neptazane and Aminophylline increased SpO_2 in hypoxia ($P < 0.02$).



Supplemental Figure 1.11. Blood lactate measured at rest, after standardized exercise (100W), and after a 12.5 km time trial in normoxia and hypoxia following oral administration of placebo, Neptazane, Aminophylline, or Neptazane with Aminophylline. Data are mean ± SE. Blood lactate concentration was always greater at completion of the time trial compared with any other point for all treatments ($P < 0.001$). The standardized exercise bout and hypoxia did not affect blood lactate ($P > 0.188$). Compared with placebo, Neptazane, and Neptazane and Aminophylline lowered blood lactate ($P < 0.021$).

CHAPTER IV – MANUSCRIPT II

Endurance Exercise in Hypoxia Following Carbohydrate Feeding: Do the metabolic benefits of sympathetic inhibition outweigh the cardio-pulmonary encumbrance?[2]

Summary

Pre-exertion skeletal muscle glycogen content is an important physiological determinant of endurance exercise performance; low glycogen stores contribute to premature fatigue. In low oxygen environments (hypoxia), the important contribution of carbohydrates to endurance performance is further enhanced as glucose/glycogen dependence is increased, however, the insulin sensitivity of healthy adult humans is decreased. In light of this insulin resistance, maintaining skeletal muscle glycogen in hypoxia becomes difficult, and subsequent endurance performance is impaired. Sympathetic inhibition promotes insulin sensitivity in hypoxia, but may impair hypoxic exercise performance, in part due to suppression of cardiac output. Accordingly, we tested the hypothesis that hypoxic exercise performance following intravenous glucose feeding in a low oxygen environment will be attenuated when feeding occurs during sympathetic inhibition. On two separate occasions (random order) 12 healthy men received one hour of parenteral carbohydrate (20% glucose solution in saline; 75g), after which they

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performed stationary cycle ergometer exercise (~65% maximal oxygen uptake) until exhaustion. Forty-eight hours prior to one visit, sympathetic inhibition, via transdermal clonidine (0.2 mg/d), began. Time to exhaustion following glucose feeding with/without sympathetic inhibition was not different (22.7±5.4 *vs*. 23.5±5.1 min; *P*=0.73). Sympathetic inhibition protects against hypoxia-mediated insulin resistance without influencing subsequent hypoxic endurance performance.

Introduction

Pre-exertion skeletal muscle glycogen content is an important physiological determinant of endurance exercise performance (5, 16, 155); low glycogen stores contribute to premature fatigue, high glycogen promotes exercise performance and extends time to exhaustion. In low oxygen environments, the important contribution of carbohydrates to endurance performance is further enhanced as glucose/glycogen dependence is appreciably increased (13, 127). While breathing a hypoxic gas mixture and/or during a hypobaric exposure, insulin sensitivity is markedly decreased in healthy adult humans (78, 103, 105). In light of this insulin resistance, maintaining skeletal muscle glycogen in high-altitude becomes difficult, thus subsequent endurance performance is impaired. We have demonstrated that inhibition of the sympathetic nervous system with the centrally acting alpha-2-adrenergic receptor agonist, clonidine, attenuates hypoxia mediated insulin resistance (105). By extension, this implies that inhibition of the sympathetic nervous system may be an effective strategy to abrogate high-altitude mediated insulin resistance and thus promote skeletal muscle glycogen maintenance and endurance exercise performance in low oxygen environments. However, the sympathetic nervous system is also a powerful regulator of cardio-pulmonary function, and sympathetic

inhibition in hypoxia may actually impair endurance exercise performance via decreased cardiac output and oxygen delivery (66, 113). The purpose of the current investigation was to address the question of whether or not the metabolic benefits of sympathetic inhibition outweigh the cardio-pulmonary encumbrance as they pertain to endurance exercise performance in hypoxia following carbohydrate feeding. We hypothesized that hypoxic exercise performance following intravenous glucose feeding in a low oxygen environment will be attenuated when feeding occurs during sympathetic inhibition.

Methods

Twelve young, healthy adult males were recruited to participate in this study. Inclusion criteria consisted of age within the range 18-40 years, body mass index within the range 18.5-30 kg/m^2, free from overt disease as determined via medical history and assessment of blood pressure and heart rate (via 12-lead electrocardiogram) at rest and during incremental exercise to volitional exhaustion, and approval from a supervising cardiologist (D.G.L or G.J.L). Exclusion criteria included current use of tobacco products or any prescribed medications, history of acute mountain sickness, asthma or any other type of lung/pulmonary dysfunction, and/or contraindications to vigorous exercise. Consequently, research participants demonstrated physiological attributes typical of young, healthy men. The experimental protocols conformed to the standards set by the Declaration of Helsinki of 1975, as revised in 1983, and were approved by the Institutional Review Board at Colorado State University. The nature, purpose, and risks of the study were explained to research participants before written informed consent was obtained.

Overview

Following screening, participants reported to the laboratory on two separate occasions; 48-hours prior to one of these visits, the sympathetic inhibitor clonidine was transdermally administered. The study visits occurred in random order and began with one hour of parenteral carbohydrate feeding, after which participants performed moderate intensity stationary cycle ergometer exercise until exhaustion. Throughout the entirety of each visit participants breathed a hypoxic gas mixture.

Participant Screening and Habituation

Body composition was assessed using dual-energy x-ray absorptiometry (DXA-IQ; Lunar Radiation Corp., Madison, WI, USA software v. 4.1). Maximal oxygen uptake (VO_{2max}) was determined in normoxia via a metabolic cart (Parvo Medics, Sandy, UT, USA) during incremental cycle ergometer exercise to volitional fatigue, as previously described (117, 118). For purposes of habituation to the exercise protocol, during a separate visit participants performed stationary cycle ergometer exercise (Velotron; Racermate Inc., Seattle, WA, USA), while breathing a hypoxic gas mixture, at an external work rate designed to evoke a metabolic rate equivalent to 65% of the their VO_{2max}, as determined in normoxia. Exercise intensity was verified with a metabolic cart (Parvo Medics, Sandy, UT, USA). Participants cycled until they were unable to maintain a pedal cadence greater than 40 rpm.

Experimental Procedures

Following screening and protocol habituation, participants reported to the laboratory on two separate occasions, after an overnight fast and 48-hour abstention from vigorous physical

activity. On arrival, participants were instrumented for measurement of heart rate (3-lead electrocardiogram), and blood pressure and oxyhemoglobin saturation (physiological monitor; Cardiocap 5, GE Datex-Ohmeda, Madison, WI, USA). Heart rate and systolic blood pressure were used to calculate the rate pressure product, a crude indicator of myocardial work. A catheter was inserted into an antecubital vein for subsequent parenteral feeding. Following a brief period of quiet rest in a semi-recumbent position, heart rate, blood pressure, and oxyhemoglobin saturation were recorded. Participants were then fitted with an airtight facemask (7450 Series; Hans Rudolph, Inc., Shawnee, KS, USA) attached to a three-way, non-rebreathing valve (2730 Series; Han Rudolph, Inc.), connected via a hose to a 100-liter non-diffusing gasbag (6000 Series; Hans Rudolph, Inc.), which was filled with precision mixed gases (15% O_2, balance N; Airgas, Denver, CO, USA). After 15 minutes, heart rate, blood pressure, and oxyhemoglobin saturation were re-recorded. Glucose (20% glucose solution in saline; 75g) was intravenously administered over one hour, after which the participants rested quietly for three hours before beginning the time to exhaustion trial. Consistent with the habituation protocol, this entailed stationary cycle ergometer exercise at an external work rate designed to evoke a metabolic rate equivalent to 65% of the their normoxic VO_{2max}. Time to exhaustion was recorded as the time during the trial at which a pedal cadence above 40 rpm could no longer be maintained.

To determine the influence of sympathetic inhibition on exercise performance in hypoxia after parenteral carbohydrate feeding, during the 48-hours prior to one of the visits (random order) transdermal clonidine (Catapres-TTS; 0.2 mg/day) was administered. Clonidine administration continued until initiation of the glucose infusion. Clonidine is a blood pressure medication; the mechanism of action is via pre-junctional stimulation of alpha-2-adrenergic

receptors. We, and others, have previously demonstrated that short-term clonidine use (2-to-7 days) results in centrally mediated peripheral sympathetic inhibition, as reflected by attenuated skeletal muscle sympathetic nerve activity (97, 98), decreased plasma norepinephrine concentration and release (95, 97, 98, 105), and increased heart rate variability (80, 153). The plasma half-life of clonidine is 21 hours (46).

