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Erythropoietin Response to Intermittent Hypoxia in Health and Type 2 Diabetes

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by

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Dedication

To my mentors, Craig, Amanda, and Sophie. For your continuous support, guidance, and help, for which none of this would be possible. To my wife, Megan. For your unwavering support and love, that has given me the opportunity to chase my dreams. To my parents, Fran and Randy. For your love and commitment to raising me, I could not have asked for better parents.

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Abstract

Erythropoietin Response to Intermittent Hypoxia in Health and Type 2 Diabetes

by

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Patients with type 2 diabetes and aging individuals experience declines in maximal oxygen consumption. Hemoglobin mass, a component of oxygen transport, strongly correlates to maximal oxygen consumption. Interventions that increase hemoglobin mass may therefore increase maximal oxygen consumption in older adults and patients with type 2 diabetes. Intermittent hypoxia, characterized by alternating periods of breathing low levels of oxygen interspersed with periods breathing normoxic air, has the potential to elicit an acute increase in erythropoietin levels and hemoglobin mass. Despite several instances of repeated exposures to intermittent hypoxia increasing red blood cell count over the course of five days to three weeks, there exists a lack of consistent stimuli across the literature, with deviations in hypoxic duration, number of cycles, and hypoxic severity. Furthermore, studies that successfully increased oxygen transport following intermittent hypoxia did not measure erythropoietin levels, the hormone regulating red cell production, thereby eliminating the possibility for protocol comparison. Therefore, the following three studies aimed to identify the shortest intermittent hypoxia protocol to increase

erythropoietin levels in healthy young individuals, and to apply this intermittent hypoxia protocol to older individuals and patients with type 2 diabetes, with the goal of potentially increasing hemoglobin mass. In the first study, we identified the shortest hypoxic protocol within the literature to increase EPO concentrations among young healthy adults. The EPO response to the 32 total hypoxic minutes was no different than a 2-hour continuous hypoxia protocol. In the second study, we demonstrated that EPO concentrations increased following the same intermittent hypoxia in middle-aged adults but found no increase to hemoglobin mass. In the third study, we demonstrated a lack of EPO response to intermittent hypoxia in patients with type 2 diabetes. Furthermore, this study was the first to report hemoglobin mass levels in patients with type 2 diabetes. Collectively, the overall findings highlight the acute effects of intermittent hypoxia on erythropoietin in health and type 2 diabetes.

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CHAPTER I. INTRODUCTION

Maximal oxygen consumption (i.e., VO_{2max}), defined by the Fick equation, that represents the heart-lung-blood-muscle responses and adaptions to the changing demands of exercise, age, and disease. Therefore, VO_{2max} defines an individual's level of cardiorespiratory fitness and corresponds to the risk of morbidity, mortality, and cardiovascular disease (1). Hemoglobin mass, a measure of oxygen transport, strongly correlates to VO_{2max} (2). As a result, sedentary individuals (3), diseases such as diabetes (4, 5), or even healthy aging (6, 7) may experience a decline in hemoglobin mass and in turn VO_{2max}. As such, novel interventions aimed at increasing hemoglobin mass could have pronounced effects on improving fitness and decreasing risk for morbidity and mortality.

Erythropoiesis, defined as the process that regulates hemoglobin mass, integrates the molecular signaling from hypoxia exposure through hypoxia inducible factors (HIFs) to the synthesis of erythropoietin (EPO) and in turn the maturation of immature red blood cells (8, 9). Traditionally, acute continuous hypoxia exposure permits the stabilization and accumulation of HIFs that in turn, leads to EPO gene transcription and production (10). However, novel methods such as acute intermittent hypoxia exposure characterized by brief hypoxic periods interspersed with brief normoxic periods may provide a more advantageous, time efficient, and safe approach to stimulate erythropoiesis, than traditional acute continuous hypoxia (11, 12). While few studies have demonstrated chronic intermittent exposure to successfully increase hemoglobin mass, no such work has measured EPO levels to gauge the hypoxic stimulation and define an optimal protocol (13-15). Therefore, an investigation into identifying an optimal intermittent hypoxia protocol that effectively increases EPO levels may lead to increases in

hemoglobin mass and potentially maximal oxygen consumption in populations that may be experiencing declining levels.

CHAPTER II. REVIEW OF LITERATURE

Maximal Oxygen Consumption and Hemoglobin Mass

Maximal oxygen consumption, defined by the Fick equation (i.e., VO_2 = cardiac output x (C_aO_2-C_vO_2)) represents the integration of the heart, lungs, blood, and muscle metabolism as each system responds to the changing demands of exercise, aging, and disease. As such, it predicts mortality to the same or potentially greater extent as traditional risk factors such as smoking, hypertension, high cholesterol, and type 2 diabetes (1). Moreover, oxygen delivery accomplished by the product of cardiac output and arterial oxygen content (CaO_2) determines the extent of oxygen volume delivery to target tissues and ultimately cardiovascular fitness levels (16). While hemoglobin concentration remains an integral part of computing arterial oxygen content (i.e., (1.39 x [Hb] x S_aO_2 + (0.003 x P_aO_2))), it remains subject to fluid volume shifts and hydration status that can yield inaccurate values. As a result, hemoglobin concentration (g/dL) demonstrates a weak relationship (i.e., r = 0.25) with maximal oxygen consumption. Hemoglobin mass, a relatively new measure that represents the total mass of hemoglobin throughout the blood, independent of fluid volume shifts or hydration, exhibits a strong relationship (i.e., r = 0.97) with maximal oxygen consumption and can be used to replace hemoglobin concentration as a means improve accuracy (2). Correspondingly, an increase to hemoglobin mass levels results in a direct elevation oxygen transport capacity. For example, a 1 gram change in hemoglobin mass corresponds to an approximate 4 mL/minute change in maximal oxygen consumption (2). While the strong relationship to maximal oxygen consumption presides, hemoglobin mass demonstrates a strong concomitant relationship with lean body mass (2). As such, an increase to lean body mass facilitates greater metabolic demand and in turn a

requirement for greater oxygen delivery, accomplished by an increase to hemoglobin mass levels (17). Furthermore, individuals with high maximal oxygen consumption accomplished through years of endurance training exhibit high levels of lean muscle mass and hemoglobin mass compared to individuals of lower fitness, untrained, or sedentary status (17, 18). Conversely, lower levels of lean muscle mass associated with healthy aging correspond to lower levels of maximal oxygen consumption (19). While the effects of hemoglobin mass with age have yet to be determined, it would seem plausible that a decline in lean muscle mass and maximal oxygen consumption would result in lower levels of hemoglobin mass due to the strong existing relationships. Therefore, methods to increase maximal oxygen consumption in several populations that may exhibit lower levels could be supported by an increase in hemoglobin mass.

Molecular Mechanisms of Hypoxic Stimulated Erythropoiesis

Hypoxia, or low partial pressure of oxygen, initiates the cascade of events that trigger the synthesis and release of erythropoietin (EPO) to facilitate erythropoiesis. Specifically, renally derived EPO synthesis coordinates the erythropoietic adaptive response to hypoxia and in turn give rise to elevations in hemoglobin mass (8). Yet, EPO levels remain under control from molecular interactions that are regulated by hypoxic mediated transcription factors, HIF-1α and HIF-2α (20). Under normoxic conditions, HIFs remain continuously translated, but are the target of rapid proteasomal degradation, and fail to translocate to cell nuclei for transcriptional activity (21). However, in the presence of hypoxemia, rapid proteasomal degradation is inhibited, thereby permitting HIFs to rapidly accumulate in a hypoxic dose-dependent manner (22) and translocate to cell nuclei, where they bind with constitutively expressed HIF-beta units. As a result, the

HIF heterodimers assemble at promotor locations of hypoxia response elements and encourage HIF-gene targeted transcription, thereby activating a wide array of adaptive biological processes (10, 23). With respect to EPO synthesis, HIF-1α remains the transcriptional factor responsible for induction, while HIF-2α has recently materialized as the main regulator responsible for renal mediated production (12). Irrespective of hypoxic stimuli, an elevation to systemic EPO concentrations directly acts to prevent apoptosis of early erythroid progenitor cells by binding to the abundant amount of EPO receptors (EPOR) located on colony-forming unit erythroids (CFU-E). Subsequently, this leads to erythrocyte maturation and a rise in hemoglobin mass within 5-6 days (9). Beyond its role in direct induction of the EPO gene, HIF-1α upregulation acts to reduce the hepatic production of hormonal hepcidin, in turn, promoting intestinal iron absorption through activation of the transmembrane protein ferroportin. Similarly, HIF-1α upregulation increases the hepatic synthesis of the plasma protein transferrin, transporting intestinal iron to the bone marrow and satisfying needs during increased erythropoietic demands. Yet, the variety of hypoxic stimulus, dose, age, and disease may contribute to disruptions of the molecular mechanisms governing EPO concentrations and erythropoiesis.

Hypoxic Stimulus

SHORT CONTINUOUS HYPOXIA

Traditional methods that have attempted to acutely raise EPO levels and potentially increase hemoglobin mass as a means to improve oxygen delivery have routinely used acute continuous hypoxia, defined as a brief consistent exposure to a hypoxic stimulus. Correspondingly, brief exposure triggering hypoxemia stimulates an

increase in HIF accumulation, EPO levels, and with enough exposure dose, may increase incidences of oxygen delivery (i.e., red blood cells, hemoglobin mass, or reticulocytes). For example, a brief hypobaric hypoxia exposure of six hours to either 3000m or 4000m produced to plasma EPO concentrations within 114 minutes and 84 minutes, respectively (24). Furthermore, continuous measurement of EPO for the remainder of the six-hour exposure resulted in a 1.8 and 3x increase to EPO concentrations from baseline with the latter resulting from the more severe altitude. In addition to this response, a calculated relationship to alveolar oxygen partial pressure and rate of EPO production indicated a more severe altitude that accompanies lower alveolar oxygen partial pressures increased EPO rate of production. This further suggests a potentially greater and quicker EPO response with greater hypoxic severity, albeit no measurement of HIFs were collected to truly ascertain this outcome. Conversely, a separate investigation using a more severe altitude of 5500m for 90 minutes reported a 55% increase to EPO levels 180 minutes after hypoxic cessation (25). Despite significantly less hypoxic exposure duration (i.e. 1.5 vs 6 hours), an analysis of EPO concentration at a common time point of 90 minutes resulted in a similar 1 mU/ml increase above baseline levels despite the greater hypoxic severity. Together the results from these works outline the requirements for EPO synthesis and specifically suggest concentrations will continue to rise after the cessation of hypoxic exposure. While an absolute 55% increase remains less than the total 1.8 and 3x increases reported in continued longer exposure (24), assessment at another common timepoint of 270 minutes resulted in only a 23% and 18% increase in EPO concentration to 4000 and 3000m, respectively. As a result, this may support Eckardt's claim of greater EPO production with greater hypoxic severity, and further point towards the notion that less hypoxic time may be required to produce elevated EPO concentrations, provided high but not severe (i.e., > 5500m) altitude is utilized. As such, Turner et al., (2017)

investigated the EPO response to three normobaric hypoxic severities corresponding to 3600, 4200, and 4800m for a 2-hour continuous exposure in young healthy males. While 4200 and 4800m produced a rise in EPO concentrations from baseline ranging from 40-50%, they were not different from each other, contradicting the previous claims. Yet, a 16% EPO concentration increase from 3600m was not enough to evoke a significant rise from baseline (26), but corresponds to the nearly identical 18% percent increase seen with similar altitude (24). Yet, the impracticality of ascent to altitude or cost of maintaining an altitude chamber makes these prior works limited in translatability. However, the altitude of these studies collectively translates to a normobaric hypoxia oxygen content ranging from 10 to 13%, which may permit more widespread usage from cheap and more practical techniques at sea level. For instance, single normobaric continuous hypoxia exposures ranging from 90-120 minutes at similar fraction of inspired oxygen levels produced nearly identical rises in EPO concentrations (11, 27). Contrary to the lack of increase among oxygen transport indices from acute hypobaric hypoxia works, Schmidt et al., (1991) observed a rise in EPO concentration that stimulated an approximate 40% increase in reticulocyte quantity after a single 90-minute normobaric hypoxia exposure in young untrained men (27). Moreover, the increase in reticulocytes persisted for 48 hours after the conclusion of hypoxia. This suggests a short normobaric hypoxic exposure supportive of EPO concentration increases and could lead to increased indices of oxygen transport such as hemoglobin mass in less total exposure time than previous hypobaric hypoxia approaches. Nonetheless, the present information amongst the literature remains supportive that continuous hypoxia ranging from 84 to 120 minutes at a moderate altitude or equivalent oxygen content under normobaric hypoxia can raise EPO levels. As only one such study has resulted in an increase to oxygen transport indicates, more work is required to validate this response.

Contrary to hypoxic severity, adequate volume of stimulus remains an important consideration for improving oxygen delivery indices. Specifically, a chronic exposure protocol that consisted of nine 90-minute sessions progressively ranging from 4000 to 5500m over the course of 21 days, triggered an approximate 58% rise in reticulocytes and a 6.5% increase in red blood cells by 11 and 18 days after the first session, respectively. Furthermore, both remained elevated 15 days beyond the 21 day intervention (28). Conversely, a greater amount of hypoxic volume (3 hours/day for 5 days of a 4 week protocol using an identical range of altitude) found significant increases in EPO, 3-hours following hypoxic exposure, but no increase to hematological indices, including direct measurement of hemoglobin mass, over the course of the 4 weeks (29).

Hemodynamic and pulmonary gas exchange responses to short continuous hypoxia

Contrary to enhanced EPO synthesis and possible effects on hemoglobin mass, continuous hypoxia stimulates an increase in sympathetic activity, which can result in an increase in blood pressure over time. Specifically, an initial peripheral chemoreceptor mediated vasodilation under acute hypoxia (30) reduces total peripheral resistance, but a concomitant gradual increase in sympathetic outflow attenuates this initial vasodilation resulting in blood pressure increases (31, 32). For example, acute continuous hypoxia lasting longer than 20 minutes at arterial oxygen saturations of 77-87%, evokes sympathetic activation outlasting hypoxia-induced peripheral vasodilation, and increases blood pressure (31). Furthermore, exposure protocols ranging from 20-60 minutes at arterial oxygen saturations of 70-80% have shown increased sympathetic activity, systolic and mean arterial pressure (33, 34). Corresponding to the increase in sympathetic outflow, Turner et al. (2017) indicates an approximate 15% rise in heart rate one hour into a 2 hour

continuous hypoxia protocol at an oxygen saturation of 75% (26). While a rapid increase in heart rate occurs as a result of the initial peripheral vasodilation in attempt to maintain mean arterial pressure, ventilation is also known to increase within minutes from the activation of peripheral chemoreceptors (35). Yet, some report no change with minute ventilation in response to 1 hour of continuous hypoxia at 70-80% (36). However, the rate of desaturation and in turn peripheral vasodilation seem to play a role in the immediate response. As such, Chacaroun et al. (2016) indicated no change in ventilation, but the authors suggest gradual adjustment, in lieu of immediate exposure to a 10% fraction of inspired oxygen, over 10 minutes lessened the peripheral chemoreceptor response.

INTERMITTENT HYPOXIA

Intermittent hypoxia, defined as brief alternating bouts of hypoxia and normoxia that vary in length (minutes) and severity has grown in popularity due to the ease of use, time efficiency, and the potential to benefits in variety of healthy and clinical populations alike. As only a few minutes are required to stabilize and initiate HIF accumulation (10), in addition to the HIF half-life ranging from 5-8 minutes in normoxia (37, 38), it would seem plausible that a total exposure time similar to continuous hypoxia approaches would be required for a rise in EPO concentrations and reticulocytes. While not yet completely understood, the exact mechanisms that contribute to a similar effective hypoxic response with less exposure time seem to act through a different molecular pathway than continuous hypoxia. For example, *in vitro* HIF-1α synthesis is upregulated 1.2x under intermittent hypoxia, while a 1.8x decline in transcription is reported from exposure to 18 hours of continuous hypoxia. Specifically, a protective negative feedback loop during continuous hypoxia (39) responds to the initial elevation in HIF-1α accumulation and

further acts to inhibit expression, preventing overactivation and inducing conservation. Conversely, HIF-1α accumulation mediated from intermittent hypoxia may fail to activate the same negative feedback loop, possibly from the interruptions with normoxia, thereby resulting in greater rate of HIF-1α transcription and further accumulation (22, 40). This suggests that over time, similar levels may be attained between methodologies. While no such work has examined these HIF responses to intermittent hypoxic episodes in human models, several murine models have concluded these exposures parallel the HIF levels observed with continuous hypoxia with shorter exposure time. For example, an intermittent hypoxia protocol consisting of six 5-minute hypoxic cycles at a 6% fraction of inspired oxygen interspersed with 5-minutes of normoxia resulted in no difference in HIF-2α levels than a similar hypoxic intensity for a continuous 2-hours (12). While the length of continuous hypoxia permitted a ~400% increase in EPO mRNA levels compared to normoxic controls, intermittent hypoxia produced a 75% increase in EPO mRNA in only 30 total hypoxic minutes (12). This further suggests that intermittent hypoxia may elevate EPO synthesis to a similar level if exposure time was matched. Interestingly, a slightly different intermittent hypoxic murine protocol with equivalent total hypoxic duration and severity (i.e., five cycles of 6 minutes breathing a 6% fraction of inspired oxygen interspersed with 6 minutes of normoxia, totaling 30 hypoxic minutes), resulted in a 6.4-fold renal EPO mRNA increase within an hour and 10-fold increase in plasma EPO levels within two hours (41). While similar in total duration to the prior work, it seems the longer cycle time may permit a greater HIF accumulation and in turn may be supportive of greater increases in EPO concentrations. Secondarily, the degree and rate of resaturation during normoxic periods has the potential provide an additive benefit to hypoxically produced increases in HIF-1α levels. Specifically, HIF-1α stabilization, accumulation, and nucleus translocation can increase as a result of increased

mitochondrial reactive oxygen species (ROS) levels generation (23). A greater HIF-1α accumulation could induce greater EPO expression in subsequent hypoxic periods. On the contrary, longer normoxic cycles may permit a greater decline in HIF accumulation due to a short half-life. As such, optimal intermittent hypoxia approaches remain disputed, but may resemble brief hypoxic exposures of a few minutes and even shorter normoxic resaturation periods in order to maintain HIF synthesis and accumulation. For example, Knaupp et al. (1992) demonstrated an intermittent hypoxic protocol consisting of 2.5 hypoxic minutes at a 10.5% fraction of inspired oxygen interspersed with 1.5 minutes of normoxia for a cumulative hypoxic duration of 108 ± 6.5 minutes increased EPO concentration by 52% from baseline in young healthy men. Similarly, a 120-minute continuous hypoxia exposure at an identical hypoxic intensity induced a 50% increase in EPO concentrations (11). While this work conveyed no advantage regarding greater EPO concentrations when hypoxic time was similarly matched, it supports the notion that EPO can be stimulated in human models from intermittent hypoxia on par with traditional continuous hypoxia. While only one other previous attempt at measuring EPO concentrations with a short intermittent exposure consisting of five hypoxic cycles of 4-minutes at a 90% oxygen saturation interspersed with 4-minute normoxic periods in young healthy physically active individuals produced no increase to EPO concentrations, a short total duration of 20-minutes may have not been enough exposure (42). Secondarily, equal normoxic periods to hypoxic periods may have eliminated the potential for HIF accumulation, with respect to HIF half-life time. More importantly, adequate volume of stimulus remains critical to production and utilization of EPO. As such, chronic investigations using a progressive 3-week program consisting of 3-5 minutes of hypoxia at fraction of inspired oxygen levels ranging from 10-14% interspersed with a consistent 3-minutes of normoxia increased red blood cell count and

hemoglobin mass by 4% in patients with and without coronary artery disease and patients with chronic obstructive pulmonary disease (13, 14). In a separate investigation, five consecutive days of an intermittent hypoxia protocol consisting of an approximate 14 cycles ranging from 3-4 hypoxic minutes at an average oxygen saturation of 85% interspersed with 2 normoxic minutes increased red blood cell count by 7.7% in healthy older adults (15). Collectively, these chronic investigations have demonstrated the potential for intermittent hypoxia's success at stimulating increases in oxygen delivery indices. Yet, they remain limited by a lack of support indicating the acute erythropoietic response to a single session of their respective protocols. While it can be inferred that increased EPO synthesis was the main driver of improved oxygen delivery indices, no direct measurement or assessment of HIFs decreases the likelihood for reciprocating and comparing their findings. Thus, it remains to be investigated as to what effect a similar or more favorable protocol would employ in young healthy, older, or other clinical populations such as type 2 diabetes.

Hemodynamic and pulmonary gas exchange responses to short intermittent hypoxia

Interestingly, intermittent hypoxia approaches have yielded varying outcomes with respect to changes in blood pressure and ventilation. Unsurprisingly, a wide variety of existing protocols that vary in cycle length, intensity, and total exposure time contributes to inconsistent responses. For example, intermittent hypoxia consisting of three or five 4-minute hypoxic cycles at a targeted arterial oxygen saturation of 90% did not have any effect on blood pressure or ventilation in young healthy individuals (42, 43). Moreover, similar results occurred from a more severe three 4-min hypoxic cycle protocol at an arterial oxygen saturation of 80% in older adults (44). However, others report when

exposed to five 6-minute hypoxic bouts at a 10% fraction of inspired oxygen or seven 5-minute hypoxic bouts at 70-80% oxygen saturation increased ventilation (36, 45, 46), but not blood pressure (45, 46). While these works collectively suggest a lack of blood pressure increases despite acute oxygen desaturation, the brief intermittent nature of the protocols may lessen peripheral chemoreceptor activation and similarly a lessen hypercapnic mediated ventilatory response. Collectively, the acute responses to brief intermittent hypoxia may preclude the use of such methodologies in clinical populations as a means to gain the favorable adaptions to hypoxia but limit the potential for a rise in blood pressure.

Hematological responses with aging

With age, declines in cardiovascular function lead to an overall decline in maximal oxygen consumption (47, 48). In general, VO_{2max} declines approximately 1% per year in men and women (49, 50). While the decline has been associated with a yearly reduction in maximal heart rate (51) that can degrade cardiac output within the Fick equation, a concomitate decline in lean muscle mass (i.e., sarcopenia) would support possible declines in hemoglobin mass. Furthermore, the majority of past works that have attempted to examine a possible decline in oxygen transport have utilized the Evans Blue Dye method, which uses measures of plasma volume and hematocrit levels to calculate red blood cell volume. This results in an indirect measure of oxygen transport, which is greatly influenced by hydration status similar to hemoglobin concentrations (52). Despite a potential for error, reduced red blood cell volume, mainly due to a lower fat free mass and decreased physical activity levels, has been reported in older men and women (3, 6). Conversely, others report no change among individuals grouped above and below forty years of age (7). Given the contradictory reports, it remains surprising that no such work has investigated the aging

response with direct hemoglobin mass measurement, provide a simple and more accurate method exists. Specifically, the modified carbon monoxide rebreathing technique, which introduces a small known body size related quantity of carbon monoxide to a closed system breathing circuit for 2 minutes, accomplishes direct assessment of hemoglobin mass levels as a result of the greater carbon monoxide binding affinity to hemoglobin than that of oxygen. Secondary to the resultant direct measurement of hemoglobin mass from this approach is the limited reliance on plasma volume, thereby eliminating the interference of hydration status and improving assessment accuracy (53). Despite no such study reporting the direct changes with age, a direct assessment of red blood cell counts as a marker of oxygen transport has been reported to decline by 19% in men from the ages of 20 to 100 years of age and decline by 9% in women who are identified in middle to low reference ranges (i.e., 4.2 to 5.4 cells/mcL) (54). Although a precise mechanism contributing to potential age-mediated decline remains additionally disputed (55), various mechanisms altering erythropoiesis such as inflammaging (20, 56, 57), impaired kidney function (58, 59), declining EPO receptor quantity (60), increased osmotic fragility of red blood cells (61), and impaired proliferation of erythroid precursors (62, 63) may play a role. In addition to these possible outcomes, measurement of basal circulating EPO concentrations may help explain potential impairments to maintaining red blood cells. For example, lower circulating EPO concentrations were present in older participants (64-67), while others indicate either elevated (68) or no change compared to young controls (69, 70). Nonetheless, some works conclude that basal EPO concentrations among aged individuals remain insufficient to correct declining hemoglobin concentrations (54, 64-66) irrespective of EPO concentration, suggesting that EPO effectiveness may be impaired with aging (62, 63, 71). Indeed, an ex vivo analysis of healthy aged individuals indicated greater EPO levels were required to overcome inflammaging restricted erythropoiesis (72). Yet, a lack

of literature, similar to a potential hemoglobin mass decline, exists that integrates EPO concentrations, potential age-mediated impairments and subsequent effects on erythropoiesis as a whole. However, approaches that utilize hypoxia as a driver of EPO synthesis may offer greater insight in to the erythropoietic determinants with age. Correspondingly, a single work has examined the aging EPO response to a single exposure to continuous hypoxia. In response to 3-hours of continuous hypoxia at a fraction of inspired oxygen of 13.5% or individualized adjustment to 80% oxygen saturation, EPO concentrations were elevated by 79% and 28% in young and older adults, respectively (73). While older adults expressed a significant increase in EPO concentrations, it remained 51% less than the exact exposure in young adults. Although no measure of oxygen transport indices were assessed following hypoxia exposure from an EPO concentration increase, this supports the notion of a possible age-mediated EPO synthesis impairment that could impact hemoglobin mass levels. However, EPO measurements were only conducted 30-minutes post hypoxia and does not eliminate the possibility that EPO synthesis may simply be slowed in an aging population. While, no analysis of HIF responses occurred, HIF-1α levels and DNA binding activity responsible for EPO gene activation (74) exhibit a decline with age (75). This may help explain for a 51% disparity in EPO concentration. In accordance with the potential HIF responses during continuous hypoxia (22, 39, 40), intermittent hypoxia may result in a more appropriate methodology to increase EPO concentrations and oxygen transport indices. While no work has measured EPO concentrations in response to intermittent hypoxia and included older individuals, it remains unanswered if a significant reduction on in EPO synthesis, similar to Torpel et al (2019), occurs.

Hematological responses with Type 2 Diabetes

Diabetes afflicts 37.3 million individuals or 11.3 % of the total United States population (76). Furthermore, only 28% meet physical activity guidelines set forth by the American Diabetes Association, which may contribute to increased risk for morbidity, mortality, and overall lower levels of cardiovascular fitness (77). As such, patients with type 2 diabetes typically display on average a 20% reduction in maximal oxygen consumption, when compared to healthy individuals matched for age, weight, and physical activity levels (78-85). While there exists no previous work that has directly reported hemoglobin mass in this population, