THESIS

SYMPATHETIC INHIBITION ATTENUATES HYPOXIA INDUCED INSULIN RESISTANCE IN HEALTHY ADULT HUMANS

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ABSTRACT

SYMPATHETIC INHIBITION ATTENUATES HYPOXIA INDUCED INSULIN RESISTANCE IN HEALTHY ADULT HUMANS

Acute and chronic exposure to hypoxia is known to decrease insulin sensitivity in healthy humans and animals, while simultaneously increasing the activity of the sympathetic nervous system (SNS). Likewise, obstructive sleep apnea (OSA) and chronic obstructive pulmonary disease (COPD) are clinical conditions characterized by hypoxia, elevated SNS activity, and a high prevalence of insulin resistance. In contrast to hypoxic exposure and hypoxic related diseases, hyperoxia (FIO_2 = 1.00) has been shown to improve insulin sensitivity while concomitantly decreasing SNS activity. Consistent with this, continuous positive airway pressure (CPAP), a common OSA treatment, has proven effective in abolishing nocturnal bouts of intermittent hypoxia, enhancing insulin sensitivity and diminishing SNS activity. Although the underlying mechanism of hypoxia induced insulin resistance remains unclear, it appears that elevated SNS activity may be a mediating factor. Therefore, we hypothesized that inhibition of the SNS would attenuate hypoxia induced insulin resistance. METHODS: 10 males (23±1 years, body mass index 24.2±0.8 kg/m^2 (mean±SE)) reported to our laboratory on 4 separate mornings, separated by a minimum of 7 days, after a 12-hour fast and 48-hour abstention from exercise. Insulin sensitivity was determined via the hyperinsulinemic euglycemic clamp technique under each of the following conditions: normoxia (F_IO_2=0.21), hypoxia (F_IO_2=0.11), normoxia and SNS inhibition (48-hour transdermal clonidine administration (Catapres-TTS; 0.2mg/day)), and hypoxia SNS inhibition. RESULTS: Oxyhemoglobin saturation was decreased (P<0.01) during hypoxia (63±2%) compared to normoxia (96±0%) and there was no significant effect of SNS

inhibition on oxyhemoglobin saturation in either normoxia or hypoxia (P>0.25). Norepinephrine was elevated in hypoxia (137±13%; P=0.02), as determined by area under curve and expressed relative to normoxia. SNS inhibition prevented the hypoxia induced increase in norepinephrine (94±14%; P=0.43). The glucose infusion rate (adjusted for fat free mass and circulating insulin), required to maintain blood glucose at 90 mg/dl (5 mmol/L) during administration of insulin, was decreased during hypoxia (128±30 nmol/kg fat free mass/pmol/L/min; P=0.03) compared to normoxia (225±23) and remained unchanged during normoxia and SNS inhibition (219±19; P=0.86), and hypoxia and SNS inhibition (169±23; P=0.23). CONCLUSION: Inhibition of the SNS attenuates hypoxia induced insulin resistance.

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

Whole body glucose homeostasis is dependent on the rate of glucose appearance (Ra) and rate of glucose disappearance (Rd). Ra is determined by glucose absorption from the intestine and production by the liver and kidneys; whereas Rd is influenced by glucose uptake and metabolism by insulin-dependent tissues and insulin-independent tissues[1,2]. Maintenance of glucose homeostasis is a balancing act achieved by alterations in Ra and Rd, with these processes being tightly regulated by pancreatic hormones and neuroendocrine signaling. During times of hyperglycemia, such as the postprandial state, insulin secretion (from pancreatic beta-cells) coupled with SNS withdrawal, decreases Ra by inhibiting hepatic glucose production and increases Rd by promoting insulin-dependent tissue glucose uptake, storage, and oxidation[2]. On the contrary, during times of hypoglycemia a combination of glucagon release (from pancreatic alpha-cells) and increased SNS activity promotes glucose Ra by stimulating hepatic glucose production and attenuates Rd by inhibiting peripheral glucose utilization[3-8].

Insulin, one of the primary glucoregulatory hormones, promotes glucose homeostasis through a series of successive steps that leads to the translocation of glucose transporter proteins (GLUT 4) to the plasma membrane and subsequent glucose uptake[9]. Primarily found in muscle and adipose tissues, GLUT 4 accounts for a majority of whole body, insulin mediated glucose disposal[10]. GLUT 4 facilitates the cellular uptake of glucose from circulation for oxidation and/or glycogen storage. Cellular oxidation of glucose results in the production of reducing equivalents and adenosine tri-phosphate that can be utilized as an energy source. Glucose can

also be stored as glycogen through a process known as glycogenesis that occurs predominantly in muscle and the liver[1,2,11].

Unfavorable perturbations to either the insulin signaling cascade or GLUT 4 translocation can result in dysregulation of glucose homeostasis and insulin resistance. Insulin resistance is a clinical condition where elevated levels of plasma insulin coincide with higher than normal plasma glucose levels[12,13]. Most commonly, insulin resistance is associated with type 2 diabetes mellitus (T2DM) that is known to have profound metabolic consequences. There are several known factors that can promote the development of insulin resistance such as visceral adiposity, high-fat diets, and inactivity. Additionally, exposure to hypoxia has also been shown to induce insulin resistance. Healthy, adult humans and animals exposed to acute, chronic or intermittent hypoxia, each demonstrate a reduction in insulin sensitivity[3,14-16]. This effect has been replicated under a variety of low oxygen conditions including: high altitude exposure[16], hypoxic gas breathing[3], and hypobaric hypoxia[15]. In addition to inducing insulin resistance, hypoxia has also been associated with increased SNS activity[3,17]. However, exposure to hyperoxia (F_IO_2=1.00), has been shown to have the opposite effect; increasing insulin sensitivity while concomitantly decreasing SNS activity[18].

In parallel, diseases of hypoxia, such as obstructive sleep apnea (OSA) and chronic obstructive pulmonary disease (COPD), have high prevalence of insulin resistance. OSA is a disease distinguished by momentary cessations of breathing (apneas) or decreases in breath amplitude (hypopneas), caused by a compromised upper airway and a decrease in brain stem respiratory motor output, sufficient enough to induce hypoxemia during sleep[19,20]. Likewise, COPD is classified as a hypoxic respiratory disease exemplified by air-flow obstruction resulting from chronic inflammation of the airways due to long-term inhalation of tobacco smoke, noxious

gases, or fine particulates[21]. Despite having slightly different pathologies, OSA and COPD share several of the same attributes including: arterial oxygen desaturation (characteristic of hypoxia), elevated SNS activity, and a reduction in insulin sensitivity[22-28]. Treatments aimed at ameliorating the hypoxic exposure characteristic of both these disease have proven effective[29,30]. Concurrent with eliminating hypoxic exposure, these treatments have favorable effects on insulin sensitivity and SNS activity. Specifically, CPAP, a common OSA treatment, has been effective in abolishing nocturnal bouts of intermittent hypoxia, enhancing insulin sensitivity, and reducing SNS activity[29], while oxygen supplementation in COPD patients has been shown to have similar effects[30].

Although the underlying mechanism of hypoxia induced insulin resistance remains unclear, the aforementioned evidence suggests elevated SNS activity may be a mediator[3]. The potential mechanism by which elevated SNS activity could mediate hypoxia induced insulin resistance is through the actions of epinephrine and norepinephrine. Epinephrine and norepinephrine are catecholamines found in systemic circulation; with elevated levels being indirectly associated with increased SNS activity. Epinephrine has been shown to promote glycogenolysis while concurrently inhibiting glucose uptake, oxidation, and storage[3-7]. Norepinephrine acts in concert with epinephrine to increase lipolysis[6,31]. Plasma lipids interfere with insulin stimulated glucose uptake, leading to a decrease in insulin sensitivity[31,32]. Therefore, targeting the sympathetic nervous system appears to be a potentially viable solution for the treatment of hypoxia induced insulin resistance.

Due to the complexities of human physiology, it appears unlikely, that elevated sympathetic nervous system activity is exclusively responsible for hypoxia induced insulin resistance. Therefore, it is important to consider other known circulating insulin sensitizing and

desensitizing factors that are regulated by hypoxia. Specific insulin (de)sensitizing that are regulated by hypoxia include but are not limited to: adiponectin[33], pigment epithelium derived factor[34], oxidized low density lipoproteins[35], tumor necrosis factor-alpha[36], and non-esterified fatty acids[37]. All have the potential to contribute to hypoxia induced insulin resistance.

There are several practical implications of identifying the mechanism(s) that underlies hypoxia induced insulin resistance. Clinically, effective treatment options for hypoxia related diseases have the potential to reduce the prevalence or development of insulin resistance. Effective treatments aimed at hypoxia will have many positive downstream effects, such as reducing medical costs and increasing quality of life. Additionally, there are many healthy individuals exposed to hypoxia on a regular basis, ranging from those who voluntarily flock to high altitude ski villages and mountain locales to recreate or soldiers that are exposed to on the job altitude extremes. These individuals may also benefit from effective hypoxia therapies that decrease the negative effects of high altitude exposure and have the potential to improve performance. Therefore, we sought to investigate whether inhibition of the sympathetic nervous system will attenuate hypoxia induced insulin resistance.

Statement of Problem

Individuals diagnosed with OSA and COPD are regularly exposed to hypoxia that elicits arterial oxygen desaturation. Additionally, OSA and COPD elevate basal SNS activity and reduced insulin sensitivity. Likewise, healthy individuals exposed to hypoxia also demonstrate elevated SNS activity and reduced insulin sensitivity. Despite this knowledge, the mechanism mediating hypoxia induced insulin resistance remains unclear. Therefore, we hope to elucidate whether elevated SNS activity and/or other circulating insulin (de)sensitizing factors are

mediating this effect. Identification of a mechanism will have clinical implication for individuals with hypoxia related disorders.

Hypothesis and Specific Aims

We hypothesized that inhibition of the sympathetic nervous system would attenuate hypoxia induced insulin resistance. Accordingly, we developed the following specific aim: to determine, in a random order, insulin sensitivity via the hyperinsulinemic euglycemic clamp technique during four conditions: normoxia (F_IO_2=0.21), hypoxia (F_IO_2=0.11), normoxia and SNS inhibition (48-hour transdermal clonidine administration (Catapres-TTS; 0.2mg/day)), and hypoxia and SNS inhibition.

Additionally, it appears unlikely that elevated SNS activity is exclusively responsible for hypoxia induced insulin resistance. Therefore, we sought to gain insight into whether other known circulating insulin sensitizing and desensitizing factors are regulated by hypoxia including: adiponectin, pigment epithelium derived factor, oxidized low density lipoproteins, tumor necrosis factor-alpha, and non-esterified fatty acid

CHAPTER II

LITERATURE REVIEW

Insulin Signaling

The insulin signaling pathway is of pivotal importance for the maintenance of whole body glucose homeostasis. Insulin is one of the primary hormones responsible for initiation of the insulin signaling cascade, that leads to the partial regulation of Ra and Rd through various intermediates[9]. Insulin receptors are found on nearly all vertebrate cells in varying densities; ranging from 40 – 200,000 receptors on a single cell[9]. The receptor is a large trans-membrane protein, 300-400 kDa in size, comprised of two alpha and two beta-subunits[38]. The two alpha-subunits, located on the extracellular surface of the cell, serve as the insulin binding domain while the beta-subunits, located on the intracellular surface of the cell, contain the insulin regulated tyrosine protein kinase[9].

When insulin is not present, the unbound extracellular alpha-subunits inhibit the intrinsic intracellular tyrosine kinase activity of the beta-subunits. However, when insulin binds to one of the alpha-subunits, this inhibition is removed, and the adjacent intracellular beta-subunit is autophosphorylated at its tyrosine residues of the regulatory domain[39]. Subsequently, autophosphorylation allows the insulin receptor to activate, via tyrosine phosphorylation, a variety of intracellular insulin receptor substrate proteins (IRS)[38,40-43]. These IRS proteins act as insulin receptor specific docking proteins that create recognition sites for additional effector molecules with Src homology 2 (SH2) domains, such as the p85 regulatory subunit of the type 1A phosphatidylinositol 3–kinase (PI(3)K)[2,38]. These IRS docking proteins serve multiple functions. They have the ability to amplify the insulin receptor signal, dissociate the intracellular

signaling cascade with the membrane bound insulin receptor, expand the number of pathways that can be regulated through one insulin receptor, and serve as a common substrate for multiple receptors, allowing the integration of multiple metabolic signals[40,44].

The insulin signaling cascade encompasses 40 known downstream intermediates[45] that involves three primary pathways: the PI(3)K/Akt pathway, the Cbl/CAP pathway, and the mitogen activated protein kinase (MAPK) cascade[2]. The PI(3)K pathway appears to play the biggest role in the maintenance of whole body glucose homeostasis. However, the MAPK and Cbl/CAP pathways are also of integral importance for the maintenance of glucose homeostasis.

PI(3)K plays a significant role in many of the metabolic processes associated with insulin including: glucose uptake, growth, protein synthesis, and glycogen synthesis[40]. Insulin stimulates glucose uptake via the PI(3)K pathway through multiple steps including: activation of IRS by the insulin receptor, binding of IRS to the PI(3)K regulatory subunit p85[46,47], phosphorylation of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) leading to increased concentrations of phoshphatidlyinositol 3,4,5-trisphosphate (PIP_3), activation of atypical protein kinase C (aPKC) and Akt through phosphoinositide-dependent kinase-1(PDK1), and GLUT 4 translocation to the plasma membrane for facilitation in cellular glucose uptake [48,49]. The activation of both aPKC and Akt has been shown to be required for the translocation of GLUT 4 to the plasma membrane[49-51]. Therefore, it is not surprising that insulin resistance is often associated with dysregulation of aPKC and Akt activation[49]. The dysregulation of aPKC and Akt in insulin resistance is supported by evidence from embryonic stem cell aPKC knockouts and adipocyte aPKC knockouts that demonstrate impaired insulin stimulated glucose uptake[52]. Likewise, Akt2 deficient mice have also been shown to become insulin resistant as a result of attenuated GLUT 4 translocation[53].

In addition to the PI(3)K/Akt pathway, the mitogen activated protein kinase (MAPK) cascade plays a substantive role in the maintenance of glucose homeostasis. Insulin stimulates MAPK activity by activating GTP-binding proteins (G-proteins) that transduce the signal through a complex series of kinase tiers[54]. There are four main variants of the MAPK signaling cascade that arise as the signal navigates through these kinase tiers that including: ERK, JNK, p38, and BMK signaling pathways[54]. The ERK cascade has been implicated in the regulation of insulin sensitivity through transcriptional control of the insulin-like receptor gene, "pointed"[55]. Similarly, activation of the p38 pathway has been shown to positively affect insulin sensitivity through insulin mediated glucose uptake but not through contraction mediated glucose uptake[56], while BMK has been shown to be attenuated with insulin resistance[57]. Contrary to the positive effects of the ERK, p38, and BMK pathways; the JNK pathway is elevated in obesity, while ablation of JNK1 mediates an increase in insulin sensitivity[58]. Thus, the MAPK signaling cascade can have a range of effects on glucose homeostasis.

In addition to the PI(3)K and MAPK pathways, the Cbl/CAP pathway also contributes to the maintenance of glucose homeostasis[59,60]. The Cbl/CAP pathway is mediated by two adaptor molecules known as CAP and APS; with Cbl and CAP forming the Cbl/CAP complex[61]. The insulin receptor activates APS, APS recruits cytoplasmic Cbl/CAP to the insulin receptor, Cbl is phosphorylated, the Cbl/CAP complex relocates to the caveolin-enriched lipid rafts of the plasma membrane, the CAP protein interacts with the lipid raft[62] leading to the recruitment of the Crk/C3G complex, C3G activates TC10, and GLUT 4 is translocated to the plasma membrane for the facilitation of cellular glucose uptake[63]. Evidence for the role of the Cbl/CAP pathway in the maintenance of glucose homeostasis comes from an obese mouse model. This obese mouse model has suggested that the decrease in insulin sensitivity associated with excess adiposity is

partially due to the down regulation of the Cbl/CAP pathway[64]. Like the previous pathways there is direct link between activation of the Cbl/CAP pathway and cellular glucose uptake, with evidence to support this. A summary of insulin signal transduction can be found in Figure 1.

The insulin receptor also has the important task of degrading insulin. After insulin has bound the insulin receptor, the receptor and substrate undergo endocytosis[9,65-67]. Upon internalization, insulin is cleaved from the insulin receptor and degraded by a protease while the unbound insulin receptor is either degraded or recycled back to the plasma membrane[9,68]. Traditionally, endocytosis has been thought of as a degradation pathway, however it is also acknowledged that endocytosis may be necessary for intracellular propagation of insulin action[69,70]. Either way, this process occurs rather frequently indicative of the relatively short, 30 minute half-life of insulin; whereas the insulin receptor is often recycled, giving it a longer half-life of approximately 10 hours[71,72]. Interestingly, the half-life of the insulin receptor tends to decline during prolonged insulin exposure through the increased activity of endocytotic lysosomal degradation[53,73]. Decreased insulin receptor concentrations have been associated with an attenuated tissue responsiveness to insulin and have been shown to contribute to the pathogenesis of insulin resistance[70].

Additionally, serine phosphorylation and protein tyrosine phosphatases (PTP's) have been shown to attenuate insulin signaling. Specifically, serine phosphorylation disrupts insulin signaling through the inhibition of tyrosine phosphorylation by fostering interactions with 14-3-3 proteins[74,75]. Likewise, PTP's can interfere with the tyrosine phosphorylation cascade essential for propagation of the insulin signal, resulting in a decreased insulin response[76].

GLUT 4 Proteins

The primary objective of the insulin signaling cascade, for the purposes of whole body glucose homeostasis, is to promote facilitative cellular glucose uptake through the translocation of GLUT 4 proteins to the plasma membrane. GLUT 4 proteins, predominantly found in muscle tissue account for a majority of whole body, insulin mediated glucose disposal. The importance of GLUT 4 is evident by the fact that: insulin-mediated glucose uptake (GLUT 4) is the rate limiting step for glucose absorption in muscle and adipose[10], heterozygous GLUT 4 knockout mice become insulin resistance[70], GLUT 4 translocation to the plasma membrane is severely disrupted in insulin resistance and T2DM[77], inadequate GLUT 4 recruitment – despite adequate expression – is associated with insulin resistance[66], and GLUT 4 over expression in diabetic mice (db/db) attenuates insulin resistance[65].

Despite the importance of GLUT 4 in the regulation of whole body glucose homeostasis, the exact mechanisms of the insulin mediated GLUT 4 translocation pathway and the intracellular sequestering locations of GLUT 4 remain elusive. Similarly, there has yet to be complete convergence between the insulin signaling pathway and subsequent activation of GLUT 4 translocation. The GLUT 4 protein itself can be found in many organelles of the cell including: the plasma membrane, sorting endosomes, recycling endosomes, the trans-Golgi network (TGN), and transport vesicles[10]. Compared to other common constitutively exocytosed proteins, the rate of non-insulin stimulated GLUT 4 exocytosis is much slower, indicating that GLUT 4 exocytosis must be restrained in some manner[10]. It is thought that GLUT 4 is either retained in an intracellular location, compartmentalized in vesicles that remain static in the absence of stimulation, involved in a dynamic intracellular transport loop that circumnavigates recycling endosomal vesicles, or a combination "multiple pools" of the three[10,46]. In support of

the "multiple pools" hypothesis, it has been demonstrated that under basal conditions only a portion, approximately 30 - 40%, of GLUT 4 proteins are localized to recycling endosomes[67] as GLUT 4 translocation occurs despite chemical ablation of these endosomes[47]. There is also evidence to support a dynamic transport loop between GLUT 4 containing endosomes and the TGN. When GLUT 4 is tracked upon endocytosis from the plasma membrane of adipocytes, it tends to localize in the vicinity of the TGN[74]. Similarly, when adipocytes are incubated 19° Celsius, the temperature that inhibits export from the TGN, insulin action is inhibited[10,75]. Additionally, in response to insulin, GLUT 4 is not only localized to the plasma membrane by TGN sorting, but also by small GLUT 4 containing vesicles. These special GLUT 4 sequestering vesicles are recognized by the presence of VAMP2, a docking and fusing protein that enables GLUT 4 to be incorporated into the plasma membrane[78]. Based upon this evidence, it appears that GLUT 4 proteins are sequestered in "multiple pools" awaiting the proper signal for translocation to the plasma membrane.

Although the insulin signal serves as a potent activator of GLUT 4 translocation to the plasma membrane, there are three unique PIP_3 phosphatases, PTEN, SHIP2, and SKIP, that each play a role in attenuating Akt activation and inhibition of GLUT 4 translocation[79]. PTEN over expression has been shown to inhibit GLUT 4 translocation and glucose uptake, while PTEN and SKIP knockout mice demonstrate insulin hypersensitivity[10,51,77]. Similarly, the phosphatase SHIP2 has also been shown to attenuate insulin stimulated glucose uptake [50]. Although each of these phosphatases negatively influence insulin sensitivity through inhibition of GLUT 4 translocation, SKIP plays the predominant role[79].

In addition to GLUT 4 being regulated prior to or during translocation, it can also be regulated after translocation. Glucose transporter activity, at the membrane, has been shown to

be altered by phosphorylation[76], nucleotide binding[80] and through variations in its oligomeric structure[81]. Analogously, the location of GLUT 4 within the membrane may also alter its ability to transport glucose as the receptor has been detected in both clathrin coated pits and the caveolae of the plasma membranes[10,82,83]. Ultimately, there are various points throughout the insulin signaling cascade and translocation of GLUT 4 that glucose uptake can be regulated.

Insulin Resistance

The potency of insulin to promote peripheral insulin dependent tissue glucose uptake is termed insulin sensitivity. Insulin sensitivity describes the effectiveness of insulin to stimulate the removal of glucose from plasma and subsequent uptake into myocytes, adipocytes, or hepatocytes for oxidation or storage[84]. Alternatively, a poor degree of insulin sensitivity is termed insulin resistance. Insulin resistance is characterized by elevated levels of plasma insulin results in higher than normal plasma glucose levels[12,13]. The development of insulin resistance initially leads to compensatory hyperinsulinemia. This is maintained, often for years, until dysfunction of pancreatic beta-cells results in an inability to compensate for peripheral tissue (muscle, adipose, and liver) insulin resistance – unable to produce enough insulin – and hyperglycemia manifests[12].

The first stage of hyperglycemia is known as pre-diabetes. For healthy individuals, normal fasting (12 hour fast) plasma glucose levels are less than 100 mg/dl (5.6 mmol/l), whereas pre-diabetes fasting plasma glucose concentrations range from 100–125 mg/dl (5.6–6.9 mmol/l). Comparably, a normal blood glucose level, two hour post an oral glucose tolerance test (ingestion of 75 grams of dissolved anhydrous glucose in water) is less than 140 mg/dl (7.8 mmol/l), while in pre-diabetes it ranges from 140–199 mg/dl (7.8–11.1 mmol/l)[85]. If pre-diabetic

hyperglycemia is exacerbated, by additional peripheral insulin resistance, pancreatic beta-cell dysfunction, and/or increased hepatic gluconeogenesis, the consequence is T2DM[12,86]. Clinically, the diagnostic criteria for the development of T2DM includes: a fasting plasma glucose level >126 mg/dl, or symptoms of diabetes and a casual plasma glucose concentration of >200 mg/dl or a plasma glucose concentration of >200 mg/dl two hours post an oral glucose tolerance test. After one positive test, a second test on a different day is often needed to confirm the diagnosis[85]. Likewise, individuals with undiagnosed T2DM may have a variety of symptoms including: frequent urination, extreme thirst, unusual hunger, fatigue, irritability, frequent infections, blurred vision, wounds and bruises that are slow to heal, tingling and numbness in the hands and feet, and recurring skin, gum, and bladder infections[87]. It is important to note that pre-diabetes, in and of itself, is not a diagnosis of T2DM but merely a risk factor for future development of the disease.

According to the 2011 Diabetes National Fact Sheet, 8.3% of the population or 25.8 million United States citizens are afflicted with diabetes, with another 79 million people being classified as pre-diabetic[88]. It has been estimated that for individuals born in the year 2000 the life-time risk of developing diabetes (predominantly T2DM) is as follows: 32.8% for Caucasian males, 38.5% for Caucasian females, 45.4% for Hispanic males, and 52.5% for Hispanic females[89]. This is important for several reasons: individuals diagnosed at the age of forty will lose approximately 13 life-years and 20 quality-of-life adjusted years[89] and have twice the risk of dying versus individuals the same age without diabetes[88]. In 2006, diabetes was the seventh leading cause of death and is associated with: heart disease, stroke, high blood pressure, blindness, kidney disease, neuropathy, and amputation. It is the leading cause of new cases of blindness and kidney failure.

Not only is T2DM detrimental to health but the economic costs associated with the disease are astronomical. It has been estimated that in 2010 the cost associated with diabetes was approximately $174 billion dollars, excluding the cost of the 79 million individuals who have pre-diabetes[87]. Thus, the development of successful preventative and treatment options for this devastating disease of insulin resistance should improve quality of life, decrease mortality, and drastically reduce associated medical costs.

Hypoxia Induced Insulin Resistance

The acute and chronic effects of exposure to conditions of low oxygen have been thoroughly documented in both animal and human models. It has been demonstrated in both models that low oxygen environments can induce insulin resistance that is characteristic of T2DM. In newborn calves, two hours of hypoxic exposure have been shown to increase levels of circulating glucose and insulin[90]. Likewise, in rats, seven days of hypoxic exposure results in increased levels of plasma insulin with no change in plasma glucose[91], while in obese mice, long-term (12 weeks) exposure to intermittent hypoxia leads to an increase in serum insulin and decrease in glucose tolerance, indicative of insulin resistance[14]. Equally, in humans acute and chronic hypoxic exposure has been shown to attenuate insulin sensitivity independent of sex[3,15,16]. Therefore, it appears that hypoxia can induce a phenotype of insulin resistance similar to that of pre-diabetes or T2DM.

It was not until the early 1990's that the study of glucose homeostasis in response to acute and chronic hypoxia in humans began to gain momentum. Initially, it was postulated that insulin sensitivity was improved in response to acute and chronic hypoxia while others believed the opposite to be true. In one study, examining glucose kinetics during a stay at 4300 meters,

with stable isotope infusion, it was determined that chronic altitude exposure improves insulin sensitivity during energy balance[92]. This was based on the determination that chronic altitude decreased fasting plasma glucose concentrations by14%, while both glucose Ra and Rd doubled compared to sea-level values despite there being no difference in plasma insulin concentrations. Additionally, chronic altitude was associated with a 153% increase in epinephrine and 136% increase in norepinephrine compared to sea-level values[92].

It appears, based upon this data, that chronic altitude improves insulin sensitivity. Despite unchanging insulin concentrations there was a decrease in fasting plasma glucose values, while Rd increased drastically compared to sea-level values. Both are indicative of improved insulin action[92]. However, it is important to note that in this study, insulin sensitivity was not measured directly via the euglycemic hyperinsulinemic clamp technique, often considered to be the gold-standard of measuring insulin sensitivity[93]. Instead, insulin action was derived from plasma glucose concentrations, glucose Ra and Rd, and levels of circulating insulin.

Around the same time period, another group showed the insulin desensitizing effects chronic hypoxic exposure to varying altitude. In this study, a group of 15 healthy men were studied at sea-level, transferred to 3500 meters for a 4 week stay, followed by a stay at 5080 meters for additional 2 weeks[94]. Upon exposure to 3500 meters there was an increase in fasting blood glucose and insulin concentrations on day 3, with insulin levels remaining elevated on day 7 of exposure, compared to sea-level values[94]. Likewise, with increasing altitude severity (5080 meters), there was an increase in plasma glucose concentrations, that was apparent for 41 days, while insulin levels remained similar to that of exposure to 3500 meters[94]. These data, contrary to the aforementioned glucose kinetics study, suggest that hypoxia exposure induces insulin resistance as evident by elevated plasma glucose concentrations in spite of elevated plasma

insulin concentrations. Ultimately, the results of these two similar, yet conflicting, altitude studies paved the way for future investigations into the effects of hypoxia on glucose homeostasis.

With these prior altitude studies in mind, another group set out to directly test the effects of altitude acclimatization upon glucose homeostasis. In order to address altitude acclimatization upon glucose homeostasis, eight healthy men were examined on three different occasions: sea level, day 2 of altitude exposure (4559 meters), and day 7 of altitude exposure (4559 meters)[16]. During these three separate occasions, measures of insulin sensitivity were carried out via the two-step hyperinsulinemic euglycemic clamp technique. Upon comparison of glucose infusion rates across the three different time points, it was determined that insulin stimulated glucose uptake was reduced by half during day 2 of altitude exposure (4.5±0.6 mg/kg/min), compared to sea-level (9.8±1.1), while it was partially restored by day 7 of altitude exposure (7.4±1.4)[16]. Meanwhile, hepatic insulin sensitivity remained unchanged across all conditions[16]. The largest reduction in glucose tolerance was found during day 2 which coincided with peak acute mountain sickness values[16]. This decrease in insulin sensitivity, on day 2, was rationalized to result from a significant increase in cortisol and norepinephrine during the same time period. Subsequent partial recovery of insulin sensitivity on day 7 was attributed to decreasing cortisol concentrations that returned to sea-level values and chronic elevated norepinephrine exposure throughout the stay at altitude. The partial recovery of insulin sensitivity as a result of decreasing cortisol concentrations is line with previous research that has implicated cortisol in induction of insulin resistance[95] through its role in decreasing the activity of glycogen synthase in skeletal muscle[96]. Likewise, norepinephrine had been shown to acutely decrease insulin sensitivity[97] while chronic norepinephrine exposure, such as day 7 of altitude exposure, has been

demonstrated to illicit an increase in insulin sensitivity[98]. In essence, this was the first study to directly demonstrate that hypoxia induces insulin resistance in man.

Despite the characterization of the effects of chronic (2-7 days) altitude exposure and glucose homeostasis in men, little was known about the acute effects of altitude exposure on glucose homeostasis. To answer this question, a group of researchers sought to determine if dysregulation of glucose homeostasis occurs in healthy individuals exposed to acute, normobaric hypoxia[3]. In a double-blind, within subject, cross-over design study, fourteen healthy males were exposed to either normoxia or a brief 30 minute (acute exposure) bout of hypoxia as insulin sensitivity was measured with the hyperinsulinemic euglycemic clamp technique. The severity of hypoxia introduced during the hypoxic intervention was sufficient to decrease arterial oxygen saturation from 96% to 75%. It was determined that hypoxia, compared to normoxia, induced a significant decrease in glucose infusion rates; that gradually began after administration of hypoxia, reached its peak 20 minutes after hypoxic exposure ended, and did not improve for 150 minutes after cessation of hypoxic exposure. Additionally, there was evidence of increased SNS activity (elevated plasma epinephrine concentrations) that coincided with the hypoxia induced insulin resistance.

Subsequently, to determine if the hypoxic exposure itself caused the observed decrease in glucose tolerance or if the hypoxia induced rise in epinephrine caused the glucose intolerance, a non-specific control was performed[3]. For the non-specific control, glucose infusion rates were compared during conditions of hypoxia to that of hypoglycemia. Previously, it had been shown that hypoglycemic induced insulin resistance is due to elevated SNS activity and is reversible through beta-adrenergic receptor antagonism[99]. Therefore, if SNS activity is greater during conditions of hypoglycemia compared to hypoxia, and epinephrine is the sole mediator of the

observed hypoxia induced insulin resistance, there should be increased insulin resistance during hypoglycemia compared to hypoxia. Despite there being significantly greater concentrations of plasma catecholamines in the hypoglycemic group, both groups displayed similar levels of glucose intolerance. Therefore, this suggested that the decrease in glucose tolerance associated with acute hypoxia is only partially mediated by increased epinephrine levels[3]. Aside from epinephrine, other mechanisms mediating glucose intolerance were proposed, such as ATP-sensitive potassium (K^+) channels that are found in both skeletal muscle and the medio-basal hypothalamus. K^+ channels have been shown to act in concordance with epinephrine to decrease skeletal muscle glucose uptake and increase hepatic glucose output and could potentially explain the decrease in insulin sensitivity observed[100,101]. Another possibility is that hypoxia could have raised plasma catecholamines enough to reach K_{max}, resulting in no further decline in insulin sensitivity despite an even greater concentration of plasma catecholamines. Either way, this study was the first to suggest that acute hypoxia induces insulin resistance that is, at least in part, mediated through an increase in plasma catecholamines.

In addition to acute and chronic hypoxia having the ability to attenuate insulin sensitivity, intermittent hypoxia has been shown to have a similar effect[102]. In a study that examined the consequences of intermittent hypoxia, healthy individuals were blindly exposed to short, repeated bouts of hypoxia over the course of eight hours. Each individual was subject to 24 hypoxic events per hour, with each event lasting long enough to significantly decrease arterial oxygen saturation[102]. During the final three hours, of the eight hour trial, subjects were administered an intravenous glucose tolerance test (IVGTT). From the IVGTT and additional blood sampling, it was determined that intermittent hypoxia induced a 17% decrease in insulin sensitivity that was associated with an increase in sympathetic nervous system activity[102].

The majority of studies have investigated hypoxia induced insulin resistance specifically in males. The reason, often cited, for excluding females in such studies is that the hormonal fluctuations stemming from their menstrual cycle confounds the result of the study with additional variables. Despite the potential confounding variables of researching females, a study was conducted that shows similar levels of glucose intolerance in females exposed to conditions of low oxygen as compared to males[15]. In this investigation, twelve healthy females were subject to 68 hours of normobaric hypoxia, followed immediately by 52 hours of hypobaric hypoxia; once with and once without alpha-1-adrenergic receptor antagonism. After 16 hours of either normobaric or hypobaric hypoxia, with and without alpha-1-adrenergic receptor antagonism, insulin sensitivity was determined by the Homeostatic Model Assessment-Insulin Resistance (HOMA-IR) and the Composite Model Insulin Sensitivity Index (C-ISI), while pancreatic beta cell function was determined by the Homeostatic Model Assessment-Beta Cell Function (HOMA-BCF)[15]. Both measures have been shown to correlate well with euglycemic and hyperglycemic clamp techniques[103]. Based upon these measures, it was established that acute hypobaric hypoxia induces insulin resistance and decreases insulin sensitivity, with alpha-1-adrenergic antagonism having no effect on insulin sensitivity during hypobaria. Furthermore, hypobaria was shown to elevate plasma epinephrine concentrations, compared to normobaric conditions[15]. Despite the concurrent decrease in insulin sensitivity and rise in epinephrine concentrations, it was concluded that epinephrine was not mediating the decrease in insulin sensitivity as alpha-1-adrenergic antagonism had no effect on insulin sensitivity[15]. However, alpha-1-adrenergic receptor antagonism is unlikely to influence sympathetic stimulation of glycogenolysis, hepatic glucose production, or inhibition of insulin release as these are primarily beta-adrenergic receptor and alpha-2-adrenergic mediated functions[104,105]. Therefore, these

results reinforce the findings of other studies conducted in men, indicating that hypoxic exposure induces a decrease in insulin sensitivity in females that may be mediated by increased sympathetic nerve activity.

Both acute and chronic hypoxic exposure attenuates insulin sensitivity. Thus, the question arises as to whether hyperoxia may have the opposite effect on insulin sensitivity. There is evidence that suggests hyperoxia will improve insulin sensitivity. This is based upon how the carotid body, a peripheral chemoreceptor, responds to varying arterial oxygen saturation. The carotid bodies are responsible for the activation of sensory afferent neurons as a result of a hypoxia induced decrease in arterial oxygen saturation[106]. Subsequently, there is increased sympathetic outflow, due to carotid body activation, that increases cardiac output in an attempt to maintain tissue oxygen delivery despite decreased arterial oxygen saturation. Likewise, hypoglycemia can mediate the same afferent carotid body response that is present in low oxygen environments, increasing sympathetic outflow[107,108] and inducing insulin resistance[109].

If insulin desensitization is mediated by increased sympathetic outflow that is ultimately controlled by the carotid bodies, it is reasonable to see why there is insulin desensitization under conditions of either hypoxia or hypoglycemia[3]. Likewise, if hyperoxic exposure desensitizes the carotid bodies then it would be expected that under conditions of hyperoxic hypoglycemia, sympathetic outflow would be reduced and there would be an attenuation of hypoglycemic induced insulin resistance. To investigate the effects of hyperoxia on insulin dependent glucose uptake, insulin sensitivity was determined via the hyperinsulinemic euglycemic clamp technique in seven healthy adults under conditions of normoxic hypoglycemia (F_IO_2=0.21; 60 mg/dl or 3.3 mmol/l) and hyperoxic hypoglycemia (F_IO_2=1.00; 60 mg/dl or 3.3 mmol/l)[110]. Insulin sensitivity

was found to be 44.2% greater during hyperoxic hypoglycemia compared to normoxic hypoglycemia[110]. In addition, the areas under the epinephrine and norepinephrine curves were decreased 62.6% and 50.7% during hyperoxic hypoglycemia compared to normoxic hypoglycemia[110]. The finding that hyperoxia improves insulin sensitivity and decreases plasma catecholamines are consistent with the idea that hyperoxia suppresses hypoglycemic carotid chemoreceptor activation, preventing the hypoglycemic induced increase in SNS activity and rescuing insulin sensitivity. Therefore, it appears that low oxygen environments stimulate carotid body afferent activity, increase sympathetic outflow, and induce insulin resistance. On the other hand, hyperoxic environments have the opposite effect through desensitization of the carotid body, decreasing sympathetic outflow that mediates an increase in insulin sensitivity.

Despite evidence that hypoxia induced insulin resistance is facilitated by increased sympathetic activation, there have been several investigators that have questioned this theory. For example, in mice exposed to 9 hours of intermittent hypoxia (F_IO_2=5–6% at 60 cycles/hour), with and without the ganglionic autonomic blocking agent hexamethonium, insulin sensitivity (hyperinsulinemic euglycemic clamp) was decreased 21% in response to hypoxia compared to normoxia. Additionally, autonomic blockade was unable to restore insulin sensitivity during hypoxia[111]. However, these results must be considered with caution as there are several limitations to the study. It appears that the dose of hexamethonium was insufficient as autonomic blockade was unable to prevent the transient reflex bradycardia associated with phenylephrine infusion[111]. Without complete autonomic blockade it is difficult to speculate on the contribution of autonomic activity to hypoxia induced insulin resistance.

Furthermore, the effect of blocking both arms of the autonomic nervous system, sympathetic and parasympathetic, may have differing consequences when compared to blocking

each arm individually. Sympathetic mediated increases in epinephrine have been shown to promote glycogenolysis while concurrently inhibiting glucose uptake, oxidation, and storage[3-7]. Likewise sympathetic mediated increases in norepinephrine is known to increase circulating concentration of NEFAs [6,31], that interfere with insulin stimulated glucose uptake, leading to a decrease in insulin sensitivity[31,32]. However, the parasympathetic system is also required for the maintenance of insulin sensitivity and therefore, appears to play a role in mediating insulin stimulated glucose uptake in hypoxia[112-114]. Specifically, the parasympathetic nervous system is responsible for the release of hepatic insulin sensitizing substance (HISS) that accounts for approximately 56% of insulin stimulated glucose uptake in muscle[115]. Although it is known that hypoxia induces an increase in sympathetic tone and a decrease in parasympathetic tone[116], the decreased but intact parasympathetic tone during hypoxia exposure may have a significant effect on insulin sensitivity. Thus, by blocking both arms of the autonomic nervous system it is impossible to conclude the extent that the sympathetic arm of the autonomic nervous system contributes to hypoxia induced insulin resistance.

In another mouse model suggesting improved glucose tolerance in response to hypoxia, the effect of intermittent hypoxia (F_IO_2=0.049; 30 seconds/min for 16 hours/day) on glucose tolerance was examined in the presence and absence of leptin. In lean mice, it was shown that acute intermittent hypoxia decreased fasting glucose levels by 20%, improved glucose tolerance, and up regulated leptin mRNA expression compared normoxic conditions. On the contrary, intermittent hypoxia in leptin deficient mice caused a 607% increase in circulating insulin levels that coincided with a large reduction in glucose tolerance when compared to normoxic conditions. This hypoxia induced increase in circulating insulin however, was completely abolished with leptin infusion prior to hypoxia[14]. The results of this study suggest that leptin

deficiency is responsible for hypoxia induced insulin resistance. Unfortunately, the lean mice exposed to hypoxia demonstrated significant weight loss during the course of the study, whereas the obese mice maintained their weight. Therefore, the improved insulin sensitivity in the lean mice was, most likely, the result of a decrease in body weight (negative energy balance) and not due to the up regulation of leptin as a consequence of intermittent hypoxic exposure[117]. Equivocally, the obese leptin deficient mouse is an inadequate model of human obesity as a majority of obese humans are hyperleptinemic as compared to the leptin deficient model[118].

In addition to utilizing mouse models, there has been further evidence in healthy and T2DM humans that suggests acute hypoxia improves glucose tolerance[119,120]. In one study, 8 healthy humans were exposed to either normoxia (362 meters) or hypoxia in an altitude chamber (4300 meters). During each trial, subjects performed an Oral Glucose Tolerance Test (OGTT). Briefly, a 75-gram sucrose solution was ingested followed by venous blood sampling at 0, 30, 60, 90, and 120 minutes. Compared to normoxia, the area under the hypoxia glucose response curve was significantly reduced suggestive of improved glucose tolerance. However, this was not due to improved insulin sensitivity as determined by HOMA-IR[119]. It is important to note that an OGTT is not a direct measure of insulin sensitivity and a definite conclusion regarding insulin sensitivity cannot be drawn from it. Likewise, the extended duration between venous blood sampling, especially during the first 60 minutes of the OGTT, could have masked differences between the two trials.

In an additional study, examining acute hypoxia in T2DM, it was found that hypoxia increased insulin sensitivity (two-compartmental minimal model analysis), while the insulin response to a glucose load during hypoxia was 22% less than compared to normoxic conditions[120]. Like in the previous OGTT study, insulin sensitivity was not directly measured by

the hyperinsulinemic euglycemic clamp technique, but was derived from a mathematical model of plasma insulin and glucose during a 4-hour intravenous glucose tolerance test (IVGTT). Thus, definitive conclusions cannot be made about insulin sensitivity. Importantly, the level of arterial oxygen desaturation achieved in this study (S_pO_2=92% vs. 98%), may not have been enough of a stimulus to elicit a sufficient sympathetic response to attenuate insulin sensitivity. Furthermore, analysis of blood glucose and insulin values took place post hypoxic exposure, during the 4-hour IVGTT. The minimal hypoxic challenge that was removed upon commencement of the IVGTT, likely had no effect on the IVGTT. Thus, insulin sensitivity in response to acute hypoxia in T2DM was not determined.

Overall, there seems to be conflicting findings as to whether hypoxia induces insulin resistance. Several of the studies that show hypoxia decreases insulin resistance, lack direct measures of insulin sensitivity or are limited by other methodological concerns such as body mass variability, inadequate measures, and an insufficient hypoxic stimulus[14,111,120]. On the contrary, several of the studies that suggest hypoxia induces insulin resistance, directly measure insulin sensitivity and limit other confounding variable such as body mass variability[3,15,16]. From the studies that have demonstrated hypoxia induced insulin resistance it appears that increased sympathetic activity is a mediating effect[3].

Hypoxia Induced Insulin Resistance: The Sympathoadrenal System and Hypothalamic-Pituitary-Adrenal Axis

In response to stress, such as hypoxia, there is increased activity of the two major neurohormonal stress systems: the sympathoadrenal system or sympathetic nervous system and the hypothalamic-pituitary-adrenal axis[121,122]. Primarily, these systems are activated under

conditions of stress in order to maintain systemic homeostasis. Initially, activation of these two stress systems appears to be advantageous however, sustained activation of either can have prodigious metabolic consequences.

The activity of the sympathoadrenal system, in response to a hypoxic stress can be measured either directly or indirectly[123,124] . Microneurography is a direct measuring technique that identifies SNS activity at the nerve by quantifying "bursts" of efferent nerve firing that originate from the hypothalamus. Commonly, the peroneal or anterior tibial nerve is the site of measurement for this procedure[125]. Despite being an extremely accurate way to measure sympathetic outflow, microneurography is an invasive, tedious, and time consuming procedure. In contrast, indirect measures of SNS activity employ the use of plasma or urinary catecholamines, heart rate variability, blood pressure, or a combination of the three as indicators of sympathetic outflow. Unlike measuring SNS activity directly, these measures are less invasive, faster, and tend correlate well with microneurography[125-128]. Therefore, the term "elevated sympathetic nerve activity" is often used interchangeably with "elevated levels of catecholamines." These two responses are interrelated as SNS activity mediates an increase in catecholamines.

Stress, such as conditions of low oxygen, has the ability to increase the activity of the sympathoadrenal system. Upon acute hypoxic exposure (5260 meters) there is an immediate increase in muscle SNS activity that remains after four weeks of acclimatization and persists for a minimum of three days upon return to sea-level[129]. Specifically, epinephrine has been shown to increase immediately upon acute altitude exposure, reaching its peak by day 2, and returning to sea-level values by day 6 or 7[3,15,17,130] . The kinetics of the norepinephrine response are a little different, with an initial rise in plasma norepinephrine occurring immediately, reaching its

peak within one week, and remaining elevated throughout the duration of exposure[17,130,131]. Likewise, elevated SNS activity in response to hypoxia has also been confirmed with microneurography[132].

The increase in sympathoadrenal activation in response to hypoxia has been attributed to maintaining adequate oxygen delivery to peripheral tissues despite concurrent arterial oxygen desaturation. Adequate oxygen delivery during hypoxia is achieved through hemo-concentration, unloading of plasma volume, and a subsequent increase in cardiac output for the maintenance of blood pressure in the face of decreasing blood volume. Although increased SNS activity in response to hypoxia sounds beneficial, chronic elevation of SNS activity has been associated with serious metabolic consequences, such as insulin resistance[5,6].

Activation of the SNS and a successive increase in plasma catecholamines is known to have substantial effects upon glucoregulation. Specifically, catecholamines have been shown to increase hepatic glucose production (gluconeogenesis and glycogenolysis) and decrease glucose uptake, resulting in an increase in circulating plasma glucose concentrations[133]. Epinephrine, released from the adrenal medulla, has been shown to promote glycogenolysis while concurrently inhibiting glucose uptake, oxidation, and storage[3-7]. The epinephrine mediated increase in glycogenolysis is achieved through activation of glycogen phosphorylase[3-5], while a reduction in glucose uptake occurs through epinephrine mediated inhibition of IRS-1 associated PI(3)K activity[7]. It has been shown that IRS-1 associated PI(3)K activity is essential for insulin mediated translocation of GLUT 4 to the plasma membrane and subsequent glucose uptake[2]. Epinephrine inhibits glucose oxidation, via glucose-6-phosphate mediated inhibition of hexokinase, while concomitantly preventing glycogenesis (glucose storage) through the inhibition of glycogen synthase[4]. The inhibition of glycogen synthase by epinephrine is

considered to be the most important factor contributing to decreased Rd and subsequent increase in plasma glucose concentrations[134].

Although the glucoregulatory effects of epinephrine have been extensively characterized, there is not as much information regarding norepinephrine. From the studies that are available, it appears that the effects of norepinephrine are only slightly different from those of epinephrine; with both acting in concert to increase plasma glucose levels. Norepinephrine and epinephrine are each known to increase lipolysis[6,31,135] by augmenting the activity of hormone sensitive lipase that is responsible for the liberation of non-esterified fatty acids (NEFAs) from adipocytes. The ensuing increase in plasma NEFAs inhibits insulin stimulated glucose uptake, via preferential fatty-acid oxidation, through a process known as the "Randle Effect[136]." Preferential fatty-acid oxidation leads to a decreased in insulin sensitivity via competitive oxidation[31,32]. Additionally, acute norepinephrine infusion has also been associated with hyperglycemia[137,138], increased hepatic glycogenolysis[139], and gluconeogenesis[140]. Thus, it is not surprising that norepinephrine is reported to be a mediator of insulin resistance[97,138,141,142]. A graphical representation of how hypoxia induces insulin resistance is shown in Figure 2.

On the contrary, some studies suggest chronic norepinephrine exposure is insulin sensitizing. Specifically, in a rat model, 10 days of continuous norepinephrine infusion (1,000 micrograms/kg/day) causes an increase in insulin sensitivity, as measured by the hyperinsulinemic euglycemic clamp technique. During 2, 6, and 200 mU/kg/h insulin infusions the glucose disposal rate was found to be 65, 60, and 13% greater during norepinephrine infused animals compared to the controls. Despite enhancement of insulin sensitivity, norepinephrine infusion did not prevent an increase in hepatic glucose production[98]. Down regulation of

beta-adrenergic receptors is most likely explanation of how chronic norepinephrine exposure can appear to improve insulin sensitivity. It has previously been shown that chronic stimulation of beta-adrenergic receptors results in receptor down regulation[143,144]. Chronic norepinephrine exposure, as it the case with this study, results in tissue desensitization that prevents the norepinephrine mediated intracellular elevations in cyclic-AMP. Ultimately, this decreases the activity of hormone sensitive lipase, preventing competitive fatty-acid oxidation, while additionally preventing the disruption of intracellular insulin signaling.

In addition to the sympathoadrenal systems contributing to glucose regulation at altitude, the hypothalamic-pituitary-adrenal axis (cortisol) has been suggested to assist in glucoregulation. In response to stress, the hypothalamus releases corticotropin releasing hormone (CRH) that acts upon the pituitary gland causing it to secrete adrenocorticotropic hormone (ACTH). ACTH targets the adrenal gland causing the release of cortisol. Cortisol increases hepatic glucose production via gluconeogenesis and glycogenolysis, decreasing glucose uptake through the inhibition of glycogen synthase activity[96,145].

In response to acute hypoxia, several studies have shown that levels of plasma cortisol remain unchanged when compared to sea-level[3,146-148]. Alternatively, several opposing studies show an increase in cortisol concentrations as a result of hypoxia[149]. For example, in individuals exposed to 2 days of high altitude exposure, plasma cortisol concentrations were elevated insulin sensitivity was decreased[16]. Although cortisol promotes insulin resistance by increasing hepatic gluconeogenesis and attenuating the activity of glycogen synthase[96,150,151], its role in hypoxic glucose regulation remains debatable.

In summary, the sympathoadrenal system is elevated under conditions of low oxygen. Epinephrine and norepinephrine play critical roles in the elevation of plasma glucose levels

under such conditions. The hypothalamic-pituitary-adrenal axis may also play a role in elevating plasma glucose levels in response to hypoxia through the actions of cortisol. However, the contributions of cortisol in elevating plasma glucose during hypoxia remain uncertain.

Hypoxia Induced Insulin Resistance: Contributions of Additional Hematologic Factors

It appears unlikely, that elevated SNS activity is exclusively responsible for hypoxia induced insulin resistance. In addition to catecholamines and non-esterified fatty acids, mediating hypoxia induced insulin resistance, there are several other circulating factors regulated by hypoxia that may influence insulin sensitivity. These factors include: adiponectin, pigment epithelium derived factor, oxidized low density lipoproteins, tumor necrosis factor-alpha.

Adiponectin is an adipokine, a protein secreted primarily from white adipose tissue, that is associated with insulin-sensitizing, anti-inflammatory, and anti-angiogenic effects [152]. Adiponectin has been shown to be decreased in insulin resistance and T2DM[153]. The insulin sensitizing effects of adiponectin occur through receptor binding (AdipoR1) and activation of AMP-activated protein kinase and proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α)[154]. Subsequently, this activation favors glucose uptake into cells and increased oxidative capacity. Interestingly, adiponectin expression and secretion is inhibited by hypoxia[155-157].

Similar to adiponectin, pigment-epithelium derived factor (PEDF) is also an adipokine that is a known regulator of insulin sensitivity, oxidative stress, inflammation and angiogenesis[158]. PEDF, unlike adiponectin, is inversely associated with insulin sensitivity[159] and elevated in insulin resistance[160]. It has been suggested that PEDF prompts insulin resistance through activation of pro-inflammatory pathways, stimulation of lipolysis, and promotion of

ectopic lipid accumulation that interfere with insulin signaling[161]. Hypoxia has been shown to up regulate mRNA and protein levels of PEDF[162].

In addition to regulating adiponectin and pigment epithelium derived factor, hypoxia also increases systemic oxidative stress[163-165]. High levels of oxidative stress have been shown to activate the JNK signaling pathway, which inhibits activation of IRS-1 and translocation of GLUT 4 to the plasma membrane[166]. Therefore, it is not surprising that insulin resistance is characterized by elevated levels of oxidative stress[167].

A final factor to be considered as a potential mediator of hypoxia induced insulin resistance is tumor necrosis factor alpha (TNF-α). TNF-α is a cytokine that is most often associated with inflammation. It has been implicated in insulin resistance as it is highly expressed in obese individuals[44], obese mice lacking tumor necrosis factor alpha receptors are protected from insulin resistance[168], and infusion of tumor necrosis alpha in rats causes insulin resistance[169]. Mechanistically, TNF-α has been suggested to induce insulin resistance through its inhibitory effects on both the insulin receptor and IRS proteins. Specifically, TNF-α has been shown to inhibit the activating tyrosine phosphorylation on the insulin receptor and IRS proteins that are crucial for the transduction of the insulin signal[44,170]. Hypoxia has been shown to positively regulate the production of TNF-α[171-173].

Clinical Conditions of Hypoxia: OSA and COPD

OSA affects approximately 3-7% of adult men and 2-5% of adult women and is characterized by momentary cessations of breathing (apneas) or decreases in breath amplitude (hypopneas) that are sufficient to induce hypoxemia and hypercapnia during sleep[19]. These hypopneas and apneas are predominantly caused by: a compromised upper airway, a decrease in

brain stem respiratory motor output, and the synchronization of these two events over time[20]. These events lead to repeated bouts of nocturnal intermittent hypoxia as defined by decreased arterial oxygen saturation[174].

The risk factors for developing OSA include: obesity, T2DM, hypertension, gender, specifics of cranial facial structures, and age[175]. However, obesity, especially visceral adiposity, has the greatest influence in promoting the development of OSA[176]. Obesity has several mechanisms by which it can contribute to the development of OSA. It can cause narrowing of the upper airway by physically decreasing lung volume and reducing the expansive "tug" that the trachea experiences from incoming air. Furthermore, adipose tissue releases various humoral factors such as leptin, TNF-α, and IL-6[20]. Leptin, in addition to influencing satiety and metabolism, can also act as a respiratory stimulant[20,177]. Impairment of leptin signaling pathways, as can be the case in obese humans, causes respiratory depression in mice and hypoventilation syndrome in humans that can result in OSA[177-179]. In addition to contributing directly to OSA, the inflammatory cytokines released from excessive adipose tissue, such as TNF-α and IL-6, can also foster the development of insulin resistance[180,181]. However, despite obesity being a primary risk factor for the development of OSA, there are many non-obese individuals that also suffer from OSA[182]. Therefore, suggesting OSA is not merely a consequence of obesity.

Similar to OSA, COPD is also a disease of hypoxia characterized by air-flow obstruction and arterial oxygen desaturation. Conditions previously known as chronic bronchitis and emphysema are now classified as COPD, all of which stem from chronic inflammation in the airways primarily due to regular exposure to tobacco smoke, noxious gases, or fine particulates.

Unlike OSA which is characterized by reversible intermittent hypoxia, COPD is a chronic disease that is progressive and irreversible[183].

The majority of individuals afflicted with either OSA or COPD display increased SNS activity[22,23,184,185]. Specifically, the hypoxic stimuli associated with OSA induces an enhanced chemoreceptor response that increases sympathetic outflow[186]. The increased sympathetic outflow is modulated by both the central and peripheral chemoreceptors. The central chemoreceptors, found within the brainstem respond to hypercapnia whereas the peripheral chemoreceptors (carotid chemoreceptors), located within the carotid bodies, respond to hypoxia. When activated, these chemoreceptors promote hyperventilation and increased sympathetic outflow[186]. With repeated bouts intermittent hypoxia as seen in OSA, the enhancement of the peripheral hypoxic chemoreflex response is magnified, resulting in an even greater increase in sympathetic nerve activity that would be exposed during hypoxia exposure[186,187]. Therefore, the severity of hypoxia, in OSA and COPD, is positively associated with the degree of increased SNS activity[188].

Elevated SNS activity in OSA patients is demonstrate by a biphasic catecholamine response. During nocturnal periods, when intermittent hypoxia is most prevalent, epinephrine levels are elevated[189]. On the other hand, during the day, when intermittent hypoxia is not present, norepinephrine levels are elevated while epinephrine remained unchanged compared to non-OSA controls[190]. This is comparable to what is found when healthy individuals are exposed to hypoxia, albeit over a much shorter time period.

One common treatment for OSA involves the use of continuous positive airway pressure (CPAP) while sleeping. CPAP is a breathing therapy that utilizes continuous pressurized air, from a face mask or nasal pillow that forces an individual to initiate each breath, thereby

eliminating hypopneas and apneas. CPAP is an effective treatment for OSA[191] and also decreases the elevated sympathetic activity typically seen in OSA[192-195]. Additionally, oxygen supplementation that eliminates hypoxic exposure in COPD patients has also been shown to favorably alter autonomic activity[30]. Thus, hypoxic exposure as a result of OSA and COPD is characterized by increased SNS activity that is attenuated upon cessation of hypoxic exposure[185,196-198].

OSA and COPD have also been associated with a high prevalence of insulin resistance and impaired glucose tolerance [24,25,199,200], independent of other factors such as age, sex, and body mass[24]. It has been demonstrated in several studies that T2DM patients, or those with impaired glucose metabolism, have an extremely high prevalence of OSA[25,201,202]. Interestingly, CPAP reduces elevated sympathetic activity in OSA patients and improves insulin sensitivity. Improvements in insulin sensitivity have been shown with only two days of CPAP treatment and these improvements remained until cessation of therapy[29,175]. Therefore, it is reasonable to suggest that the hypoxic exposure as the result of OSA or COPD increases SNS activity and this increased SNS activity is partially mediating the high prevalence of insulin resistance found in these populations. Likewise, the improvements in insulin sensitivity associated with CPAP appear to be facilitated by a decrease in SNS activity. There appears to be a logical link between hypoxia, elevated SNS activity, and insulin resistance.

Alternatively, it is possible that the hypothalamic-pituitary-adrenal axis response to hypoxic stress could be mediating the high prevalence of insulin resistance in OSA and COPD patients. Specifically, under periods of stress the hypothalamic-pituitary-adrenal axis promotes the secretion of cortisol. Cortisol is known to have potent glucoregulatory effects by increasing hepatic glucose production and decreasing glucose uptake[96,145]. However, there is conflicting

data regarding serum cortisol concentrations in OSA and COPD patients. Several studies have demonstrated non-significant differences in serum cortisol concentrations of OSA and COPD patients compared to healthy controls[203,204], while others have found serum cortisol concentrations to be elevated in OSA and COPD patients[205,206]. Therefore, the extent to which cortisol could be mediating the effect of insulin resistance in OSA and COPD population remains to be seen.

In summary, OSA and COPD are characterized by hypoxia, increased SNS activity, and a high prevalence of insulin resistance. Hypoxia is known to enhance the peripheral chemoreceptor response that is responsible for increasing sympathetic outflow in OSA and COPD. CPAP, a common treatment option for OSA, eliminates nocturnal bouts of intermittent hypoxia, prevents arterial oxygen desaturation, decreases SNS activity, and improves insulin sensitivity. This suggests that elevated SNS activity is mediating the high prevalence of hypoxia induced insulin resistance found in these populations.

Summary

Insulin, one of the primary glucoregulatory hormones, promotes glucose homeostasis through a series of successive steps that lead to the translocation of glucose transporter proteins to the plasma membrane and subsequent glucose uptake[9]. Unfavorable perturbations to either the insulin signaling cascade or GLUT 4 translocation can result in dysregulation of glucose homeostasis and insulin resistance[2]. Specifically, acute and chronic exposure to hypoxia is known to reduce insulin sensitivity while simultaneously increasing the activity of the sympathetic nervous system[3,15,16]. Additionally, obstructive sleep apnea (OSA) and chronic obstructive pulmonary disease (COPD) are clinical conditions characterized by hypoxia, elevated

SNS activity, and a high prevalence of insulin resistance[201]. In contrast to hypoxic exposure and hypoxic related diseases, hyperoxia has been shown to improve insulin sensitivity while concomitantly decreasing SNS activity[18]. Consistent with this, continuous positive airway pressure (CPAP), a common OSA treatment, has proven effective in abolishing nocturnal bouts of intermittent hypoxia, enhancing insulin sensitivity and diminishing SNS activity[29]. Although the underlying mechanism of hypoxia induced insulin resistance remains unclear, it appears that elevated SNS activity may be a mediating factor[3]. It is unlikely, that elevated sympathetic nervous system activity is exclusively responsible for hypoxia induced insulin resistance. Therefore, it is important to consider other known circulating insulin sensitizing and desensitizing factors that are regulated by hypoxia. Identification of the mechanism(s) underlying hypoxia induced insulin resistance may provide effective treatment options for hypoxia-related disease. Effective treatments have the potential to reduce the prevalence or development of insulin resistance, thereby reducing medical costs and increasing quality of life.

CHAPTER III

METHODS AND PROCEDURES

Subjects

We studied a total of 12 healthy, adult males. Recruitment criteria included: being between the ages of 18 and 65 years, non-smokers or had not smoked in the previous 2-years, normotensive (<140/90 mmHg), normoglycemic (fasting blood glucose concentration <100 mg/dl (<5.5 mmol/l)), resting heart rate of greater than 40 beats per minute, were not taking medications or supplements that may have confounded the interpretation of data, and were willing to abstain from exercise for 48 hours prior to testing. The participants were classified as healthy, based upon a medical history questionnaire, and were free of any cardiac abnormalities as determined by a 12-lead beat-by-beat electrocardiogram and blood pressure measurements both at rest and during an incremental exercise test. The nature, purpose, and risks of the study were explained to each subject before written informed consent was obtained. The experimental protocol conformed to the standards set forth in the *Declaration of Helsinki* and was approved by the Institutional Review Board at Colorado State University.

Overall Experimental Design

All measurements were made at the Colorado State University Human Performance and Clinical Research Laboratory. The hypothesis was addressed using a randomized repeated measures design. Independent variables included normoxia/hypoxia and intact SNS/inhibited SNS via 48-hour transdermal clonidine administration. The primary dependent variable, insulin sensitivity, was determined by the hyperinsulinemic euglycemic clamp technique[93,159].

Following screening procedures (health history questionnaire, graded exercise test, 12-lead electrocardiogram, and measures of body composition) insulin sensitivity was determined, in random order, during four conditions: normoxia (F_IO_2=0.21), hypoxia (F_IO_2=0.11), normoxia and SNS inhibition (48-hour transdermal clonidine administration (Catapres-TTS; 0.2mg/day)), and hypoxia and SNS inhibition. Each trial was separated by a minimum of 7 days and subjects were not blinded to conditions.

Screening Procedures

Fat mass and fat-free mass were measured using dual-energy X-ray absorptiometry (Hologic, Discovery W, QDR Series, Bedford, MA, USA). Body height was measured to the nearest 1 millimeter and body weight to the nearest 100 grams using a stadiometer and beam scale (Detecto, Webb City, MO, USA). Body mass index was calculated as body mass/height^2 (kg/m^2). Maximal oxygen uptake (VO_{2max}) was determined with a metabolic cart (Parvo Medics, Sandy, UT, USA) during a progressive, maximal cycle ergometer (Velotron Dynafit Pro, RacerMate, Inc., Seattle, WA, USA) test. Briefly, subjects cycled on a cycle ergometer at an increasing work rate until exhaustion. A valid VO_{2max} was determined by whether three of the following four criteria were satisfied: heart rate within 10 beats/min of their age-related maximum[207], plateau in VO_{2max} despite increasing work rate, RER>1.10, and a rating of perceived exertion $\geq$ 18[208].

Normoxic/Hypoxic Gas Breathing

For all trials, subjects reported to the laboratory between the hours of 0600 and 0800 following a 12 hour fast, 48 hour abstention from exercise, and after having consumed a

standardized meal (Ensure and Power Bar) in addition to their normal diet the evening before (verbal confirmation). Upon arrival, subjects were weighed (Detecto, Webb City, MO, USA), instrumented for determination of beat-by-beat heart rate (3-lead electrocardiogram), blood pressure (automated physiological monitor: Cardiocap 5, GE Datex-Ohmeda, Madison, WI USA), and oxygen saturation (Pulse oximetry: Cardiocap 5, GE Datex-Ohmeda, Madison, WI USA).

Normoxia and hypoxia were simulated with administration of a 21% (normoxic) or 11% (hypoxic) oxygen dry gas mixture (Airgas Intermountain, Inc., Denver, CO, USA) on the day of the euglycemic hyperinsulinemic clamp procedure; commencing 15 minutes prior to, and continuing throughout the duration of the procedure. All measurements were performed between 0600 and 1300 in a dimly lit room at a comfortable temperature (approximately 23°C) with subjects quietly resting in a semi-recumbent position. An intravenous catheter was placed in an antecubital and contralateral dorsal hand vein, and baseline venous blood samples were obtained from the contralateral dorsal hand vein.

Thereafter, subjects began breathing either a normoxic or hypoxic gas mixture. Subjects inspired via an airtight facemask (7450 Series; Hans Rudolph, Inc., Shawnee, Kansas, USA) attached to a three-way, non-rebreathing valve (2730 Series: Hans Rudolph, Inc., Shawnee, Kansas, MO, USA), connected via hose to a 100 liter, non-diffusing gas bag (6000 Series: Hans Rudolph, Inc., Shawnee, Kansas, MO, USA) filled with either the normoxic or hypoxic gas.

After 15-minutes of baseline gas breathing, the measurement of insulin sensitivity commenced. Subjects continued to breathe either the normoxic or hypoxic gas mixture for the remainder of the 180-minute protocol. Heart rate, blood pressure, and oxyhemoglobin saturation were monitored continuously and recorded every 15 minutes.

Insulin Sensitivity via Hyperinsulinemic Euglycemic Clamp Technique

The clamp is considered, by many, to be the gold-standard measurement of insulin sensitivity and has been previously utilized in the lab[93,159,209]. Briefly, antecubital catheters were utilized for infusion of insulin and glucose, while contralateral dorsal hand vein catheters, warmed via a heated blanket (Kaz, Inc., Hudson, NY, USA) were utilized for arterialized-venous blood sampling. A descending dose (127–40 mU/(m body surface area)^2/min) of regular insulin (Humulin, Eli Lilly and Co., Indianapolis, IN, USA) was administered over the first 10 min, followed by a continuous infusion (40 mU/(m body surface area)^2/min) from 10 to 180 min. Administration of a 20% dextrose solution was initiated at 4 minutes (2 mg/(kg body mass)/min) and adjusted as necessary to maintain blood glucose at a concentration of 90 mg/dl (5 mmol/l) throughout the clamp. Arterialized venous blood samples (~1 ml) were obtained every 5 minutes and blood glucose concentration was analyzed immediately using an automated device (2300 STAT Plus Glucose Lactate Analyzer, YSI Inc., Yellow Springs, OH, USA). Insulin sensitivity was determined from the mean rate of glucose infusion during the last 30 minutes of the clamp and expressed as milligrams of glucose per kilogram body weight per minute.

Inhibition of the Sympathetic Nervous System

Inhibition of the SNS began 48-hours prior to two of the four laboratory visits. The SNS was inhibited through the administration of transdermal clonidine to the upper arm (Catapres-TTS; 0.2 mg/day). Clonidine is a common hypertension medication that is typically administered in doses ranging from 0.1mg/day to 0.3mg/day[210,211]. Its mechanism of action is via pre-junctional stimulation of alpha-2-adrenegic receptors, including those located in the locus coeruleus of the brain stem, resulting in centrally mediated peripheral sympathoadrenal

inhibition, as reflected by decreased norepinephrine release[212] and attenuated muscle SNS activity[213,214]. Subjects were supplied with emergency contact information and were required to report for a follow-up blood pressure measurement 24 hours after clonidine administration, to be monitored for adverse effects, such as hypotension and skin irritation.

Blood Collection and Analysis

Venous blood samples were also collected at time points: -15 minutes (baseline prior to administration of supplemental gas), 0 minutes, 30 minutes, 60 minutes, 120 minutes, and 180 minutes. All times corresponding to the start of the clamp. Of the -15 minute, 0 minute, and 180 minute venous blood samples: approximately 10 ml was preserved with K3 ethylenediaminetetraacetic acid (Vacuette EDTA tubes - Non-ridged (pull cap), Greiner Bio-One North America, Inc., Monroe, NC, USA), approximately 10 ml was transferred into a tube containing a silica clot activator, polymer gel, silicone-coated interior (Vacutainer SST Tube with Silica Clot Activator, Polymer Gel, Silicone-Coated Interior, BD, Franklin Lakes, NJ, USA), and approximately 5 ml was preserved with ethylene glycol tetraacetic acid/glutathione). Of the 30 minute, 60 minute, and 120 minute venous blood samples: approximately 5 ml was preserved with ethylene glycol tetraacetic acid/glutathione. Samples preserved with K3 ethylenediaminetetraacetic acid and glycol tetraacetic acid/glutathione were collected in chilled tubes, placed immediately on ice and centrifuged (-4°C and 3600 rpm) within 60 minutes of collection to isolate plasma. Samples collected in silica clot activator, polymer gel, silicone-coated interior plasma tubes, were collected and remained at room temperature for approximately 30 minutes after sample collection. Samples were then centrifuged (-4°C and

3600 rpm) within 60 minutes of collection to isolate serum. Subsequently, all samples were stored at –80°C until analysis.

Enzyme-linked immunosorbent assays (ELISA) were used to measure, in duplicate, plasma concentrations of C-peptide, adiponectin, and pigment epithelium derived factor (all Millipore Corporation, Billerica, Massachusetts, USA), insulin (ALPCO Diagnostics, Salem, New Hampshire, USA), oxidized low density lipoproteins (ALPCO Diagnostics, Salem, New Hampshire, USA), tumor necrosis factor-alpha (R&D Systems, Inc., Minneapolis, Minnesota, USA), catecholamines (Rocky Mountain Diagnostics, Colorado Springs, Colorado, USA), and non-esterified fatty acids (Wako Diagnostics, Richmond, Virginia, USA).

Statistical Analysis

This study was designed as a randomized, repeated measures study. Therefore, we examined potential differences in insulin sensitivity due to hypoxia/normoxia and intact/inhibited SNS using repeated measures analysis of variance (ANOVA). If differences were detected, a Newman-Keuls post hoc analysis was performed to determine the location of these differences. Plasma catecholamines, compared to control (normoxic gas breathing, intact SNS) were analyzed by calculating the area under the curve, via the trapezoidal method, above baseline. Subsequently, these values were compared using repeated measures ANOVA. Other circulating factors including C-peptide, adiponectin, pigment epithelium derived factor, oxidized low density lipoproteins, tumor necrosis factor-alpha, and non-esterified fatty acids were also analyzed with repeated measures ANOVA including time (-15, 0, 180 minutes) as an additional factor. Results are reported as mean±SE and statistical significance was set at $P<0.05$.

CHAPTER IV

RESULTS

Subjects

There were 12 subjects recruited for the study and a total of 10 subjects completed the entire protocol. Two of the initial 12 subjects were excluded from the study due to symptoms associated with hypoxia intolerance including: nausea and headache. These individuals recovered quickly, upon cessation of hypoxic gas breathing, but were excluded from the study. The remaining group of 10 subjects was: young, normal weight, normal body mass index, normotensive, normal fasting blood glucose, and were of average aerobic fitness. Details of these subject characteristics are shown in Table 1.

Oxyhemoglobin Saturation

Hypoxic gas breathing caused a decrease ($P<0.01$) in oxyhemoglobin saturation, compared to normoxia (Figure 3), whereas clonidine had no effect ($P>0.25$).

Plasma Catecholamines

Plasma catecholamine concentrations were determined prior to and throughout the clamp procedure under each of the four conditions: normoxic gas breathing ($FIO_2=0.21$), hypoxic gas breathing ($FIO_2=0.11$), normoxic gas breathing and clonidine administration ($FIO_2=0.21$; Catapres-TTS 0.2mg/day-48 hours), and hypoxic gas breathing and clonidine administration ($FIO_2=0.11$; Catapres-TTS 0.2mg/day-48 hours). During basal or baseline conditions, prior to

normoxic/hypoxic gas breathing, clonidine decreased norepinephrine (P=0.05) while there was no significant change in epinephrine (P=0.16).

Overall, the area under the norepinephrine curve was increased with hypoxia (137±13%; P=0.02) compared to normoxia, while clonidine prevented the hypoxia induced increase norepinephrine (94±14%; P=0.43). The area under the epinephrine curve was increased in hypoxia (371±40%; P<0.001) compared to normoxia. However, clonidine was ineffective at preventing the hypoxia induced increase in epinephrine (346±82%; P<0.001). Plasma catecholamine data is shown in Figures 4 and 5.

Heart Rate and Blood Pressure

At baseline, prior to either normoxic or hypoxic gas breathing, clonidine decreased resting heart rate (P<0.04) and resting systolic blood pressure (P<0.05) while diastolic blood pressure remained unchanged (P<0.08).

During the clamp with hypoxic gas breathing, heart rate was higher (P<0.001) compared to the normoxic clamp. Clonidine decreased the hypoxic clamp induced elevation in heart rate (P=0.006).

The hypoxic clamp did not have an effect on diastolic blood pressure compared to the normoxic clamp (P=0.10). Clonidine administration, compared to non-clonidine administration, decreased diastolic blood pressure during the hypoxic and normoxic clamps (P<0.001). Systolic blood pressure remained unchanged under hypoxic and normoxic clamps, with or without administration of clonidine (P>0.41). Heart rate and blood pressure data, under all conditions, are shown in Table 2.

The Hyperinsulinemic Euglycemic Clamp and Insulin Sensitivity

Insulin sensitivity was determined by the clamp procedure with greater glucose infusion rates indicative in greater insulin sensitivity. The glucose infusion rates (adjusted for fat free mass and circulating insulin concentration) required to maintain blood glucose concentration at 5 mmol/l during administration of insulin under: normoxic gas breathing (F_IO_2=0.21), hypoxic gas breathing (F_IO_2=0.11), normoxic gas breathing and clonidine administration (F_IO_2=0.21; Catapres-TTS 0.2mg/day for 48 hours), and hypoxic gas breathing and clonidine administration (F_IO_2=0.11; Catapres-TTS 0.2mg/day for 48 hours) are shown in Figure 6.

Compared to normoxia, insulin sensitivity was decreased during hypoxia (*P*=0.03). In contrast, insulin sensitivity remained unchanged during normoxia and sympathetic inhibition (*P*=0.86), and hypoxia and sympathetic inhibition (*P*=0.23).

At the end of the clamp (180 minutes) there was no difference in blood glucose concentrations between all conditions (Normoxia: 90.1 mg/dl±0.7 (5.00±0.04 mmol/l); Normoxia-Clonidine: 90.5±0.5 (5.02±0.03); Hypoxia: 89.9±1.1 (4.99±0.06); Hypoxia-Clonidine: 90.8±0.7 (5.04±0.04); *P*=0.77) suggestive of successful glucose clamping.

Plasma insulin concentrations at the end of the clamp were approximately 10-fold higher than when compared to basal, pre-clamp concentrations (*P*<0.001), with there being no interaction between time, hypoxia and sympathetic inhibition (Figure 7; *P*=0.22). The increase in end-clamp plasma insulin concentrations is attributed to exogenous insulin administration as there was no difference in pre-clamp and end-clamp concentrations of C-peptide (Figure 8; *P*>0.41). It is known that C-peptide is a good indicator or pancreatic insulin release, with increasing endogenous insulin production being mirrored by an equimolar increase in C-

peptide[215]. Similar to plasma insulin concentrations, there was no interaction between time, hypoxia and sympathetic inhibition with C-peptide (Figure 8; $P>0.84$).

Additional Hematologic Factors

We also examined additional circulating hematological factors that are traditionally known to be modulated by hypoxia and/or have an effect on insulin sensitivity including: non-esterified fatty acids (Figure 9), pigment epithelium derived factor (Figure 10), oxidized low density lipoproteins (Figure 11), tumor necrosis factor-alpha (Figure 12), and adiponectin (Figure 13). However, it was found that none of these factors were influenced by time, hypoxia and/or sympathetic inhibition (all $P\geq0.058$).

CHAPTER V

DISCUSSION

This study was designed to elucidate whether elevated SNS activity is a mediator of hypoxia induced insulin resistance. During the hypoxic clamp, the glucose infusion rate was attenuated by approximately 50%, compared to the normoxia and normoxia/clonidine interventions. However, during the hypoxia/clonidine trial, the differences in glucose infusion rates were abolished compared to the normoxia and normoxia/clonidine interventions. Based upon these findings, effective SNS inhibition and improved insulin sensitivity with SNS inhibition, we are confident in our conclusion that inhibition of the sympathetic nervous system attenuates hypoxia induced insulin resistance.

The observation of hypoxia induced insulin resistance in our study is consistent with previous studies that have demonstrated insulin resistance in healthy adult humans and animals exposed to acute, chronic, and intermittent hypoxia[3,14-16]. Although there have been several studies that have suggested elevated SNS activity as a mediator of hypoxia induced insulin resistance[3], few have directly addressed this issue. Of those that have examined elevated SNS activity as a mediator of hypoxia induced insulin resistance, the results are inconclusive. For example, in one study, despite a concurrent rise in epinephrine concentrations and a decrease in glucose tolerance, it was concluded that epinephrine was not mediating the decrease in insulin sensitivity as alpha-1-adrenergic antagonism was unable to ameliorate the effect[15]. However, alpha-1-adrenergic antagonism is unlikely to influence sympathetic stimulation of glycogenolysis, hepatic glucose production, or inhibition of insulin release as these are primarily beta-adrenergic and alpha-2-adrenergic receptor mediated functions[104,105]. Similarly, in mice

exposed to 9 hours of intermittent hypoxia, with and without the ganglionic autonomic blocking agent hexamethonium, insulin sensitivity was decreased in response to hypoxia (compared to control, normoxic conditions) and was not restored with autonomic blockade[111]. However, the dose of hexamethonium administered was ineffective, indicative of its inability to prevent the transient reflex bradycardia associated with phenylephrine infusion[111]. Without complete autonomic blockade it is difficult to speculate on the contribution of autonomic activity to hypoxia induced insulin resistance. In addition, the effect of blocking both arms of the autonomic nervous system, sympathetic and parasympathetic, may have differing consequences, compared to blocking each arm individually. The parasympathetic system is required for maintaining insulin sensitivity and therefore plays a critical role in mediating insulin stimulated glucose uptake in hypoxia[112]. Although there have been several studies that have attempted to address the role of the sympathetic nervous system in hypoxia induced insulin resistance, most of these studies have shortcomings.

Taking into consideration the ineffectiveness of peripheral alpha-1-adrenergic antagonism and ganglionic autonomic blockade, we chose to direct our aim towards central inhibition of the sympathetic nervous system, with the anti-hypertensive drug clonidine. Clonidine, as previously mentioned, acts centrally upon pre-synaptic alpha-2-adrenergic receptors in the brainstem allowing potassium influx of the pre-synaptic neuron, hyperpolarization, and inhibition pre-synaptic neurotransmitter release[216]. Administration of clonidine, at comparable dosing to that used in our study, has been shown to directly decrease skeletal muscle sympathetic nerve activity[143,214], inhibit norepinephrine release, and decrease plasma norepinephrine concentrations[143,212]. In agreement with these previous studies, we

observed similar decreases in basal plasma norepinephrine concentrations, heart rate and systolic blood pressure, and attenuation of the norepinephrine and heart rate responses to hypoxia.

Upon close examination of glucose infusion rates across conditions, we demonstrated that hypoxia causes a 50% decrease in glucose infusion compared to normoxia. However, when comparing the glucose infusion rates during hypoxia/clonidine to that of normoxia, although there is no significant difference between the two conditions, it appears that glucose infusion rates during hypoxia/clonidine is approximately 75% of that during normoxia. However, extreme caution must be taken when making this comparison as there was no significant difference in glucose infusion rates between conditions.

The incomplete abrogation of hypoxia induced insulin resistance in the presence of clonidine can be explained by variations in plasma catecholamine levels. Despite clonidine effectively preventing the hypoxia induced rise in norepinephrine, it did not inhibit the hypoxia induced rise in epinephrine. This is in accordance with previous findings[143]. Epinephrine is potent mediator of glucose homeostasis as it can induce insulin resistance by promoting glycogenolysis while inhibiting glucose uptake, oxidation, and storage[3-7]. Specifically epinephrine's effects are mediated through the activation of glycogen phosphorylase[3-5], and inhibition of IRS-1 associated PI(3)K activity[7], hexokinase, and glycogen synthase activities[4]. Therefore, the failure of clonidine to inhibit the hypoxia induced rise in epinephrine may explain why we did not see a complete abrogation of glucose infusion rates when comparing conditions of normoxia to that of hypoxia/clonidine. The inhibition of hypoxia induced increases in both plasma epinephrine and norepinephrine could be addressed in future studies with the administration of a tyrosine hydroxylase inhibitor such as alpha-methyl-p-tyrosine.

The positive effect on glucose infusion rates under conditions of hypoxia/clonidine appears to be the result of clonidine induced inhibition of hypoxia stimulated norepinephrine release. Norepinephrine is known to invoke lipolysis[6,31] by increasing the activity of hormone sensitivity lipase which is responsible for the release of non-esterified fatty acids from adipocytes into systemic circulation. The subsequent increase in plasma non-esterified fatty acids interferes with insulin stimulated glucose uptake, leading to a decrease in insulin sensitivity[31,32]. However, in the current investigation we did not demonstrate a significant (P=0.058) interaction between time, hypoxia, and sympathetic inhibition for non-esterified fatty acids. Despite not reaching a level significance, clonidine's insulin sensitizing actions, potentially, resulted from inhibition of norepinephrine release, subsequent inhibition of lipolysis, and attenuation of plasma non-esterified fatty acid levels.

In addition to catecholamines and non-esterified fatty acids mediating hypoxia induced insulin resistance, we also considered several other factors that are known to be regulated by hypoxia and may have the potential to influence insulin sensitivity. These factors include: adiponectin, pigment epithelium derived factor, oxidized low density lipoproteins (marker of oxidative stress), tumor necrosis factor-alpha (marker of inflammation). Despite each of these circulating factors being regulated by hypoxia and directly or indirectly associated with insulin resistance, none were affected by hypoxia or sympathetic inhibition. There is the possibility that, although none of these factors reached significance, additive small changes in each factor could be enough to induce insulin resistance.

Aside from circulating cytokines, oxidative stress, and inflammation having the potential to cause variations in glucose uptake, hepatic and muscle glycogen content can also have an effect. Blood glucose it typically maintained within narrow limits. During periods when blood

glucose is elevated the activity of glycogen synthase is increased, stimulating the removal of glucose from the blood and promoting storage as glycogen in liver and muscle tissue. In addition, acute bouts of exercise are known to deplete muscle glycogen stores, increase the activity of glycogen synthase, and ultimately result in increased post-exercise insulin sensitivity[217]. With this in mind, we attempted to ensure equal muscle and liver glycogen content across all trials for each subject. This was accomplished by having subjects refrain from exercise for 48-hours prior to each intervention, having them fast for 12-hours prior to the commencement of each study, and providing a standardized meal the evening before each intervention. Although we attempted to limit variability in glycogen content between trials, we did not obtain muscle or liver tissue for direct measurements. This could be addressed in future studies.

There are several additional limitations that warrant discussion. As mentioned, the hyperinsulinemic euglycemic clamp technique is considered the gold standard for the measurement of insulin sensitivity[209]. However, the one-step clamp, utilized for the purposes of our study, does not provide information about hepatic insulin sensitivity or renal insulin sensitivity. It is known that epinephrine stimulates hepatic[218] and renal gluconeogenesis[219]. Therefore, it is possible that the observed hypoxia induced insulin resistance is the result of epinephrine mediated gluconeogenesis. However, this appears unlikely, given the approximate ten-fold increase in circulating insulin levels during the euglycemic hyperinsulinemic clamp procedure. For future direction this could be addressed through the incorporation of a two-step clamp and labeled glucose infusion.

In addition to the lack of data on hepatic insulin sensitivity, we also lack data on a peripheral mechanism of hypoxia induced insulin resistance. Specifically, we did not examine

muscle or adipose tissue for possible alterations in the multifaceted steps of the insulin signaling pathway and GLUT 4 translocation. Muscle and adipose tissue biopsies with subsequent analysis would be effective in addressing this issue.

Despite examining several circulating factors associated with insulin sensitivity and hypoxia we did not address cortisol levels. Previous research has implicated cortisol in induction of insulin resistance[95] through its role in decreasing the activity of glycogen synthase in skeletal muscle[96]. Cortisol concentrations have been shown to be elevated during exposure to hypoxia[16]. Therefore, it is possible that elevated cortisol levels in response to hypoxia could be in part responsible for hypoxia induced insulin resistance.

In order to avoid the monthly hormonal fluctuations of females, our subject recruitment was limited to males. Therefore, our results can only be directly applied to males. However, based upon previous observations of hypoxia induced insulin resistance in women, we do not expect there to be any sex differences[15].

Sympathetic inhibition was achieved through transdermal clonidine administration. Instead of a relative dose, we used an absolute dose of clonidine (0.2mg/day) for each individual. Ideally, we would have standardized our clonidine administration, relative to body surface area, body mass, or fat-free mass. Typically, when clonidine is administered as an anti-hypertensive, a low dose is initially administered and adjusted upwards until the desired response is achieved. However, this is rather impractical for our current experiment due to the short duration of clonidine administration. Regardless, differences in body mass, body composition, and transdermal absorption may contribute to variability in our outcome variables.

In regards to our hypoxic stimulus, we utilized normobaric hypoxia. For this, we had subjects breathe either a 21% or 11% dry gas oxygen mixture, from a mask attached to a non-

diffusing gas bag that was not humidified. This is in contrast to hypobaric hypoxia or altitude exposure, where there are differences in barometric pressure and the air is often humidified. Despite these disparities, we expect there to be minimal difference between the varying forms of hypoxic stimuli. Specifically, it has been shown that normobaric hypoxia[3], hypobaric hypoxia[15], and altitude exposure[16] are each able to induce insulin resistance.

In spite of these limitations, which can be addressed in future studies, our data has several important clinical implications. The diseases OSA and COPD are two clinical conditions that are characterized by hypoxia and elevated sympathetic activity[23,185] and have a high prevalence of insulin resistance and impaired glucose tolerance[24,25,199,200], independent of other factors such as age, sex, and body mass[24]. Currently, the most common treatments for OSA and COPD include: CPAP and administration of supplemental oxygen. CPAP is an effective OSA treatment as it eliminates bouts of intermittent hypoxia[191], decreases sympathetic activity[192-194], and improves insulin sensitivity[29,175]. Supplemental oxygen administration in COPD patients has also been shown to positively alter autonomic activity[30] and improve insulin sensitivity[220]. Further, our data support the hypothesis that hypoxia induced insulin resistance is mediated by elevated sympathetic activity. In addition to CPAP and supplemental oxygen administration, clonidine could serve as a potential therapeutic to ameliorate the decline in insulin sensitivity associated with OSA and COPD.

Moreover, there are millions of individuals exposed to acute hypoxia on a regular basis. Individuals voluntarily flock to high altitude ski villages or mountain locales to recreate on a year-round basis. Furthermore, in times of conflict, such as the war in Afghanistan, soldiers are acutely exposed to altitude extremes that can induce insulin resistance. Hypoxia induced insulin resistance can deplete glycogen stores and limit performance[221]. It is of utmost importance for

soldiers to perform at maximal capacity under such extremes to ensure their survival. Therefore, our data provide a mechanism that partially explains the decrease in insulin sensitivity at altitude, providing a potential target for future interventions aimed at decreasing symptoms of high altitude exposure and improving performance.

Table 1. Physiological characteristics of the research participants (*n* = 10 men).

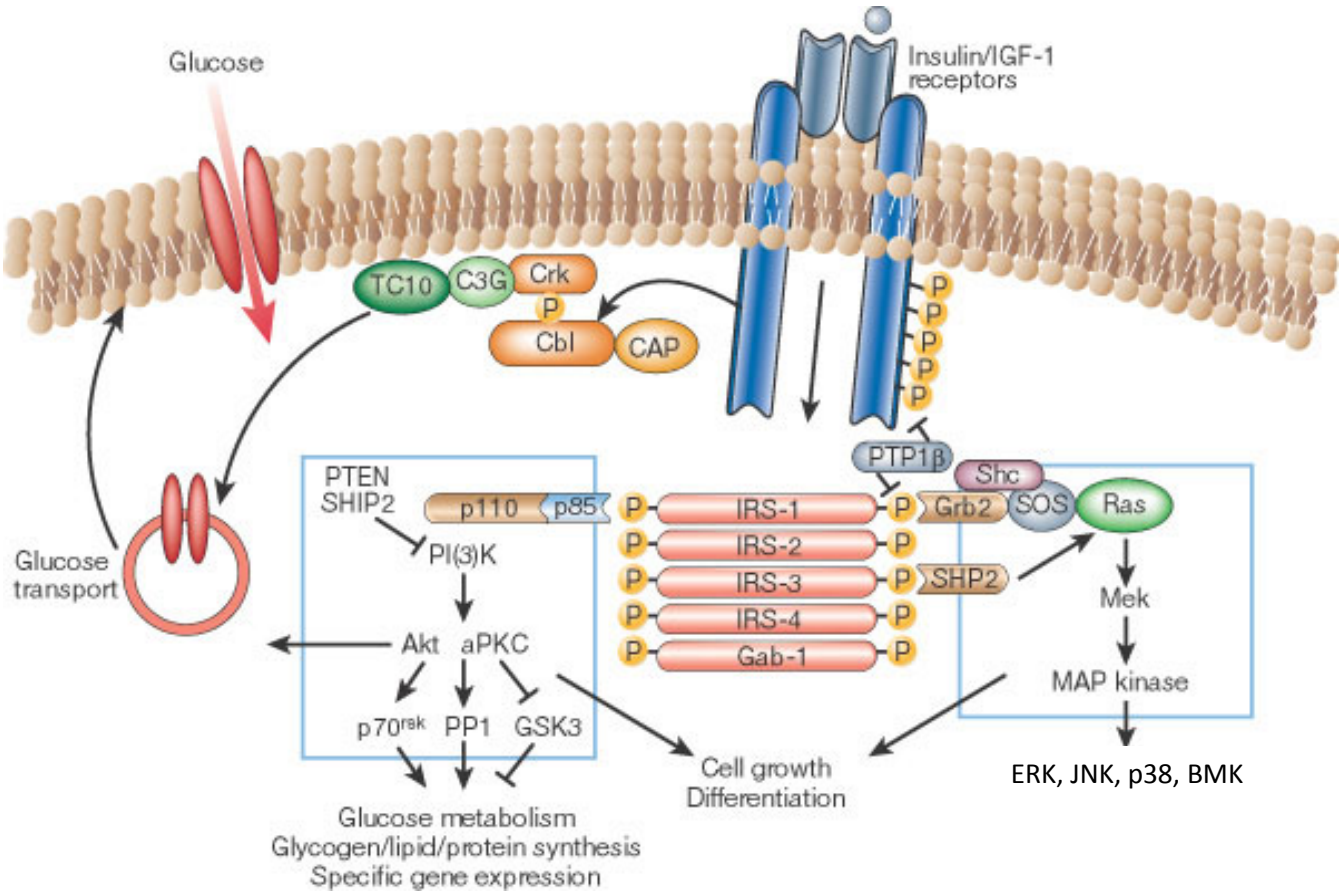
Variable	Mean ± SE
Age (years)	23 ± 1
Height (m)	1.80 ± 0.01
Body mass (kg)	78.6 ± 2.6
Body mass index (kg/m^2)	24.2 ± 0.8
Fat mass (kg)	15.2 ± 0.9
% Body fat	18.5 ± 1.2
Fat free mass (kg)	59.9 ± 1.7
Maximal oxygen uptake (ml/kg/min)	46.8 ± 2.5
Resting heart rate (beats/min)	59 ± 3
Resting blood pressure (mmHg)	120/72 ± 3/2
Fasting blood glucose concentration (mg/dl)	81.3 ± 1.3

Data are mean ± SE.

Table 2. Heart rate and blood pressure data, prior to and during normoxic and hypoxic gas breathing, with and without sympathetic inhibition (48-hour transdermal clonidine administration). * Prior to normoxic or hypoxic gas breathing, clonidine decreased resting heart rate (P<0.04) and resting systolic blood pressure (P<0.05). ** During the hypoxic clamp, heart rate was higher (P<0.001), compared to the normoxic clamp. † Clonidine lowered the hypoxic clamp induced elevation in heart rate (P=0.006). ‡ Clonidine lowered diastolic blood pressure during the hypoxic and normoxic clamps (P<0.001), compared to non-clonidine administration.

	Normoxia (F_IO_2 = 0.21)				Hypoxia (F_IO_2 = 0.11)			
	No Clonidine		Clonidine		No Clonidine		Clonidine	
	Prior to	During	Prior to	During	Prior to	During	Prior to	During
HR(beats/min)	59 ± 3	68 ± 2	55 ± 4*	61 ± 3	57 ± 2	84 ± 4**	49 ± 2*	76 ± 3†
SBP(mmHg)	120 ± 3	124 ± 3	110 ± 3*	114 ± 3	123 ± 3	122 ± 2	113 ± 3*	125 ± 9
DBP(mmHg)	72 ± 2	71 ± 2	67 ± 3	64 ± 3‡	75 ± 3	68 ± 2	66 ± 2	63 ± 2‡

Data are mean ± SE. "Prior to" data collected before breathing normoxic or hypoxic gas. "During" data are averaged values collected throughout the entire euglycemic hyperinsulinemic clamp procedure (180 minutes). SBP: Systolic blood pressure. DBP: Diastolic blood pressure.



Saltiel and Kahn, 2001

Figure 1. Summary of insulin signal transduction. Upon activation and autophosphorylation, the insulin receptor activates downstream targets including IRS-1 family proteins, Cbl/CAP and MAPK. Activation of these pathways leads to the regulation of glucose metabolism through alterations in GLUT 4 translocation, protein synthesis, enzyme activity, and gene expression[2].

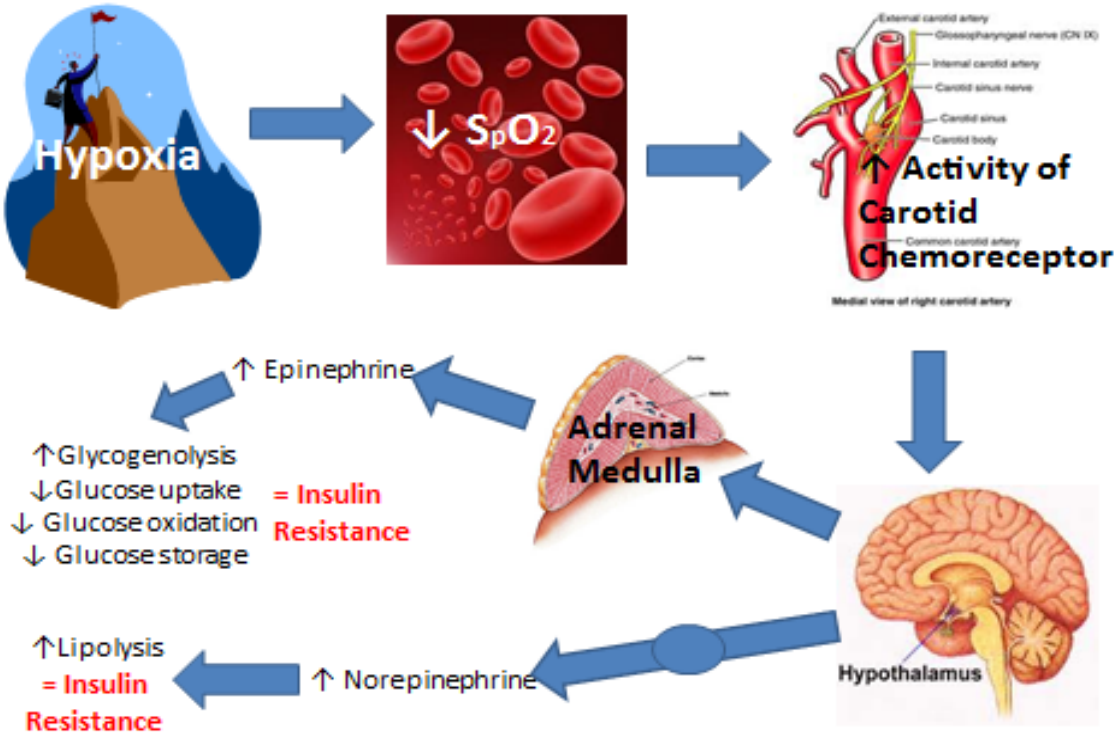


Figure 2. Hypoxia causes insulin resistance by decreasing arterial oxygen saturation. The decrease in arterial oxygen saturation is sensed by the carotid chemoreceptor located at the base of the branch point of the internal and external carotid arteries. Activation of the carotid chemoreceptor sends sensory afferent signals to the hypothalamus. The afferent signals are processed and efferent SNS activity is transduced along efferent neurons. Stimulation of the adrenal medulla leads to the secretion of epinephrine whereas transduction of the SNS signal to skeletal muscle targets leads to norepinephrine spillover. Epinephrine and norepinephrine have high affinity for beta-adrenergic receptors. Beta-adrenergic receptor stimulation induces lipolysis and glycogenolysis while concurrently inhibiting glucose uptake, glucose oxidation, and glucose storage.

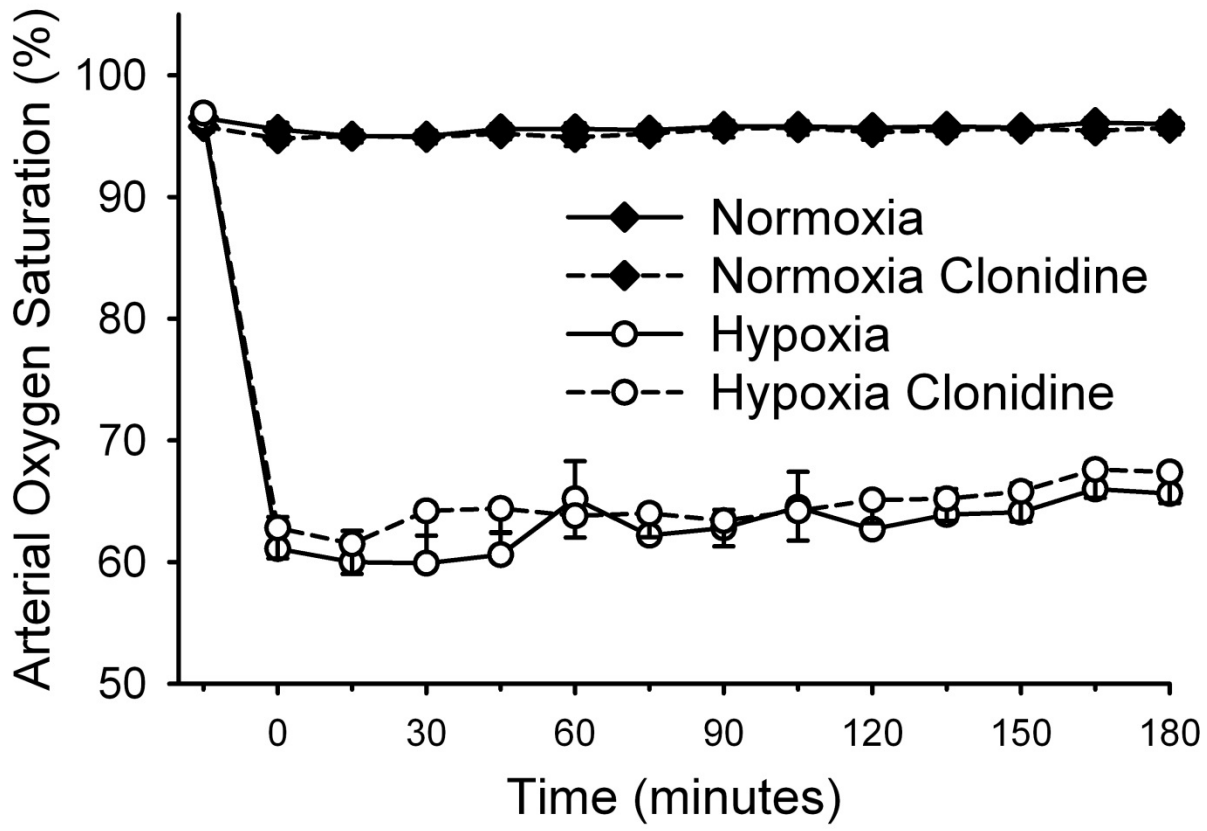


Figure 3. Arterial oxygen saturation (pulse oximetry) prior to (-15 min) and during (0 – 180 min) normoxia (F_IO_2=0.21) and hypoxia (F_IO_2=0.11), with and without sympathetic inhibition (48-hour transdermal clonidine), during the euglycemic hyperinsulinemic clamp procedure. Hypoxic gas breathing significantly decreased oxyhemoglobin saturation (P<0.01) compared with normoxia at all time points. Clonidine had no effect on arterial oxygen saturation (P>0.25). Data: mean ± SE.

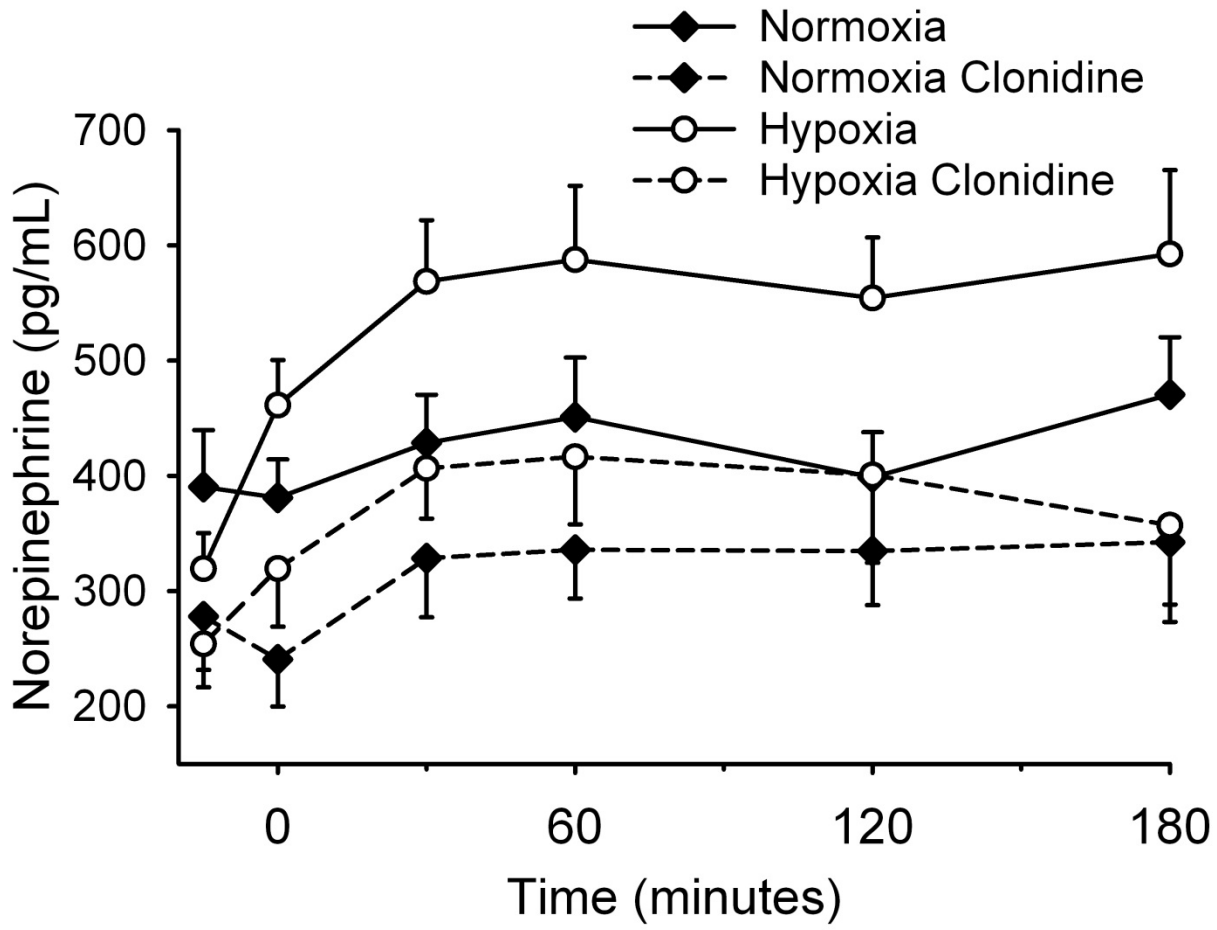


Figure 4. Plasma norepinephrine prior to (-15 min) and during (0 – 180 min) normoxia (F_IO_2=0.21) and hypoxia (F_IO_2=0.11), with and without sympathetic inhibition (48-hour transdermal clonidine), during the euglycemic hyperinsulinemic clamp procedure. The area under the norepinephrine curve (relative to the normoxic response) was increased with hypoxia (137±13%; *P*=0.02); clonidine prevented the hypoxia induced increase (94±14%; *P*=0.43). Data: mean ± SE.

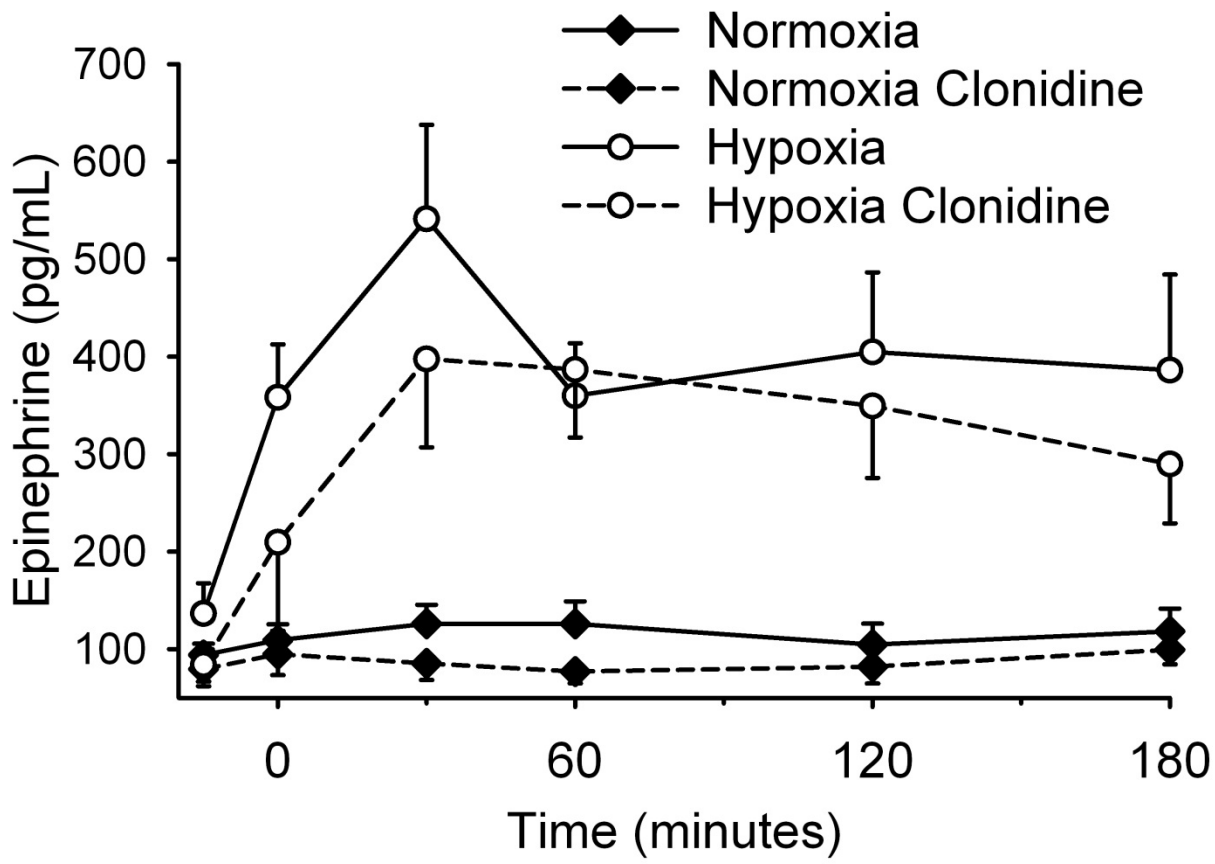


Figure 5. Plasma epinephrine prior to (-15 min) and during (0–180 min) normoxia (F_IO_2=0.21) and hypoxia (F_IO_2=0.11), with and without sympathetic inhibition (48-hour transdermal clonidine), during the euglycemic hyperinsulinemic clamp procedure. The area under the epinephrine curve (relative to the normoxia response) was significantly increased in hypoxia (371±40%; *P*<0.001). Clonidine did not affect the hypoxia induced increase in epinephrine (346±82%; *P*<0.001). Data: mean ± SE.

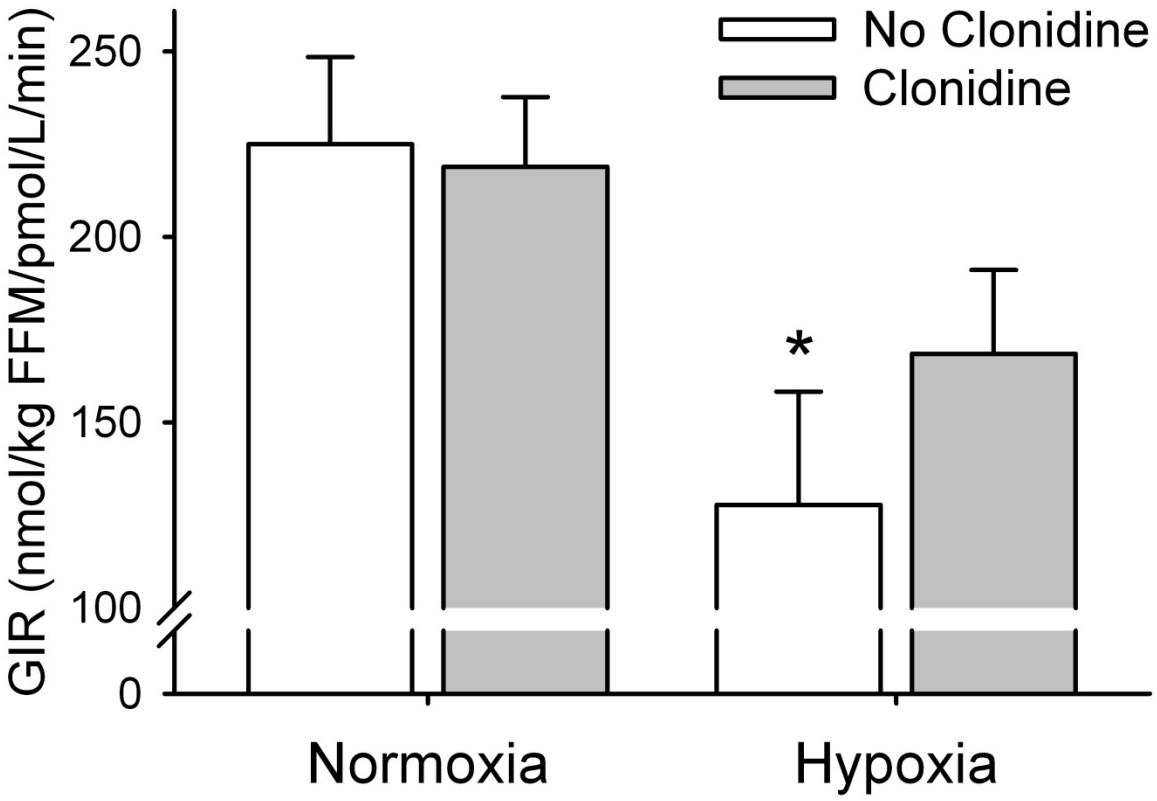


Figure 6. The rate of intravenous glucose administration (adjusted for fat free mass and circulating insulin concentration) required to maintain blood glucose concentration at 90 mg/dl (5 mmol/L) during standardized intravenous insulin administration during normoxia (F_IO_2=0.21) and hypoxia (F_IO_2=0.11), with and without sympathetic inhibition (48-hour transdermal clonidine). Data: mean ± SE. * Denotes difference to normoxia without clonidine (P<0.05). GIR: Glucose infusion rate.

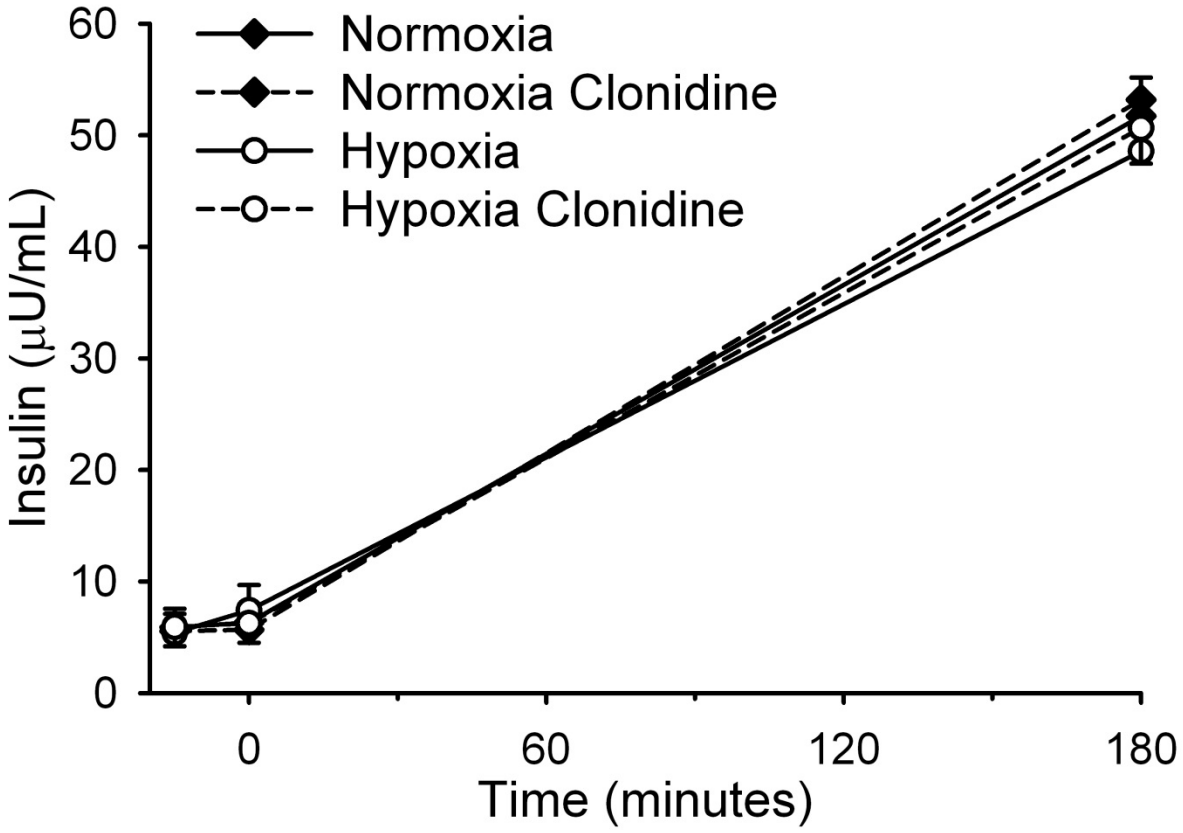


Figure 7. Plasma concentrations of insulin prior to (-15 min) and during (0 and 180 min) normoxia (F_IO_2=0.21) and hypoxia (F_IO_2=0.11), with and without sympathetic inhibition (48-hour transdermal clonidine), during the euglycemic hyperinsulinemic clamp procedure. Data: mean ± SE. *P* Values reflect interaction between time, F_IO_2 and clonidine (*P*=0.22).

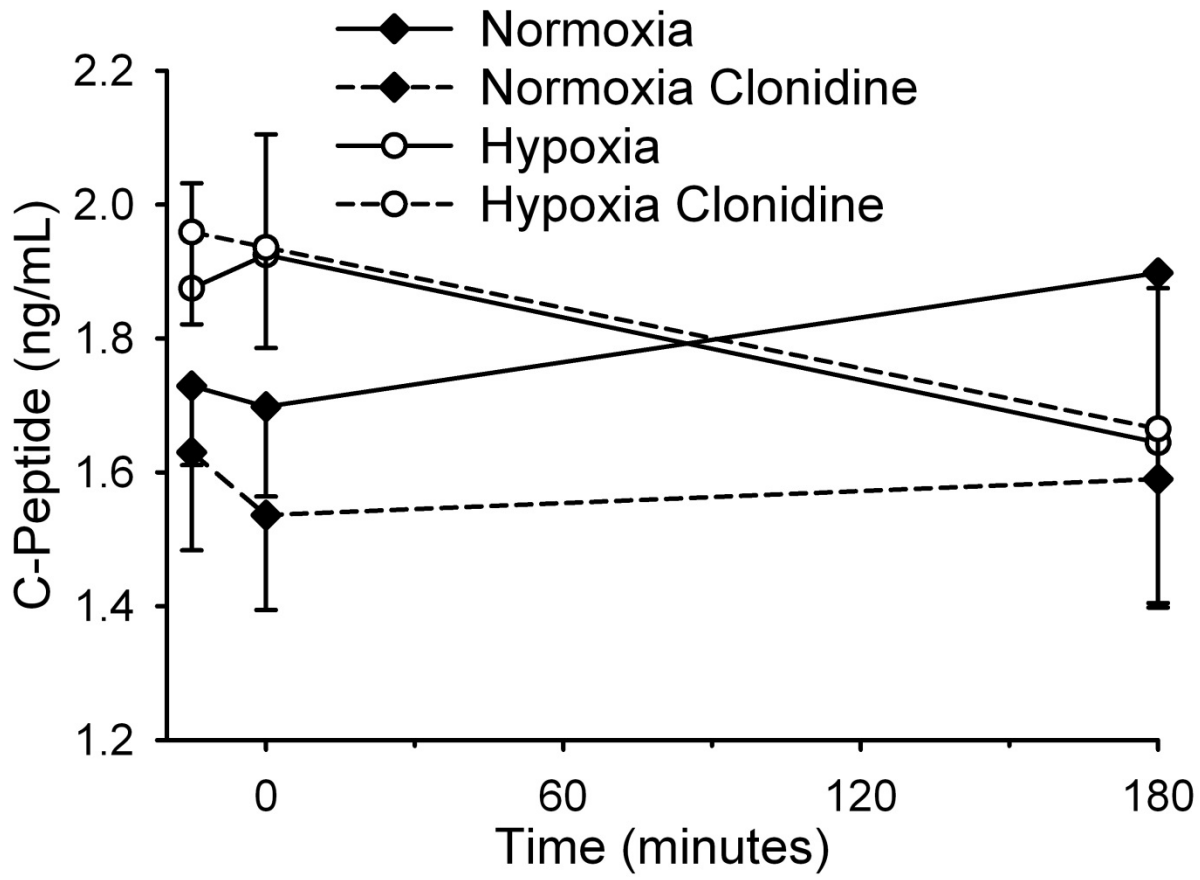


Figure 8. Plasma concentrations of C-peptide prior to (-15 min) and during (0 and 180 min) normoxia (F_IO_2=0.21) and hypoxia (F_IO_2=0.11), with and without sympathetic inhibition (48-hour transdermal clonidine), during the euglycemic hyperinsulinemic clamp procedure. Data: mean ± SE. *P* Values reflect interaction between time, F_IO_2 and clonidine (*P*=0.84).

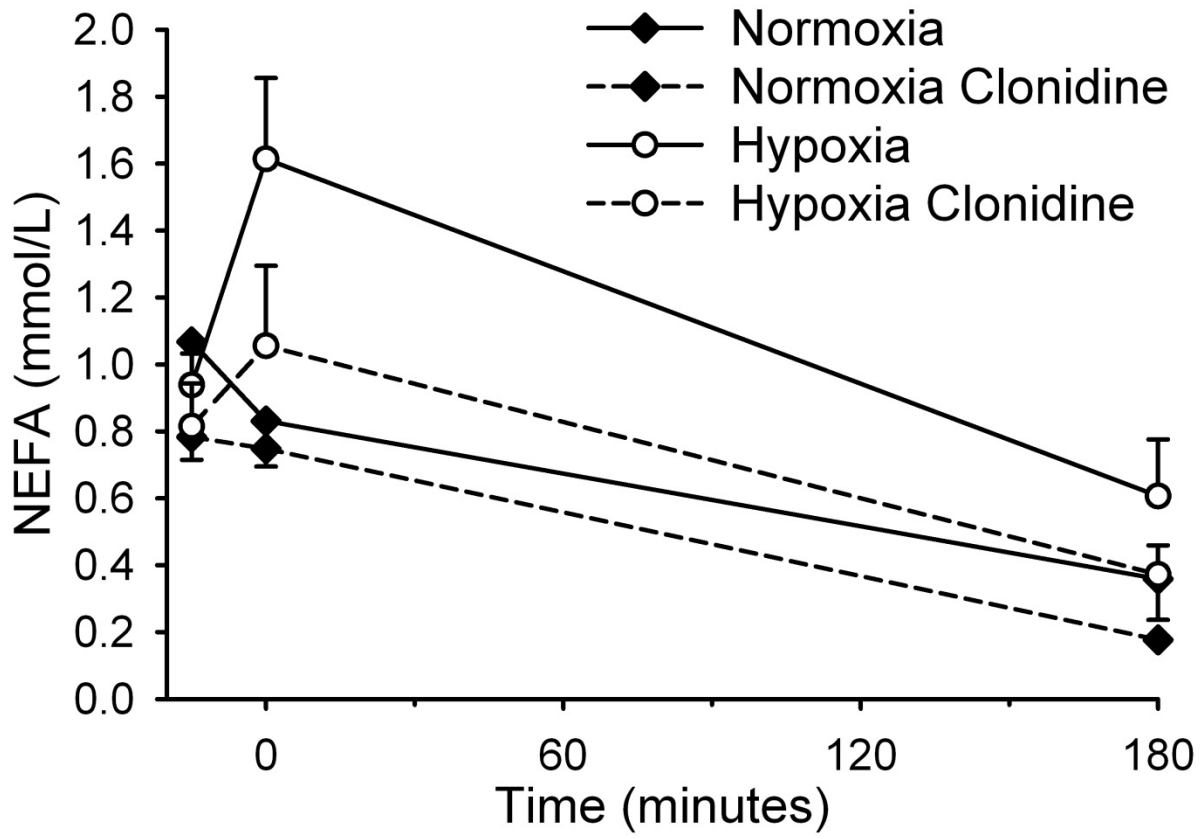


Figure 9. Plasma concentrations of non-esterified fatty acid (NEFA) prior to (-15 min) and during (0 and 180 min) normoxia (F_IO_2=0.21) and hypoxia (F_IO_2=0.11), with and without sympathetic inhibition (48-hour transdermal clonidine), during the euglycemic hyperinsulinemic clamp procedure. Data: mean ± SE. *P* Values reflect interaction between time, F_IO_2 and clonidine (*P*=0.058).

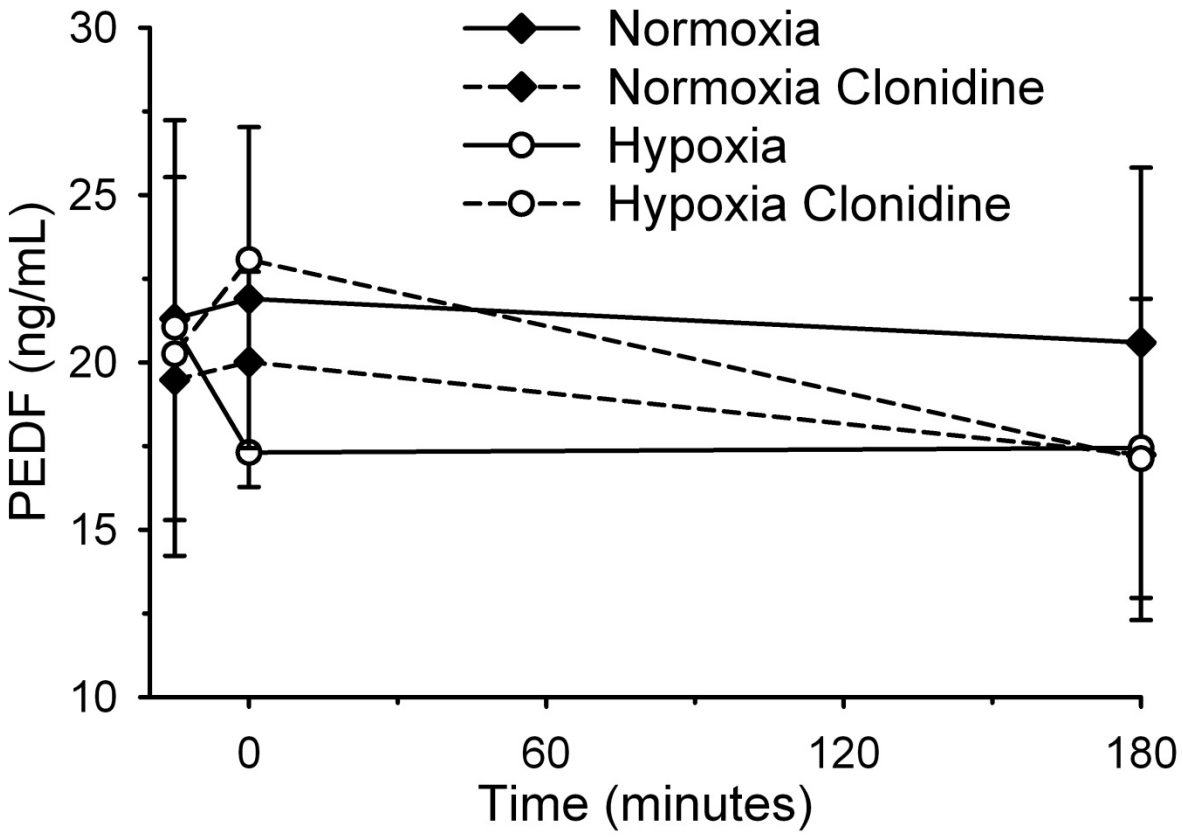


Figure 10. Plasma concentrations of pigment epithelium derived factor (PEDF) prior to (-15 min) and during (0 and 180 min) normoxia (F_IO_2=0.21) and hypoxia (F_IO_2=0.11), with and without sympathetic inhibition (48-hour transdermal clonidine), during the euglycemic hyperinsulinemic clamp procedure. Data: mean ± SE. *P* Values reflect interaction between time, F_IO_2 and clonidine (*P*=0.30).

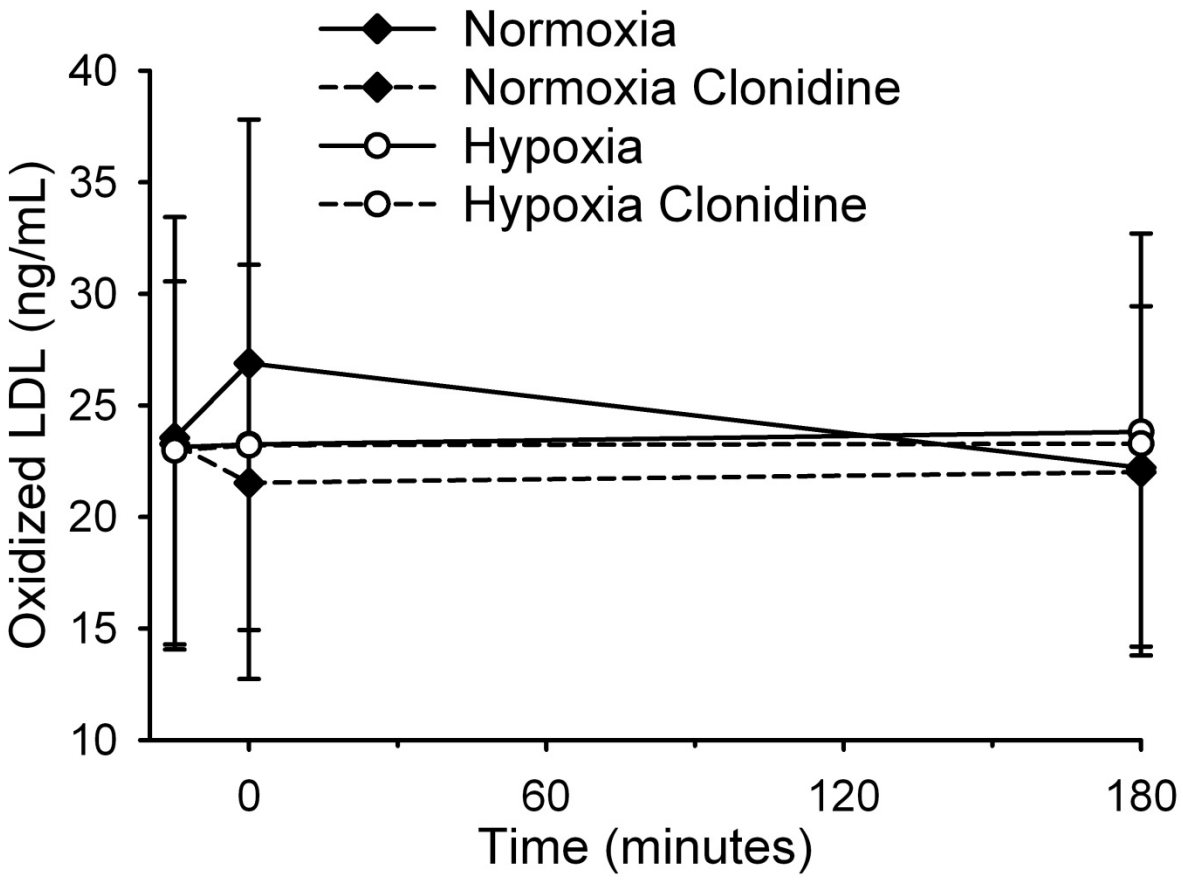


Figure 11. Plasma concentrations of oxidized low density lipoprotein (oxidized LDL) prior to (-15 min) and during (0 and 180 min) normoxia (F_IO_2=0.21) and hypoxia (F_IO_2=0.11), with and without sympathetic inhibition (48-hour transdermal clonidine), during the euglycemic hyperinsulinemic clamp procedure. Data: mean ± SE. *P* Values reflect interaction between time, F_IO_2 and clonidine (*P*=0.47).

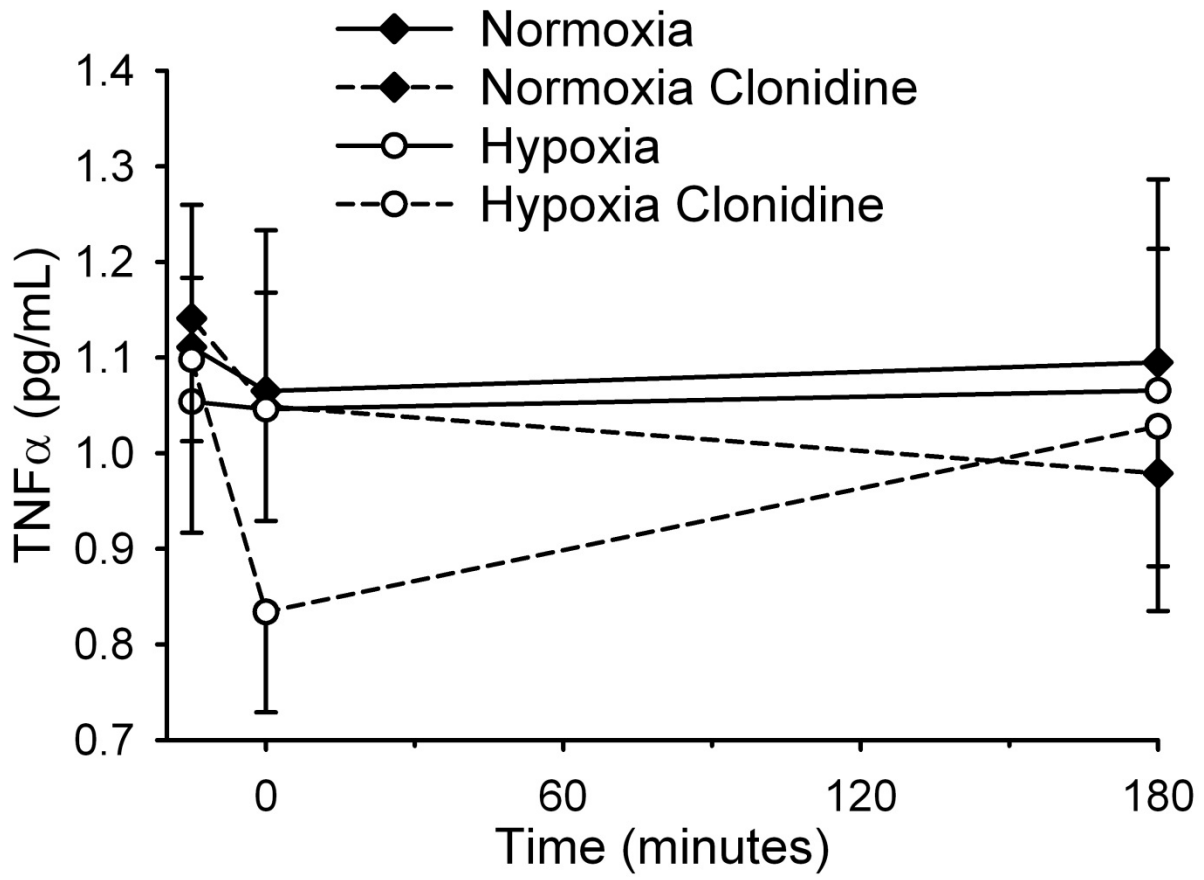


Figure 12. Plasma concentrations of tumor necrosis factor alpha (TNFα) prior to (-15 min) and during (0 and 180 min) normoxia (F_IO_2=0.21) and hypoxia (F_IO_2=0.11), with and without sympathetic inhibition (48-hour transdermal clonidine), during the euglycemic hyperinsulinemic clamp procedure. Data: mean ± SE. *P* Values reflect interaction between time, F_IO_2 and clonidine (*P*=0.51).

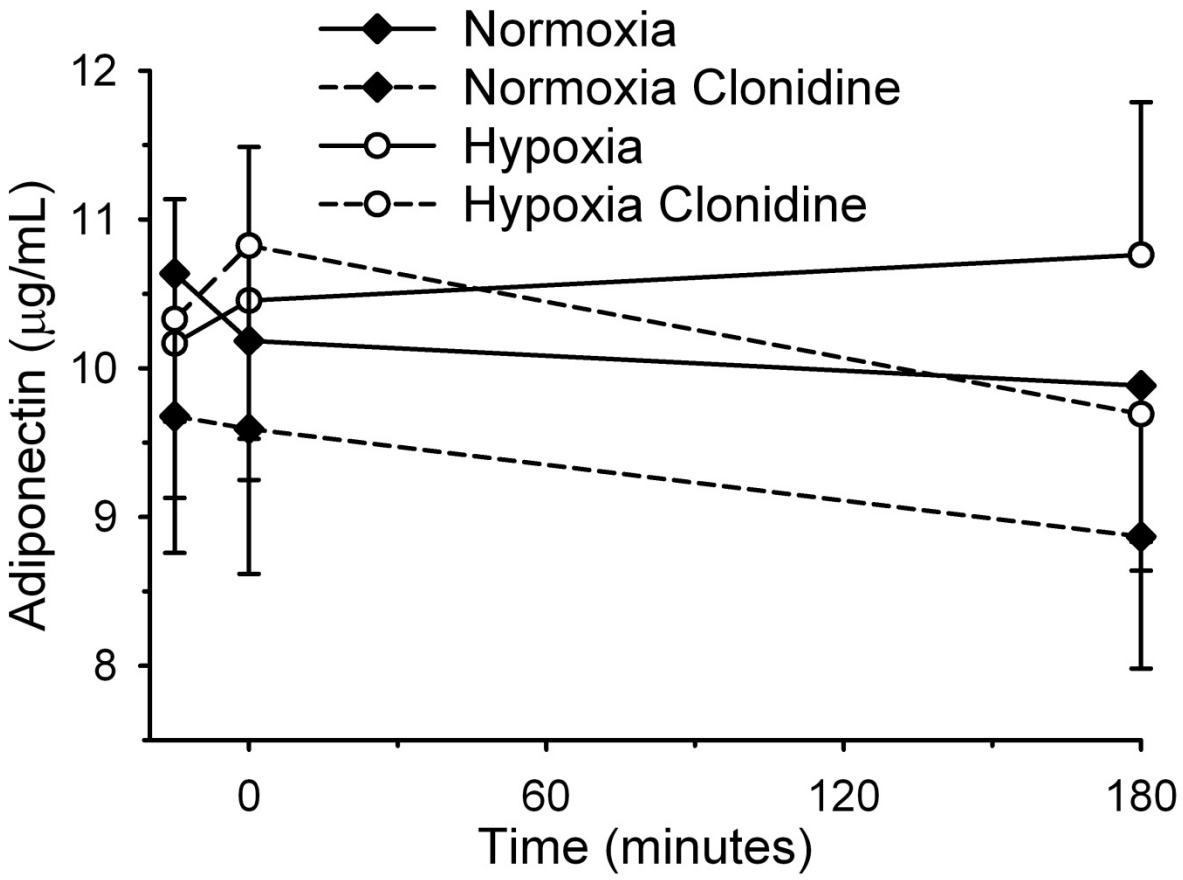


Figure 13. Plasma concentrations of adiponectin prior to (-15 min) and during (0 and 180 min) normoxia (F_IO_2=0.21) and hypoxia (F_IO_2=0.11), with and without sympathetic inhibition (48-hour transdermal clonidine), during the euglycemic hyperinsulinemic clamp procedure. Data: mean ± SE. *P* Values reflect interaction between time, F_IO_2 and clonidine (*P*=0.56).

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APPENDIX I

CONSENT FORM

Consent to Participate in a Research Study
Colorado State University

TITLE OF STUDY: Does Short-Term Inhibition of the Sympathetic Nervous System Attenuate Hypoxia-Induced Insulin Resistance in Adult Humans?

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WHY AM I BEING INVITED TO TAKE PART IN THIS RESEARCH?
You are an adult man or woman aged between 18 and 65 years. You do not smoke. You are not pregnant. Your resting heart rate is greater than 40 beats per minute. You have normal blood pressure (< 140/90 mmHg).

WHO IS DOING THE STUDY?
Christopher Bell, Ph.D., an assistant professor in the Department of Health and Exercise Science at Colorado State University will perform this research. A cardiologist, Dr. Dennis Larson, and trained graduate and undergraduate students will assist Dr. Bell.

WHAT IS THE PURPOSE OF THIS STUDY?
When people are exposed to a low-oxygen environment (for example, at high-altitude) their ability to control the concentration of sugar (glucose) in their blood is decreased. One possible reason for this is the increased in the activity of the nervous system. The purpose of this study is to see if decreasing the activity of the nervous system can reverse the decrease in the control of blood sugar seen in a low oxygen environment.

WHERE IS THE STUDY GOING TO TAKE PLACE AND HOW LONG WILL IT LAST?
All of the procedures (unless otherwise stated) will take place in the Human Performance Clinical/Research laboratory (HPCRL) in the Department of Health & Exercise Science (Moby Complex).
This whole research project will take place over a period of approximately one year. You will be asked to be involved for approximately 2 months. The total time of your participation is approximately 18 hours.

WHAT WILL I BE ASKED TO DO?

After responding to a questionnaire and completing some initial tests you will be asked to visit the HPCRL on 4 separate mornings. During these visits we will measure your ability to control blood sugar while you breathe air that contains either the same amount of oxygen as normal room air (21%) or air that contains less oxygen (11%).

For 48-hours prior to and during two of these visits you will wear a patch on your shoulder that releases a medication into your body. This medication will decrease the activity of your nervous system. These visits will be performed in a random order.

You will also be asked to make four additional brief visits for blood pressure checks and patch collection.

Below is a detailed description of all of the procedures; a member of the research team will fully explain each procedure and its duration.

Screening Visit – 2 Hours
The first visit to the HPCRL will be a screening visit. During this visit we will make sure that participation in this study is right for you.
This visit will include the following procedures:

Medical Questionnaire
You will be asked to answer several pages of questions related to your health, any illness you may have or have had, and medications you use or have used in the past.

Pregnancy Test
If you are female you will be required to have a sample of your urine tested for the presence of human chronic gonadotropin (HCG), a hormone that indicates whether you may be pregnant. This will require approximately 1 cup of your urine. If you are pregnant or the test indicates that you are pregnant you will not be able to participate in this study.

Blood Pressure
We will measure your blood pressure using a standard blood pressure cuff (the same as in a doctor's office). Blood pressure will be measured during all of the tests performed in the lab with the exception of body composition. There are no known risks associated with this procedure.

Body Composition
We will measure how much fat you have in your body using a test called dual energy x-ray absorptiometry (DEXA). The DEXA test requires you to lie quietly on a padded table while a small probe gives off low-level x-rays and sends them over your entire body. This test gives very accurate measurements of your body fat and bone mineral density. We will also measure the circumference of your waist and hip using a tape measure.

Exercise Stress Test
You will be asked to perform a vigorous exercise test. This test will tell us if your heart is healthy. You will be asked to walk on a motorized treadmill or ride an exercise cycle (cycle ergometer) for approximately 10-15 minutes. The exercise will become more difficult every 2

minutes. While you are walking/riding we will measure your heart rate with an electrocardiogram (ECG) and your blood pressure with a cuff placed around your upper arm. We will also ask you to wear a nose clip (something that stops you breathing through your nose) and ask you to breathe through a mouthpiece. This will let us measure the gases you breathe. Depending on your age, a physician may supervise the test. If we do not think your heart is healthy you will be referred to your primary care physician for further testing. There is a chance that you may not be allowed to take part in our study.

Remaining Visits
You will be asked to complete four more long visits and four very quick visits. Each of the big visits will last approximately 4 hours. The quick visits will last ~ 5 minutes. During the big visits we will measure your ability to control your blood sugar.

Blood Sugar Tests
This procedure is formally known as the hyperinsulinemic euglycemic clamp and often shortened to "The Clamp". This procedure measures the ability of your body to control sugar. This test will take place early in the morning after a 12-hour fast, and abstention from alcohol, caffeine and exercise. We will inject sugar (glucose) and insulin (a naturally occurring substance produced by your body) into one of your veins. From another vein we will sample a very small amount of blood (~ 1 ml or 0.2 teaspoons) every 5-minutes for three hours (total amount of blood sampled ~ 36 ml or 7.3 teaspoons). We will measure the amount of sugar in each of these blood samples. We will continue to inject insulin and sugar into your veins to try to keep the concentration of sugar in your blood the same. In order to prepare for the blood sugar tests, you will be asked to refrain from exercising for 48 hours, and avoid alcohol and caffeine for 12 hours. The night before the blood sugar test you will be asked to eat a standardized meal (a high-energy drink (e.g. Ensure) and a high-energy snack (e.g. Powerbar)).

Breathing Special Air
During each of the blood sugar tests you will wear a facemask. This facemask will be connected to a tube. The tube will be connected to a large balloon. The balloon will be filled with special air from a gas tank. On two visits the air will contain the same amount of oxygen as normal room air (~21%). On the other two visits the air will contain less oxygen than normal room air (~11%). You will start breathing from the balloon 15 minutes before the beginning of the blood sugar test, and keep breathing from the balloon throughout the remainder of the blood sugar test.

Decreasing The Activity of Your Nervous System
For two days prior to-, and during two of the blood sugar tests you will wear a clonidine patch. Clonidine is a medication used to treat people with high blood pressure. You will be asked to wear a patch on your arm/shoulder that allows clonidine to slowly leak from the patch, through your skin and into your blood. After wearing the clonidine patch for a day, you will be asked to visit the HPCRL so that we can measure your blood pressure. This blood pressure visit will last ~ 5 minutes.

ARE THERE REASONS WHY I SHOULD NOT TAKE PART IN THIS STUDY? You will not be allowed to participate in these studies for any of the following reasons:

1) You are pregnant.
2) You are a nursing mother.
3) You suffer from a disease of the cardiovascular or pulmonary systems
4) You currently use tobacco or have used it regularly within the previous two years.
5) Your fasting blood glucose concentration is greater than 100 mg/dL (we will measure this for you).
6) You regularly use any prescription medications other than birth control.
7) You have asthma or any other type of lung/respiratory dysfunction.
8) Your resting oxygen saturation < 95% (we will measure this for you).
9) You are unwilling to abstain from exercise for 48 hours prior to testing.
10) You use anticoagulant therapy or have a known or suspected bleeding disorder.
11) A contraindication was identified during the screening visit (e.g. positive stress test).

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?
It is not possible to identify all potential risks in research procedures, but the researcher(s) have taken reasonable safeguards to minimize any known and potential, but unknown, risks. The Human Performance Clinical Research Laboratory has emergency supplies including a medicine trolley equipped with heart machines and supplemental oxygen. The investigator has a great deal of experience with all of the procedures. Some of the procedures for which you are being asked to volunteer have a number of associated risks:

Body Composition
The risks associated with the DEXA are very low. The maximum radiation dose you will receive in this study is less than 1/3000th of the federal and state occupational whole body dose limit allowed to radiation workers. Put another way, you will receive less than 1.3 mrem from this scan and you already receive approximately 450 mrem /year from normal background radiation dose in Colorado. The more radiation you receive over the course of your life, the more the risk increases of developing a fatal cancer or inducing changes in genes. The radiation dose you receive from this scan is not expected to significantly increase these risks, but the exact increase in such risks is not known. There are no discomforts associated with this procedure. Women who are or could be pregnant should receive no unnecessary radiation and should not participate in this study.

Exercise Tests
There is a very small chance of an irregular heartbeat during exercise (< 1% of all subjects). Other rare risks of a stress test are heart attack (< 5 in 10,000) and death (<2 in 10,000). Wearing a mouthpiece and nose-clip can sometimes cause dryness in the mouth and mild discomfort.

Blood Collection
When the needle goes into a vein, it may hurt for a short period of time (a few seconds). Also there may be minor discomfort of having the needle/plastic tube taped to your arm. In about 1 in 10 cases, a small amount of bleeding will occur under the skin that will cause a bruise. The risk of forming a blood clot in the vein is about 1 in 100, and the risk of significant blood loss is 1 in 1,000. Additionally, there is a risk that you may faint while having blood collected or having the catheter inserted in your vein.

Blood Sugar Test
The procedure involves placement of a catheter (hollow plastic needle) inside a vein thus the usual risks of blood collection apply (minor discomfort, bruising, fainting and blood clot (rare)). In addition there is a risk of hypoglycemia (low blood sugar); symptoms include hunger, nervousness and shakiness, perspiration, dizziness or light-headedness, sleepiness, confusion, difficulty speaking, and feeling anxious or weak. Although hypoglycemia can happen suddenly it can usually be treated very quickly by terminating the insulin infusion and continuing the glucose infusion, returning blood sugar concentration back to normal. To reduce the risk of hypoglycemia, blood glucose concentration is measured every 5 minutes. If blood glucose concentration falls below 65 mg/dL insulin administration will be terminated and glucose infusion continued until normal concentration (70 – 100 mg/dL) is resumed; this usually occurs very quickly (~ 5 minutes).

Breathing Special Air Containing Less Oxygen Than Normal Room Air
The risks associated with breathing a gas that contains less oxygen than normal room air include nervousness and shakiness, perspiration, dizziness or light-headedness, sleepiness, confusion, difficulty speaking, and feeling anxious or weak. We will measure your heart rate, oxygen saturation (how oxygen you have in your blood), and blood pressure; if oxygen saturation falls below 70%, heart rate increases by more than 50 beats per minute above rest, or blood pressure increases by more than 35 mmHg above rest, the test will be terminated. Should you decide you no longer wish to continue breathing the special air, the facemask is very easy to remove. Supplemental air (100% oxygen) will be available on request or if needed. Symptoms associated with low oxygen can be treated very, very quickly by breathing normal room air and/or supplemental oxygen.

Decreasing The Activity of Your Nervous System
We will be giving you a similar amount of clonidine that is usually given to people with high blood pressure. This drug will lower your heart rate and blood pressure. There is a small risk of hypotension (low blood pressure). The symptoms of blood pressure dropping too low are dizziness, fainting, or in rare instances heart block. If your mean blood pressure falls below 90 mmHg, or if your heart rate falls below 40 beats per minute we will stop giving you the clonidine. There is also a small risk of dry mouth, tiredness or weakness, and irritation (itchy skin) at place where you wear the patch. To help avoid any unpleasant side effects we will be taking a number of precautions:

1) We measure your heart rate and blood pressure after 1 day of wearing the clonidine patch.
2) You will be given a list of people and their telephone numbers who you should contact if you feel unwell while you are wearing the patch.
3) You will be given a credit card sized laminated tag to carry while you are wearing the patch. The tag will read, “Clonidine Alert. I am a participant in a clinical research investigation. I currently receive 0.2 mg of transdermal clonidine per day. For information please call Dr. Christopher Bell 970-491-7522”.

ARE THERE ANY BENEFITS FROM TAKING PART IN THIS STUDY?
There are no direct benefits in participating, however you will receive a copy of your results and information pertinent to your body composition (i.e. height and weight), and metabolic and cardiovascular risk factors. For example, in blood we will measure concentrations of insulin and glucose. You will be provided with a copy of your DEXA scan; you may wish to have this interpreted by a medically qualified professional. Finally, this study has the potential to identify a physiological mechanism of high-altitude induced insulin resistance, and subsequently provide a practical pharmacological intervention.

DO I HAVE TO TAKE PART IN THE STUDY?
Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled.

WHAT WILL IT COST ME TO PARTICIPATE?
Other than transport to and from the lab, your participation should incur no costs.

WHO WILL SEE THE INFORMATION THAT I GIVE?
We will keep private all research records that identify you, to the extent allowed by law.

Your information will be combined with information from other people taking part in the study. When we write about the study to share it with other researchers, we will write about the combined information we have gathered. You will not be identified in these written materials. We may publish the results of this study; however, we will keep your name and other identifying information private.

Your identity/record of receiving compensation (NOT your data) may be made available to CSU officials for financial audits.

CAN MY TAKING PART IN THE STUDY END EARLY?
Your participation in the study could end if you become pregnant, or if you miss any of the scheduled appointments.

WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY? On completion of the study you will receive $300. Should your participation in the study end early, you will still receive feedback pertaining to your health and fitness.

WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH? The Colorado Governmental Immunity Act determines and may limit Colorado State University's legal responsibility if an injury happens because of this study. Claims against the University must be filed within 180 days of the injury.

WHAT IF I HAVE QUESTIONS?
Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind now. Later, if you have questions about the study, you can contact the investigator, Christopher Bell at physiology@cahs.colostate.edu or 970-491-3495. If you have any questions about your rights as a volunteer in this research, contact Janell Barker,

Human Research Administrator at 970-491-1655. We will give you a copy of this consent form to take with you.

This consent form was approved by the CSU Institutional Review Board for the protection of human subjects in research on (Approval Date).

WHAT ELSE DO I NEED TO KNOW?
Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing 8 pages.

_____ _____
Signature of person agreeing to take part in the study Date

_____
Printed name of person agreeing to take part in the study

_____ _____
Name of person providing information to participant Date

_____
Signature of Research Staff