Statistical Analysis

One-way repeated measures analysis of variance was used to determine the influence of sympathetic inhibition on the primary outcome variable: exercise time to exhaustion following carbohydrate feeding. The level of statistical significance was set at $P < 0.05$. Data are expressed as mean ± SE.

Results

Two of the 12 men recruited for participation were unable to complete the study due to hypoglycemic responses to glucose administration, one in the control condition and the other during clonidine administration. Selected physiological characteristics of the remaining 10 participants are presented in Table 2.1. As a group, the participants were normal weight (based on body mass index) and were of average aerobic fitness (based on VO_{2max}).

Heart rate, blood pressure, and oxyhemoglobin saturation in normoxia with and without prior clonidine administration are presented in Table 2.2. Additionally, the average of these variables over the four hours at rest in hypoxia with and without prior clonidine administration are presented in Table 2.2 as well.

Basal Response to Hypoxia

Compared with measurements made in normoxia, oxyhemoglobin saturation was decreased in hypoxia ($P < 0.001$) while heart rate was increased ($P = 0.003$). Hypoxia decreased systolic blood pressure ($P = 0.041$) but did not affect diastolic blood pressure ($P = 0.741$).

Basal Response to Clonidine – Evidence of Sympathetic Inhibition

Clonidine administration decreased systolic ($P = 0.037$), diastolic ($P = 0.023$), and mean arterial ($P = 0.015$) blood pressure in normoxia. Oxyhemoglobin saturation was unaffected.

Basal Response to Clonidine and Hypoxia – Evidence of Sympathetic Inhibition

Clonidine attenuated the heart rate response to hypoxia (albeit, not significantly; $P = 0.071$) and decreased both systolic ($P = 0.009$) and diastolic blood pressure ($P = 0.023$). The inhibitory influence of clonidine on the sympathetic nervous system was also evident in the mean arterial pressure and rate pressure product responses in normoxia and hypoxia (Figure 2.1): clonidine decreased mean arterial pressure ($P = 0.015$) and the rate pressure product ($P = 0.024$) in hypoxia. Clonidine did not influence the effect of hypoxia on oxyhemoglobin saturation ($P = 0.275$).

Endurance Exercise Performance

There was no difference in exercise time to exhaustion with and without prior clonidine administration (Figure 2.2; $P = 0.73$). The mean difference between the two conditions was 49.2 ± 2.3 seconds and the average work rate was 177 ± 8 Watts. Four of the ten participants

extended their time to exhaustion with prior clonidine use (magnitude of increase in time to reach volitional exhaustion was greater than one minute), while an equal number of participants demonstrated earlier fatigue (magnitude of decrease in time to reach volitional exhaustion was greater than one minute). The magnitude of difference between conditions for the remaining two participants was less than one-minute. During exercise there was no effect of clonidine on heart rate (Control 165 ± 3 *vs*. Clonidine 163 ± 3 beats/min; $P = 0.484$) or oxyhemoglobin saturation (Control 69 ± 3 *vs*. Clonidine 74 ± 2%; $P = 0.145$).

Discussion

We have addressed the question, do the metabolic benefits of sympathetic inhibition outweigh the cardio-pulmonary encumbrance as they pertain to endurance exercise performance in hypoxia following carbohydrate feeding. The novel finding of our investigation was sympathetic inhibition does not extend time to exhaustion during exercise in hypoxia, but importantly, it does not inhibit exercise performance either. We suggest that while sympathetic inhibition may inhibit cardio-pulmonary function, the cardio-pulmonary encumbrance is potentially offset by improved glucose regulation.

In light of the increased reliance on skeletal muscle glycogen during exercise in hypoxia/hypobaria (11, 13, 127), pre-exertional glucose regulation and glycogen synthesis in low-oxygen environments are of obvious interest, especially in the context of sympathetically mediated hypoxia-induced insulin resistance (12, 78, 103, 105). In this regard, the influence of the carotid bodies on metabolic regulation has become the subject of increased scrutiny. Traditionally considered from the sole perspective of cardio-pulmonary regulation via chemoreceptors (91), recent animal and human data have provided support for a metabolic role

of the carotid bodies. For example, in addition to detecting changes in arterial oxygen content, carotid bodies are sensitive to changes in circulating glucose and insulin concentrations (71, 147). Further, surgical resection of carotid bodies prevents high fat diet induced insulin resistance in a rodent model (114), highlighting the contribution of the carotid bodies to the development of metabolic disorder. In humans, desensitization of the carotid bodies via hyperoxic gas breathing lowers sympathetic activity and augments insulin sensitivity (61, 146). Collectively, these observations might support the use of supplemental oxygen to promote/maintain metabolic health at high-altitude, however this is often an impractical and cumbersome strategy. Alternatively, a pharmacological approach to inhibit the sympathetic nervous system and improve glucose regulation may have merit. We have previously shown that pharmacological inhibition of the sympathetic nervous system with clonidine attenuates hypoxia-mediated insulin resistance (105). Therefore, clonidine administration prior to prolonged hypoxic exposure could promote physiological function by augmenting insulin sensitivity and facilitating the maintenance of skeletal muscle glycogen stores.

Conversely, during exercise the sympathetic nervous system plays a vital role in physiological regulation, contributing to cardio-pulmonary function and substrate mobilization and delivery. Accordingly, inhibition of the sympathetic nervous system during exercise in hypoxia may seem counter-intuitive. Previous studies have demonstrated impaired cardiac output and aerobic exercise performance with sympathetic inhibition (65, 113). In the current study, we inhibited the sympathetic nervous system for 48 hours prior to hypoxic parenteral glucose feeding and subsequent hypoxic endurance exercise. When considered in the context of our previous study, our current data suggest that while 0.2 mg/day of clonidine over 48 hours is sufficient to attenuate hypoxia-mediated insulin resistance and promote glucose regulation (105),

this degree of inhibition may not be potent enough to inhibit endurance exercise performance in hypoxia. That is, during hypoxic exercise, the increased central sympathetic drive may be sufficient to overcome the inhibitory actions of the administered clonidine dose. In support of this, during clonidine administration resting blood pressure and rate pressure product were reduced, but during exercise heart rate and time to fatigue were unaffected.

There are several issues and potential limitations in the current study that warrant brief discussion. First, although in the current study we have indirect evidence to support a sympatho-inhibitory effect of clonidine at rest (e.g. lower systolic, diastolic and mean arterial pressures) we did not confirm that clonidine improved glucose regulation in hypoxia. However, based on consistent data from three independent laboratories, there is no reason to suspect that clonidine did not facilitate glucose regulation in hypoxia (105, 114, 146). Second, participants did not complete an exercise trial in normoxia, thus we were unable to compare our hypoxic data to a normoxic control. The detrimental effects of hypoxia on endurance exercise performance are well established and have been described repeatedly over the previous 50+ years (17, 33, 79, 92). Accordingly, we did not believe burdening our participants with an additional exercise trial was warranted. Instead, the primary comparison within the current study was the influence of our intervention *vs*. control on endurance exercise performance in hypoxia. Third, this was an open-label study. That is, the research participants were not naïve as to clonidine *vs*. control. This decision was based on our past experiences with clonidine administration. In two separate studies (98, 105), 100% of the research participants correctly identified clonidine *vs*. placebo. While it is plausible that participants' expectation may have influenced our primary outcome, that is, if clonidine is believed to be ergogenic then a participant may (sub-) consciously increase their effort during the exercise trial, or vice versa; our data would suggest otherwise in that we

observed an equal number of "positive responders" *vs*. "negative responders." Further, the research participants were not provided with a full explanation of the rationale for the study, that is, clonidine was presented in a neutral light (neither ergogenic nor ergolytic). Finally, all of the participants in the study were long-term residents of Fort Collins, CO, situated at an altitude of ~1500m. Thus it is possible that as a result of long-term acclimatization the influence of hypoxia on glucose regulation and/or exercise performance may have been less than that of a sea level dwelling adult. We feel this is unlikely and that the exposure to hypoxia was physiologically significant; the magnitude of decrease in oxyhemoglobin saturation during rest and exercise support this notion.

In conclusion, sympathetic inhibition via clonidine administration did not favorably or adversely impact endurance exercise performance in hypoxia following carbohydrate feeding. From this investigation, it appears that the metabolic benefits of sympathetic inhibition with respect to hypoxia-induced insulin resistance may outweigh the potential decrement to cardio-pulmonary function. This exploratory study examined the potential of a metabolic rather than a traditional cardio-pulmonary strategy to promote hypoxic exercise tolerance. The neutrality of our data does not rule out the need for future explorations of integrative, multi-system strategies for facilitating endurance exercise performance in hypoxia.

Table 2.1. Select characteristics of research participants.

	$n = 10$
Age (years)	28 ± 2
Height (m)	1.77 ± 0.20
Weight (kg)	78.5 ± 2.1
Body mass index (kg/m^2)	25.1 ± 0.5
% Body Fat	19.9 ± 1.5
Fat free mass (kg)	61.9 ± 1.7
Maximal oxygen consumption (ml/kg/min)	45.5 ± 2.7
Maximal heart rate (beats/min)	178 ± 3
Fasting blood glucose (mmol/L)	4.58 ± 0.11

Data are mean ± SE.

Table 2.2. Resting Hemodynamic responses to hypoxia with and without clonidine.

	Normoxia Control	Normoxia Clonidine	Hypoxia Control	Hypoxia Clonidine
SpO_2 (%)	96 ± 0	96 ± 1	83 ± 1*	84 ± 1*
HR (beats/min)	58 ± 2	60 ± 3	65 ± 2*	62 ± 2*
SBP (mmHg)	133 ± 3	130 ± 4†	133 ± 4	119 ± 3†**
DBP (mmHg)	78 ± 2	74 ± 3†	79 ± 3	74 ± 3†

Data are mean ± SE. SpO_2: Oxyhemoglobin saturation. HR: Heart rate. SBP: Systolic blood pressure. DBP: Diastolic blood pressure. *Different from normoxia ($P < 0.01$). †Different from control ($P < .05$). ** Significant interaction between hypoxia and clonidine ($P = 0.009$).

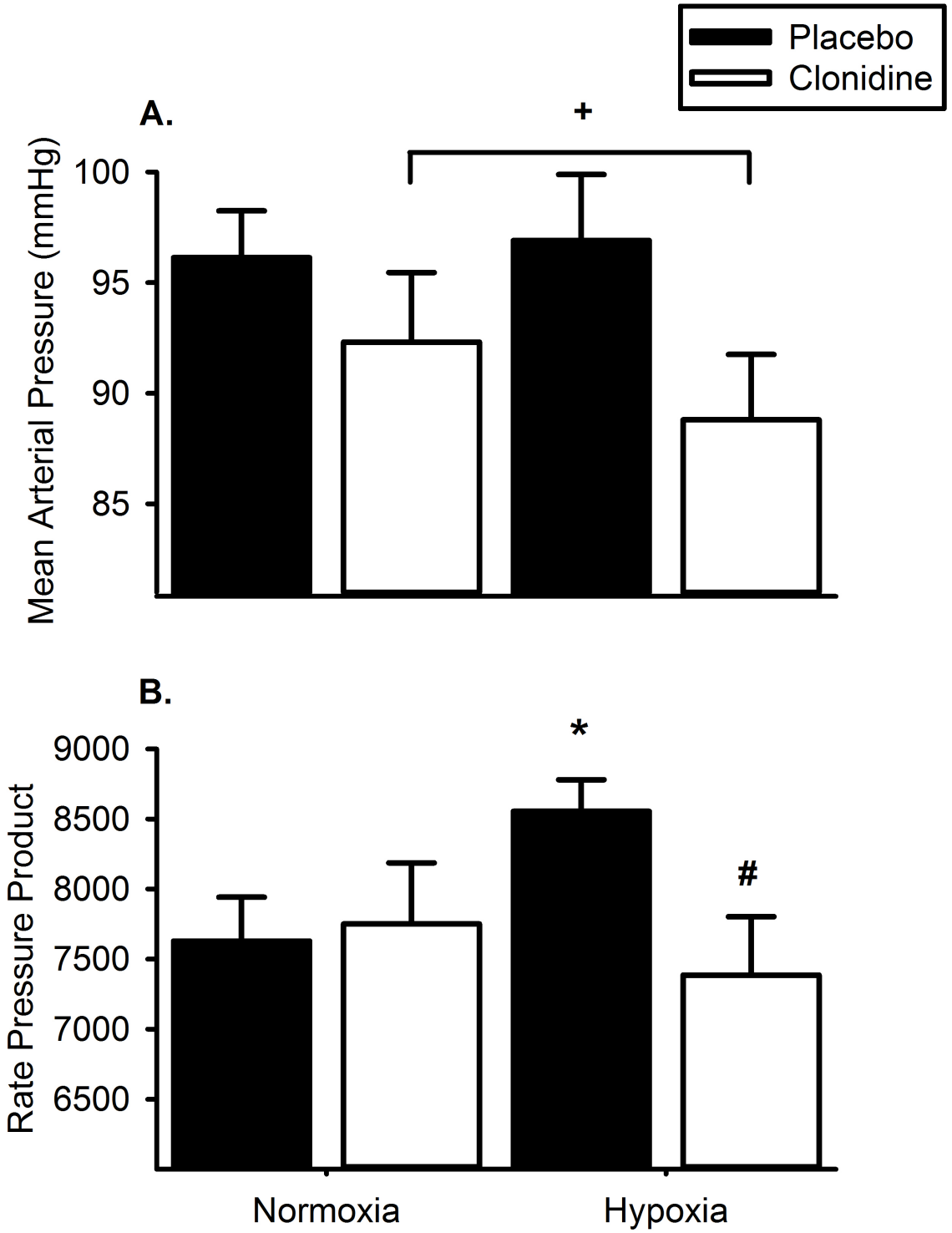

Figure 2.1. Mean arterial pressure (A) and rate pressure product (B) at rest with and without prior clonidine administration in normoxia and hypoxia. Data are mean ± SE. $^{+}$Main effect of clonidine (P = 0.015). *Different than normoxia-control (P = 0.028). #Different than hypoxia-control (P = 0.024).

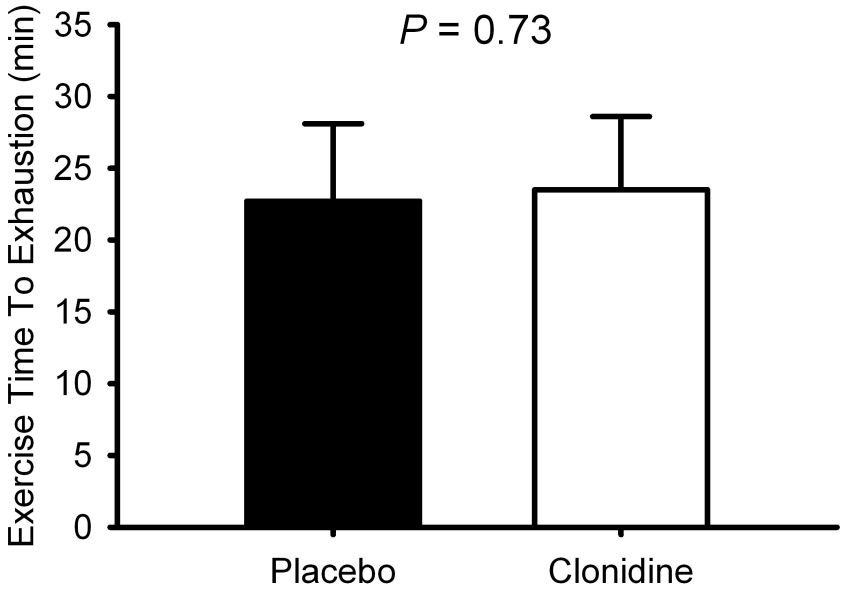

Figure 2.2. Cycle exercise time to exhaustion in hypoxia following the intravenous glucose infusion with and without prior clonidine administration. Data are mean ± SE.

CHAPTER V – MANUSCRIPT III

Sugar Highs in High-Altitude: Administration of metformin to promote endurance exercise performance in hypoxia[3]

Summary

Resting muscle glycogen is an important physiological determinant of endurance exercise performance, especially in high-altitude where glucose/glycogen dependence increases. Previously, we have demonstrated that insulin sensitivity is decreased in young, healthy men in hypoxia, thus making maintenance of skeletal muscle glycogen difficult. Accordingly, we tested the hypothesis that metformin would prevent the hypoxia-associated decrement in exercise performance following a high-carbohydrate meal in hypoxia. Ten males (age: 27±2 years; %body fat: 18.7±2.8; maximal oxygen uptake: 49.5±3.9 ml/kg/min; mean±SE) completed three visits in random order, one in normoxia and two in hypoxia (FiO_2=0.15) following three days of placebo or metformin (500mg, BID). Eighteen hours preceding each visit, participants completed a glycogen depleting exercise protocol (10x30s sprints) and were given a low-carbohydrate dinner (1200 kcals, <10% carbohydrate). The following morning, participants consumed a high-carbohydrate breakfast (1225 kcals, 70% carbohydrate) and rested quietly for 3.5 hours, before completing a 12.5 km time trial on a stationary cycle ergometer. Skeletal

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muscle (vastus lateralis) samples were collected immediately prior to breakfast and the time trial. Hypoxia decreased resting oxyhemoglobin saturation in both hypoxic conditions ($P<0.001$). Following breakfast, neither hypoxia nor metformin affected glucose area under the curve ($P=0.991$), however the insulin response associated with the glucose excursion was reduced with metformin (insulin AUC placebo: 6716±1089 *vs.* metformin: 5789±865 AU; $P=0.039$). Metformin increased glycogen content (+99%; $P=0.001$) following breakfast in hypoxia, and glycogen content also increased in normoxia (+54%: $P=0.039$) but not in hypoxia with placebo (+24%; $P=0.147$). Hypoxia decreased time trial performance (normoxia: 22.1±0.4 *vs.* hypoxia-placebo: 24.9±1.1 min; $P=0.001$) and metformin attenuated this hypoxia-mediated decrement (23.9±0.4 min), although this difference did not attain statistical significance (+2.9±1.0 min *vs.* +1.8±0.3 min; $P=0.288$). In healthy, adult men, metformin promotes glycogen synthesis and may improve endurance exercise performance in hypoxia.

Introduction

Rapid ascent in altitude for recreational and professional purposes challenges human physiological function and leaves travelers, such as military personnel, at a disadvantage when needing to complete physically demanding tasks (38). As pre-exertional skeletal muscle glycogen content is a primary determinant of endurance exercise performance (5, 38, 110, 131) and reliance on carbohydrate is increased when oxygen availability is limited (11, 13, 127), maintenance of glucose homeostasis is imperative for functional success at high-altitude. Glucose regulation, however, is disrupted when arterial oxygen content is compromised (78, 103, 105). Pharmacological inhibition of the sympathetic nervous system attenuates hypoxia-mediated insulin resistance (105) offering a potential strategy for improving metabolic health at

increased altitude, however, the cardio-pulmonary encumbrance of sympathetic inhibition (65, 113) makes it an inappropriate strategy for promoting exercise performance at high-altitude. An alternative approach to promote physiological function in hypoxia via augmentation of insulin sensitivity would be to administer an insulin sensitizer that does not influence cardio-pulmonary function. Metformin, a pharmaceutical utilized to treat adults with type 2 diabetes, promotes glucose control by inhibiting hepatic gluconeogenesis (58) and increasing skeletal muscle glucose uptake (3), augmenting skeletal muscle glycogen content. Administration of metformin during travel to high-altitude may restore human function by promoting maintenance of skeletal muscle glycogen. Accordingly, we tested the hypothesis that prior/concurrent metformin administration would attenuate the hypoxia-mediated decrement in endurance exercise performance.

Methods

Ten healthy, adult males were recruited to participate in this study. Inclusion criteria consisted of age within the range 18-40 years, free from overt disease as determined via medical history and assessment of blood pressure and heart rate (via 12-lead electrocardiogram) at rest and during incremental exercise to volitional exhaustion, and approval from a supervising cardiologist (D.G.L or G.J.L). Exclusion criteria included current use of tobacco products or any prescribed medications, impaired liver function, history of acute mountain sickness, asthma or any other type of lung/pulmonary dysfunction, and/or contraindications to vigorous exercise. Consequently, research participants demonstrated physiological attributes typical of young, healthy men. Colorado State University (Fort Collins campus) is situated at an altitude 1,525 m (~5,000 ft) above sea level, thus the normoxic conditions may be considered mildly hypobaric

(typical barometric pressure ~ 640 mmHg). All research participants had been residents of Fort Collins for a minimum of 12 months and refrained from travel to sea level destinations prior to and during study participation. The experimental protocols conformed to the standards set by the Declaration of Helsinki of 1975, as revised in 1983, and were approved by the Institutional Review Board at Colorado State University. The nature, purpose, and risks of the study were explained to research participants before written informed consent was obtained.

Overview

Following screening, participants reported to the laboratory on three separate occasions; 18-hours prior to each of these visits, participants completed a high-intensity exercise protocol to deplete skeletal muscle glycogen. The study visits occurred in random order: one in normoxia, the other two in hypoxia following three days of either placebo or metformin administration. All study visits included collection of skeletal muscle samples before and 3.5 hours following a high-carbohydrate breakfast. After the second skeletal muscle biopsy participants completed a standardized exercise bout and a 12.5 km cycle time trial.

Participant Screening and Habituation

Body composition was assessed using dual-energy x-ray absorptiometry (DXA-IQ; Lunar Radiation Corp., Madison, WI, USA software v. 4.1). Maximal oxygen uptake (VO_{2max}) was determined in normoxia via a metabolic cart (Parvo Medics, Sandy, UT, USA) during incremental cycle ergometer exercise to volitional fatigue, as previously described (117, 118). For purposes of habituation to the exercise protocol, participants performed a 15-minute warm-

up at 100W followed by a 12.5 km time trial on an electrically braked stationary cycle ergometer (Dynafit Velotron; Racermate Inc., Seattle, WA, USA) during a separate visit in normoxia.

Experimental Procedures

Following screening and protocol habituation, participants completed three trials in random order: normoxia, hypoxia-placebo, and hypoxia-metformin. The hypoxia-placebo and hypoxia-metformin trials were preceded by double-blind administration of placebo (719mg of maltodextrin) or metformin (500mg) for four days (one dose on day one, two doses on days two and three, one dose during the experimental protocol in hypoxia). Eighteen hours prior to the normoxic and hypoxic visits, participants completed a high-intensity exercise protocol to deplete skeletal muscle glycogen: ten 30-second sprints against a fixed resistance equal to 7.5% of their body mass on an electrically braked bike (Dynafit Velotron; Racermate Inc., Seattle, WA, USA)). Participants were given a low-carbohydrate meal (1200 kcal, <10% kcal from carbohydrate) to consume at their home that evening, and they returned to the laboratory the subsequent morning following a 12-hour fast and abstention from any additional exercise. Upon arrival, participants were situated in either a clinical laboratory or an environmental chamber within which the inspired oxygen concentration could be manipulated (Colorado Altitude Training, Louisville, CO, USA). In the clinical laboratory, the inspired oxygen concentration was normoxic ($FiO_2 = 0.21$), and during the hypoxic visits, there was a reduction in the ambient oxygen content ($FiO_2 = 0.15$) for the entire duration of the trial. Once in the assigned laboratory environment, participants were instrumented for measurement of heart rate (3-lead electrocardiogram), blood pressure, and oxyhemoglobin saturation (SpO_2; pulse oximeter; physiological monitor; Cardiocap 5, GE Datex-Ohmeda, Madison, WI, USA). An intravenous

catheter was inserted into an antecubital vein for subsequent and repeated blood sampling and was kept patent via a saline drip. Participants rested in a seated position for 15 minutes before an initial venous blood sample was acquired. Also at that time, a skeletal muscle sample of the vastus lateralis was obtained under local anesthesia (1% lidocaine) using a Bergstrom needle. Blood samples were preserved with ethyl-enediamine-tetra-acetic acid and kept temporarily on ice before being transferred to a chilled centrifuge. Subsequent plasma aliquots were stored at -80°C for determination of insulin and glucose concentration. Skeletal muscle samples were flash frozen and stored at -80°C for determination of muscle glycogen content and glycogen synthase protein expression.

After baseline tissue samples were obtained, participants consumed a high-carbohydrate meal (1225 kcal, 70% kcals from carbohydrate) and the final dose of the test drug if it was a hypoxic trial. Participants were given 15 minutes to consume all of the provided items to ensure consistent timing between meal consumption and subsequent tissue samples. Participants then rested quietly for 3.5 hours during which time venous blood was sampled frequently and heart rate, blood pressure, and oxyhemoglobin saturation were recorded every 30 minutes. Water was consumed ad libitum. A second skeletal muscle biopsy sample was obtained 3.5 hours after the beginning of the high-carbohydrate meal from the same leg as the pre-meal sample (proximal to the initial incision).

Following the second skeletal muscle biopsy, participants completed 15 minutes of standardized exercise on a computer controlled, electrically braked stationary cycle ergometer (Dynafit Velotron; Racermate Inc., Seattle, WA, USA); resistance was set to 100 W. Based on the physiological characteristics and habitual physical activity of the research participants, this workload represented low-to-moderate intensity exercise that could easily be completed in both

normoxia and hypoxia. On completion, participants were permitted a brief (up to 5 minutes) break before beginning an exercise time-trial, the goal of which was to perform stationary cycle ergometer exercise equivalent to a distance of 12.5 km (7.75 miles) as quickly as possible. During the time trial, all time cues were hidden from the participants but feedback regarding distance cycled was provided continuously. Heart rate, blood pressure, oxyhemoglobin saturation, and ratings of perceived exertion (8) were recorded at regular intervals during both the standardized exercise bout and the time trial.

Tissue Analysis

Blood glucose concentration was analyzed using an automated device (2300 STAT Plus Glucose Lactate Analyzer, YSI Inc., Yellow Springs, OH, USA) and plasma insulin concentration via ELISA (ALPCO Diagnostics, Salem, NH, USA).

Skeletal muscle glycogen content was determined via enzymatic method (104). Briefly, samples were removed from -80°C, weighed, and immediately placed in 0.5mL 2N HCl. They were boiled at 100°C for 2 hours, cooled, and neutralized with 1.5mL 0.67N NaOH. A volume of this muscle extract was added to an enzymatic glucose reactant mixture (50mM Tris Base, 25mM HCl, 1mM $MgCl_2$, 0.5 mM Dithiothreitol, 0.3mM ATP, 0.05mM NADP, 1U/mL Hexokinase, 0.1U/mL Glucose-6-Phosphate Dehydrogenase), and muscle glycogen was calculated based on the fluorescence of NADPH (ex: 360nm; em: 460nm).

Glycogen synthase protein expression was determined in skeletal muscle homogenates obtained from powderized tissue samples lysed with phosphatase and protease inhibitors (HALT). All samples were standardized by total protein and analyzed by SDS-PAGE as previously described (126). Phosphorylated (Serine 641) and total glycogen synthase protein

content (Cell Signaling Technology, 3891 and 3893; Boston, MA, USA) was determined via western blot. Equal protein loading was verified with Ponceau-S staining and actin protein content (Santa Cruz Biotechnology Inc., sc1616; Dallas, TX, USA).

Indices of Insulin Sensitivity

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting plasma glucose and insulin concentrations ((fasting glucose (mmol/L) × fasting insulin (μU/mL))/22.5) (94). Insulin sensitivity was estimated using the Matusda index from the plasma glucose and insulin concentrations observed prior to and following the high-carbohydrate meal (10 000/[(fasting glucose (mg/dL) × fasting insulin (μU/mL)) × (mean glucose (mg/dL) × mean insulin (μU/mL))]^{½}) (93).

Statistical analysis

If the assumptions of parametric statistics were met, two-way repeated measures analysis of variance (ANOVA) was used to determine the influence of hypoxia and metformin administration on the metabolic parameters and exercise performance data. If these assumptions were not satisfied, a non-parametric alternative (Holm-Sidak) was employed. The level of statistical significance was set at $P < 0.05$. Data are expressed as mean ± SE.

Results

Participants

Select characteristics of the ten participants are presented in Table 3.1. As a group, they were normal weight (based on body mass index) and were of average aerobic fitness (based on VO_{2max}).

High-Intensity Exercise

Power output from the high-intensity exercise was recorded to verify the physiological stimulus was similar before all experimental conditions. Mean power values were not different across interventions/treatments (normoxia: 645 ± 32 *vs.* hypoxia-placebo: 640 ± 33 *vs.* hypoxia-metformin 638 ± 34 Watts; $P = 0.824$).

Baseline Blood Values

Fasting measurements of blood glucose and insulin and calculated HOMA-IR values are presented in Table 3.2. There was no effect of experimental condition on fasting glucose ($P = 0.094$), insulin ($P = 0.605$) or HOMA-IR ($P = 0.481$).

Basal Response to Hypoxia and Metformin

Measurements of heart rate, blood pressure, and oxyhemoglobin saturation were averaged over the resting period; mean values are presented in Table 3.2. There was no effect of experimental condition on heart rate ($P = 0.889$), systolic blood pressure ($P = 0.917$), or diastolic blood pressure ($P = 0.666$). Hypoxia decreased oxyhemoglobin saturation ($P < 0.001$); there was no effect of metformin administration on this response ($P = 0.0826$).

Response to High-Carbohydrate Meal

Following the high-carbohydrate meal, there was a significant increase in circulating glucose and insulin (Figure 3.1; both $P < 0.001$). The blood glucose response to the meal was dependent on experimental condition (although not significantly; $P = 0.062$); preliminary examination of responses by time point revealed blood glucose was lower 45 minutes after the high-carbohydrate meal in hypoxia with metformin administration compared with normoxia (Figure 3.1A; 119 ± 8 *vs.* 99 ± 7 mg/dL; $P = 0.01$). The cumulative glucose excursion following the high-carbohydrate meal was unaffected by experimental condition (Figure 3.1B; $P = 0.991$). There was no effect of hypoxia or metformin on insulin concentration at any specific time point (Figure 3.1C; $P = 0.276$); however, the insulin area under the curve was diminished following metformin administration compared with placebo (Figure 3.1D; $P = 0.039$). Insulin sensitivity assessed via Matsuda index was unaffected by condition (normoxia: 10.4 ± 2.7 *vs.* hypoxia-placebo: 10.2 ± 3 *vs.* hypoxia-metformin: 13.6 ± 3.9; $P = 0.328$).

Skeletal Muscle Glycogen Synthesis

Glycogen synthase S^{641} phosphorylation: total glycogen synthase protein content (inversely related to the activity of glycogen synthase) increased following the high-carbohydrate meal in hypoxia with metformin administration (Figure 3.2A; $P = 0.003$), however it was unaffected by the meal in normoxia ($P = 0.467$) or hypoxia with placebo ($P = 0.132$). Additionally, post-meal glycogen synthase phosphorylation was greater in hypoxia with metformin compared with normoxia ($P = 0.003$).

There was no difference in pre-meal glycogen content between the three conditions (Figure 3.2B; $P > 0.09$). Glycogen content increased with the high-carbohydrate meal in normoxia ($P = 0.039$) and hypoxia with metformin ($P = 0.001$) but did not change in hypoxia with placebo ($P = 0.147$). Noteworthy, post-meal glycogen content in hypoxia with metformin was positively associated with glycogen synthase inhibition (phosphorylation: total ratio: r = 0.77; $P = 0.042$). This relationship was not present in normoxia (r = 0.02; $P = 0.964$) or hypoxia with placebo (r = -0.24 $P = 0.61$).

Standardized Exercise Bout

Mean values of hemodynamic variables measured during the standardized exercise bout are presented in Table 3.3. There was no effect of condition on heart rate ($P = 0.737$) or systolic ($P = 0.259$) or diastolic blood pressure ($P = 0.771$). Oxyhemoglobin saturation was decreased during hypoxic exercise ($P < 0.001$); metformin did not influence this response ($P = 0.401$). Further, experimental condition did not influence blood lactate concentration at any time point ($P = 0.889$). Blood lactate increased from rest (normoxia: 1.7 ± 0.2 *vs.* hypoxia-placebo: 1.9 ± 0.1 *vs.* hypoxia-metformin 1.8 ± 0.2) during the standardized exercise bout (Table 3.3; $P < 0.001$). There were no differences in rating of perceived exertion during the standardized exercise bout (Table 3.3; $P = 0.691$).

Time trial Performance

Hypoxia impaired time trial performance (Figure 3.3; normoxia: 22.1 ± 0.4 *vs.* hypoxia-placebo: 24.9 ± 1.1 min; $P = 0.001$) and metformin attenuated this hypoxia-mediated decrement

(23.9 ± 0.4 min), although this difference did not attain statistical significance (+2.9 ± 1.0 *vs.* +1.8 ± 0.3 min; $P = 0.288$).

Heart rate, blood pressure, blood lactate, and rating of perceived exertion all increased during the time trial compared with standardized exercise ($P < 0.01$); mean responses are presented in Table 3. Hypoxia attenuated the rise in heart rate ($P = 0.019$); however the heart rate response in normoxia was not different compared with hypoxia-metformin ($P = 0.125$). Systolic and diastolic blood pressure were unaffected by experimental condition during the time trial ($P > 0.38$). Hypoxia decreased oxyhemoglobin saturation ($P < 0.001$); metformin did not influence this effect ($P = 0.118$). Blood lactate concentration and rating of perceived exertion were greater at the completion of the time trial in contrast to the end of the standardized exercise bout (both $P < 0.001$). Rating of perceived exertion was unaffected by experimental condition ($P = 0.714$).

Discussion

The purpose of this investigation was to determine if a metabolic approach to promote human physiological function in hypoxia would improve endurance exercise performance in a low oxygen environment. Prior/concurrent metformin administration improved glucose tolerance and promoted skeletal muscle glycogen accumulation following a high-carbohydrate meal in hypoxia. These metabolic improvements, however, were not associated with a reduction in the hypoxia-mediated decline in endurance exercise performance.

Arterial oxygen content is reduced and human physiological function is impaired at high-altitude; this functional decrement has been confirmed by attenuated endurance exercise performance (17, 33, 79, 92). Data from the current investigation support these previous

findings; time trial performance was slower, and oxyhemoglobin saturation was decreased in our model of simulated high-altitude. Traditional approaches for improving human performance at high-altitude have focused on cardio-pulmonary mechanisms to combat hypobaria-mediated declines in arterial oxygen content. These approaches have included carbonic anhydrase inhibitors (9, 32, 42), beta-adrenergic receptor agonists (142), xanthine derivatives (39), and phosphodiesterase inhibitors (56, 72), and the resultant effects have been inconsistent. A non-cardio-pulmonary based approach to restore human function in high-altitude warranted investigation. We, and others, have demonstrated a hypoxia-mediated attenuation of insulin sensitivity (78, 103, 105). As pre-exertional skeletal muscle glycogen content is a determinant of endurance exercise performance (5, 110, 131), and reliance on carbohydrate (glucose/glycogen) is increased when arterial oxygen content is compromised (11, 13, 127), combating hypoxia-mediated insulin resistance is one potential strategy to promote human physiological function in high-altitude.

Chemoreceptor activation, via the decline in arterial oxygen content, stimulates sympathetic nervous system activity, which acts through several cardio-pulmonary mechanisms to maintain arterial oxygen content (65, 66). The sympathetically mediated cardio-pulmonary response has metabolic consequences, specifically, disrupted glucose regulation and impaired insulin sensitivity (62). Previous work from our laboratory demonstrated that pharmacological inhibition of the sympathetic nervous system restored insulin sensitivity in hypoxia (105); however, this approach is not a feasible strategy to promote endurance exercise performance in high-altitude because sympathetic inhibition impairs cardiac output and aerobic exercise performance in normoxia (65, 113) and, in hypoxia, the metabolic benefits of sympathetic inhibition do not compensate for the cardio-pulmonary encumbrance (125).

An alternative method to address hypoxia-mediated insulin resistance, without incurring cardio-pulmonary impediment, would be beneficial for military personnel and travelers physically active at high-altitude as it would promote maintenance of skeletal muscle glycogen content. Metformin is frequently prescribed to improve glucose regulation in adults with type 2 diabetes (60); importantly, it is without known cardio-pulmonary effects. Metformin is purported to improve glucose homeostasis via suppression of hepatic glucose production (58) and augmented skeletal muscle glucose uptake (3), both targets of sympathetically mediated disruptions in glucose regulation and insulin resistance (59, 103, 111, 130, 144). Our preliminary data suggest that metformin improved glucose tolerance, indicated by the reduction of insulin area under the curve and the more rapid decline of blood glucose 45 minutes after the beginning of the high-carbohydrate meal in hypoxia compared with normoxia. Especially important in the context of endurance exercise performance, glycogen content increased with metformin administration following the high-carbohydrate meal in hypoxia but did not change in hypoxia with placebo. The increase in glycogen synthesis following metformin administration, though, did not improve endurance exercise performance in hypoxia compared with placebo. An effect of metformin on endurance exercise performance may have been more appreciable had we selected a different metric of performance (e.g. a longer distance time trial or cycle time to exhaustion test). The 12.5 km time trial, however, was sufficient to illustrate a hypoxia-mediated decrement in this investigation, and when utilized previously, it captured the effect of hypoxia and a pharmaceutical treatment (124).

Phosphorylation of glycogen synthase was elevated in hypoxia with metformin following the high-carbohydrate meal indicating reduced activity of this enzyme that determines glycogen synthesis. There was no difference in glycogen synthase phosphorylation after the high-

carbohydrate meal in normoxia or hypoxia with placebo. Regulation of glycogen synthase activation is complex (21) and includes negative feedback control by glycogen content (63, 100). A positive association between post-meal glycogen synthase phosphorylation and glycogen content in hypoxia with metformin suggests this glycogen content-mediated negative regulation of glycogen synthase activity. Further, these results highlight the limitation of a static measurement (protein expression) at two time points to measure a dynamic process (glycogen synthesis) that occurs over many hours.

There are three additional considerations that warrant brief discussion. First, there was no metformin control condition in normoxia. We did not anticipate metformin would impact glucose handling in healthy participants who were not metabolically challenged (i.e. in normoxia), therefore, this condition was eliminated from our experimental design to reduce participant burden and minimize their metformin exposure. It is possible that responses in our data set, however, represent an effect of metformin independent of hypoxia-mediated insulin resistance. The fall in blood glucose following the peak response to the high-carbohydrate meal occurred to a greater extent in hypoxia with metformin compared with the response in normoxia. There was not a difference in blood glucose at this time point in hypoxia with placebo compared with normoxia. Additionally, glycogen synthase phosphorylation was not affected by hypoxia alone, just metformin administration. Further experiments should include this additional control. Second, the participants in this investigation received 1000mg of metformin per day (~15mg/kg) while previous investigations of metformin and exercise performance utilized 2000mg of metformin per day (~ 30mg/kg) (10, 89). It is possible that had we utilized a larger dose in the current study, the data may have provided more conclusive evidence for the use of metformin to promote metabolic health and human performance in hypoxia. However, gastro-intestinal

distress is a common side effect of metformin administration. In this regard, a larger dose of metformin would have increased the risk of participants suffering this side effect, which could have had a negative impact on endurance exercise performance. Finally, all of the participants in the study were long-term residents of Fort Collins, CO, situated at an altitude of ~1500m. Thus, it is possible that as a result of long-term acclimatization the influence of elevated altitude on glucose regulation and/or exercise performance may have been less than that of a sea level dwelling adult. We feel this is unlikely and that the exposure to hypoxia was physiologically significant; the magnitude of decrease in oxyhemoglobin saturation during rest and exercise support this notion.

Rapidly transitioning to high-altitude environments challenges human physiology, especially during exercise/exertion. Promoting glucose homeostasis and the maintenance of skeletal muscle glycogen is one strategy to combat the hypoxia-mediated decrement in human function as glycogen content is a primary determinant of endurance exercise performance. These preliminary data suggest that metformin augments glycogen synthesis in response to a high-carbohydrate meal in hypoxia. Metformin administration may be an alternative strategy to cardio-pulmonary approaches to promote human physiological function in high-altitude.

Table 3.1. Select participant characteristics.

Variable	Mean ± SE
n	10 men
Age (years)	27 ± 2
Body Mass (kg)	81.5 ± 5.0
Body Mass Index (kg/m^2)	26.0 ± 1.6
% Body Fat	18.7 ± 2.8
Fat Mass (kg)	17.2 ± 3.3
Fat Free Mass (kg)	64.2 ± 2.1
Maximal Oxygen Uptake (L/min)	3.87 ± 0.14
Maximal Oxygen Uptake (ml/kg/min)	49.5 ± 3.9
Maximal Respiratory Exchange Ratio	1.15 ± 0.0
Maximal Heart Rate (beats/min)	174 ± 3
Maximal Work Rate (Watts)	366 ± 22

Table 3.2. Resting Variables in Normoxia and Hypoxia.

	Normoxia	Hypoxia-placebo	Hypoxia-metformin
Variable	Mean ± SE	Mean ± SE	Mean ± SE
Heart Rate (bpm)	67 ± 2	74 ± 2	71 ± 3
Systolic blood pressure (mmHg)	122 ± 4	126 ± 3	125 ± 3
Diastolic Blood Pressure (mmHg)	73 ± 2	75 ± 2	72 ± 2
Oxyhemoglobin Saturation (%)	97 ± 1	87 ±1*	85 ±1*
Fasting Blood Glucose (mg/dL)	74.6 ± 2.8	78.4 ± 2.4	74.1 ± 2.4
Fasting Plasma Insulin (μU/ml)	8.8 ± 3.5	8.4 ± 3.5	5.9 ± 3.4
HOMA-IR	0.81 ± 0.06	1.69 ± 0.72	1.06 ± 0.60

*Different compared with Normoxia ($P < 0.001$).

Table 3.3. Hemodynamic responses to exercise

		Normoxia	Hypoxia-placebo	Hypoxia-metformin
Time Point	Variable	Mean ± SE	Mean ± SE	Mean ± SE
100W	Heart Rate (bpm)	118 ± 4	121 ± 4	122 ± 5
	Systolic blood pressure (mmHg)	141 ± 5	141 ± 4	144 ± 4
	Diastolic Blood Pressure (mmHg)	68 ± 4	67 ± 3	66 ± 3
	Oxyhemoglobin Saturation (%)	94 ± 1	81 ±1*	80 ±1*
	Lactate (mmol/L)	1.9 ± 0.2	2.4 ± 0.3	2.3 ± 0.2
	RPE	9 ±1	9 ± 1	9 ± 1
TT	Heart Rate (bpm)	170 ± 4	160 ± 4†	163 ± 3
	Systolic blood pressure (mmHg)	166 ± 4	161 ± 5	164 ± 6
	Diastolic Blood Pressure (mmHg)	63 ± 4	62 ± 3	62 ± 3
	Oxyhemoglobin Saturation (%)	91 ± 1	77 ±1*	76 ±1*
	Lactate (mmol/L)	9.8 ± 1.1	8.8 ± 0.9	9.3 ± 0.9
	RPE	16 ± 0	16 ± 0	16 ± 0

*Different compared with Normoxia ($P < 0.001$). †Different compared with Normoxia ($P = 0.019$). 15 minutes of standardized exercise: 100W. 12.5km time trial: TT.

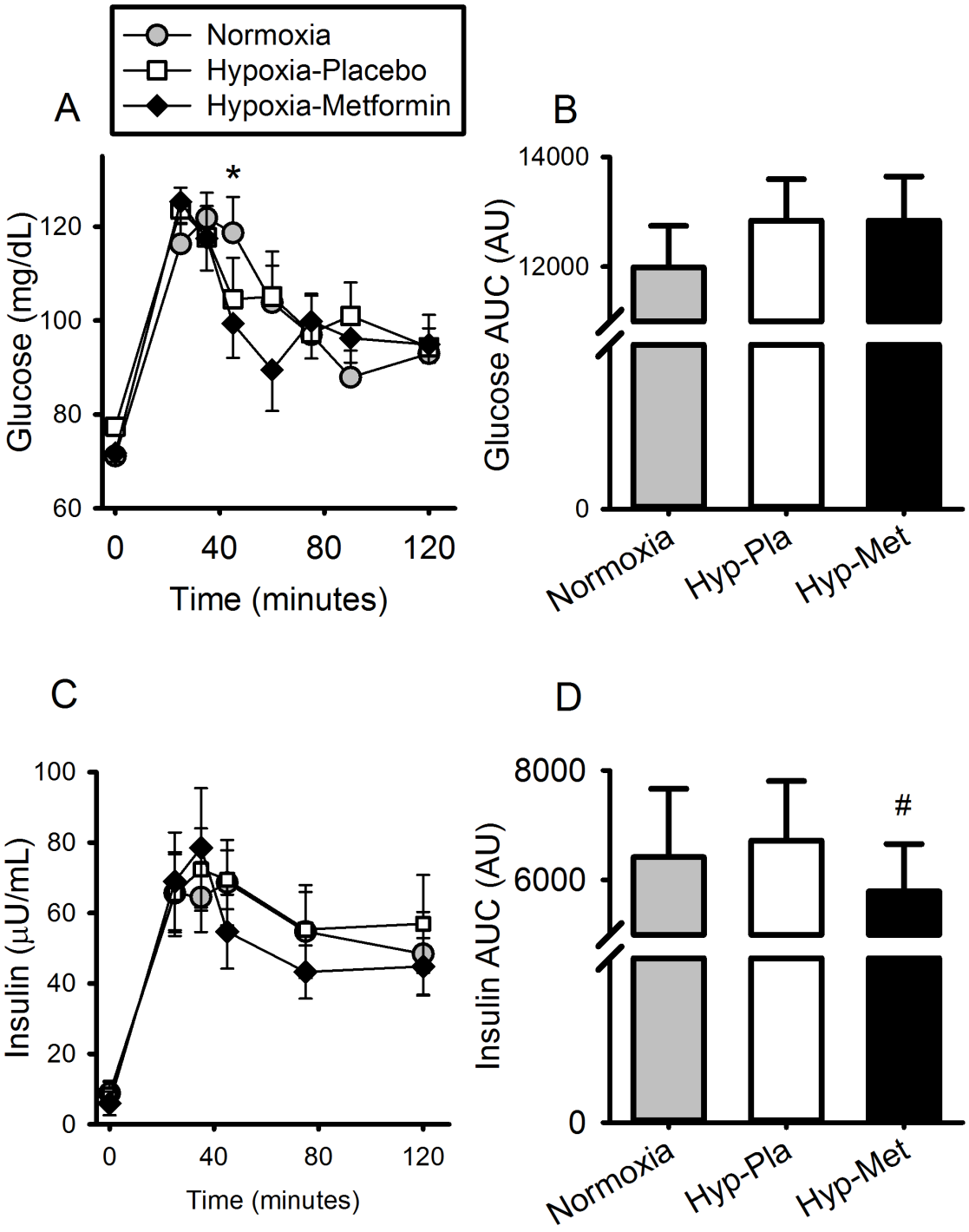

Figure 3.1. Blood glucose and insulin responses to a high-carbohydrate meal in normoxia and hypoxia. Both glucose and insulin increased following the meal in all three conditions ($P < 0.001$). *Different from normoxia ($P = 0.01$). #Different from hypoxia-placebo ($P = 0.039$). Hypoxia-placebo: Hyp-Pla. Hypoxia-metformin: Hyp-Met.

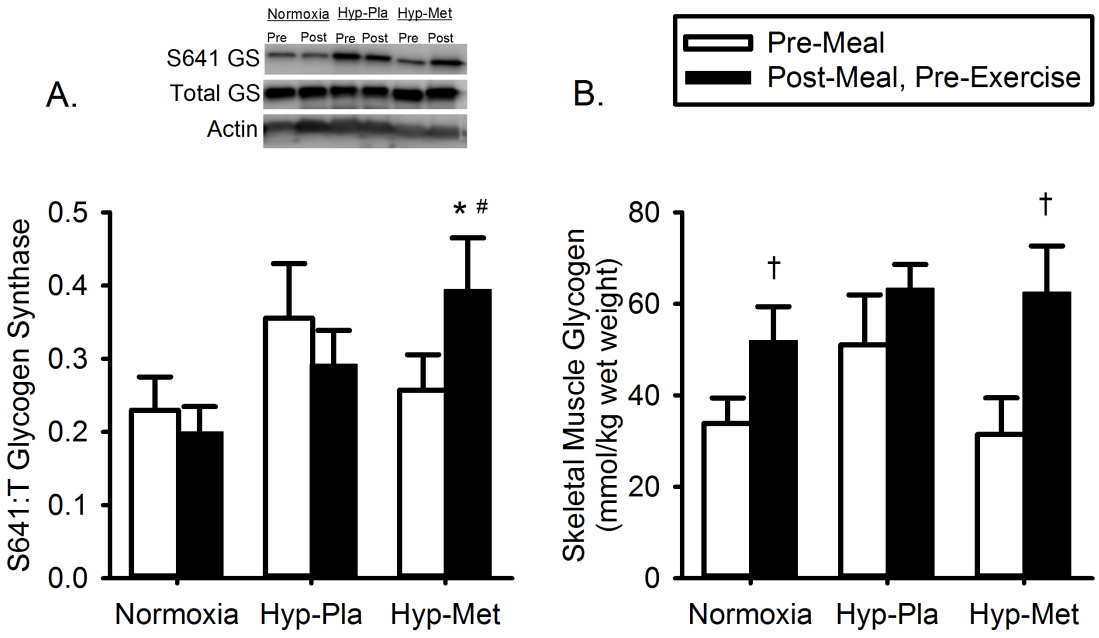

Figure 3.2. Skeletal muscle glycogen synthase phosphorylation and glycogen content prior to and following a high-carbohydrate meal in normoxia and hypoxia. *Different from Hyp-Met pre-meal ($P = 0.003$). $^{\#}$Different from normoxia post-meal, pre-exercise ($P = 0.003$). †Different from pre-meal ($P < 0.039$). Hypoxia-placebo: Hyp-Pla. Hypoxia-metformin: Hyp-Met.

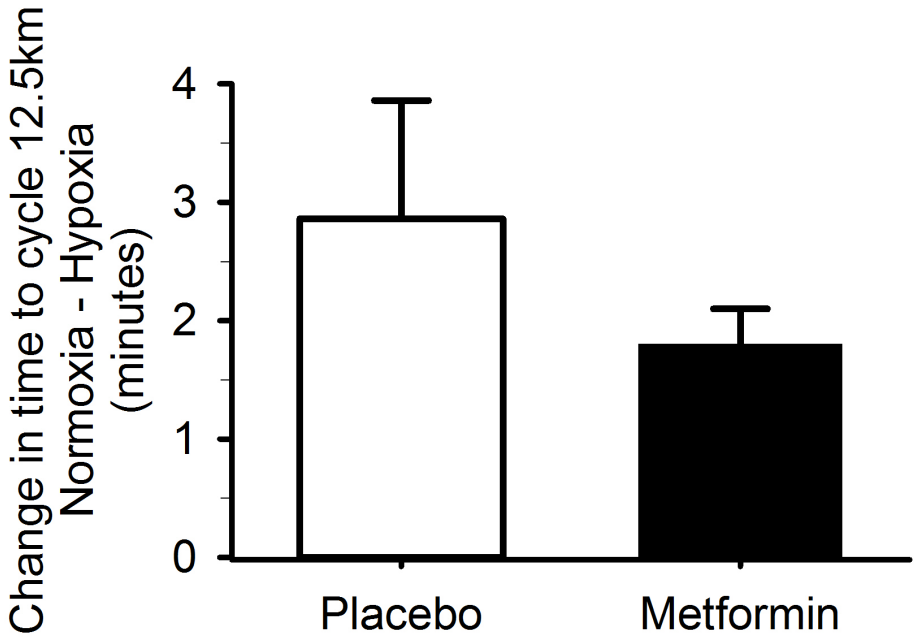

Figure 3.3. Change in 12.5 km time trial performance from normoxia to hypoxia following administration of placebo or metformin.

CHAPTER VI – OVERALL CONCLUSIONS

Rapidly transitioning to high-altitude poses a functional challenge for human physiology. Military personnel are frequently deployed in this manner without ample opportunity for acclimatization to the increase in altitude. The swift nature of these deployments leaves soldiers' safety and the success of their missions potentially compromised. Several pharmacological interventions have previously been described to promote human physiological function in high-altitude, however, these approaches have been developed to meet civilian needs and are not always appropriate for the military. Here, we have investigated three novel applications of established pharmaceuticals that are more applicable to military requirements to promote physiological function in high-altitude.

The conventional approach to improving human physiological function in high-altitude is to attenuate the hypobaria-mediated decline in arterial oxygen content. Previously investigated pharmaceuticals that target arterial oxygen content are not appropriate for active military needs, however, in contrast Aminophylline and Neptazane may be suitable. We hypothesized that Aminophylline, Neptazane, and/or the combination of the two would attenuate the hypoxia-mediated decrement in endurance exercise performance. We found that the magnitude of decrease in time trial performance from normoxia to hypoxia was smaller with the combination of Aminophylline and Neptazane compared with placebo. When administered individually, neither Aminophylline nor Neptazane proved to be ergogenic. The abrogated decline in endurance exercise performance in hypoxia was potentially mediated via augmented oxyhemoglobin saturation. These results support concomitant administration of Aminophylline and Neptazane to promote functional performance of military personnel in high-altitude.

An alternative approach to improve endurance exercise performance in high-altitude is to target the indirect and unfavorable metabolic consequences of reduced arterial oxygen content, specifically, increased insulin resistance. In previous work, we demonstrated that sympathetic inhibition with the anti-hypertensive medication clonidine attenuated hypoxia-mediated insulin resistance. As pre-exertional skeletal muscle glycogen content is a determinant of endurance exercise performance, maintaining glucose regulation in high-altitude is of obvious benefit to military personnel. However, sympathetic inhibition has cardio-pulmonary ramifications with respect to endurance exercise performance. Accordingly, in the second study we hypothesized that prior clonidine use would impair endurance exercise performance in hypoxia. Despite an attenuated sympathetic response to hypoxia, endurance exercise performance was not impaired with prior clonidine administration. Clonidine may be an acceptable strategy to promote metabolic health in high-altitude but is not suitable for active military deployments.

In contrast to sympathetic inhibition, an insulin sensitizing pharmaceutical without known cardio-pulmonary consequences may be an alternative strategy to promote endurance exercise performance in high-altitude. Therefore, the final hypothesis was prior/concurrent metformin administration would attenuate the hypoxia-mediated decrement in endurance exercise performance. Preliminary data suggest metformin may promote endurance exercise performance in hypoxia compared with placebo, and this effect is potentially mediated by improved glucose handling and augmented activation of skeletal muscle glycogen synthase. Metformin is a feasible strategy for military personnel to improve metabolic health and endurance exercise performance in high-altitude.

The experiments included in this body of work highlight novel applications of established pharmaceuticals to promote human physiological function in high-altitude. However, there are

general limitations of these investigations that need to be addressed. First, the experimental designs were developed to ascertain the effect of short-term administration of the various pharmaceuticals on endurance exercise performance. Our results do not provide information regarding the effect of long-term administration on human performance. Related, given adequate time to acclimatize, human function is partially restored in high-altitude. It is possible that these pharmacological treatments could inhibit the natural adaptations to hypobaria that occur with chronic exposure. Describing the effect of longer duration administration of these pharmaceuticals and their impact on endurance exercise performance is a direction of future work pertinent to the goal of promoting human function in high-altitude. Second, only males were recruited for these experiments. While we have no reason to suggest these data are not applicable to females, future experiments should include female participants to determine if our findings are sex-specific. Finally, military personnel must maintain cognitive function to make combat related decisions. Our data pertain to the performance of stationary cycle ergometer exercise and do not provide information regarding the impact of Aminophylline, Neptazane, clonidine, or metformin on cognitive proficiency in high-altitude. Experiments designed to determine the impact of these pharmacological treatments are necessary to illustrate their effect on cognitive function. The data presented in this body of work establish novel strategies, applicable to the military population, to promote rapid acclimatization at high-altitude, and identify important new directions for future experiments.

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APPENDIX I – CURRICULUM VITAE

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Education

Ph.D. Human Bioenergetics	2014 (December)	Colorado State University
M.S. Exercise Physiology	2007	Ball State University
B.S. Exercise Science	2005	University of Kansas

Professional Experience

2010-present, Graduate Research Assistant – Integrative Biology Laboratory, Colorado State University, Fort Collins, CO

2007-2010, Exercise Physiologist – Inpatient and Outpatient Cardiopulmonary Rehabilitation, Vanderbilt University Medical Center, Nashville, TN

2006-2007, Graduate Assistant – Outpatient Cardiopulmonary Rehabilitation, Ball Memorial Hospital, Muncie, IN

2005-2007, Graduate Research Assistant, Human Performance Laboratory, Ball State University, Muncie, IN

2003-2005, Undergraduate Research Assistant, Energy Balance Laboratory, University of Kansas, Lawrence, KS

Publications

Scalzo RL, Peltonen GL, Binns SE, Klochak AL, Szallar SE, Wood LM, Larson DG, Luckasen GJ, Irwin D, Schroeder T, Hamilton KL, Bell C. Endurance Exercise in Hypoxia Following Carbohydrate Feeding: Do the metabolic benefits of sympathetic inhibition outweigh the cardio-pulmonary encumbrance? *In review: High Altitude Medicine and Biology*.

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Pfeiffer RL, Stevenson TG, Kaminsky LA, Nagelkirk PR. Ratings of perceived exertion and pain between two different strength training modalities. American College of Sports Medicine, 2005, Indianapolis, IN.

Nagelkirk PR, **Pfeiffer RL**, Harber MP, Kaminsky LA. Fibrinolytic responses to resistance training in women. American College of Sports Medicine, 2005, Indianapolis, IN.

Professional Presentations and Invited Lectures

2014, Sprint Interval Training: Experiments in the fast lane. Rocky Mountain American College of Sports Medicine Annual Meeting. Denver, CO.

2012, Sprint Interval Training & Nuclear Factor (Erythroid-Derived 2)-Like 2 (Nrf2) Activator Supplementation. Office of Naval Research. San Diego, CA.

Honors and Awards

2012, Student Research Grant - Rocky Mountain American College of Sports Medicine

2010, University Programs for Research and Scholarly Excellence Fellowship - Colorado State University

2001-2004, Scholar-Athlete – University of Kansas

Professional Memberships

2011- present, American Physiological Society

2006- 2012, American College of Sports Medicine

Certifications

American College of Sports Medicine – Clinical Exercise Specialist

American Heart Association – Advanced Cardiac Life Support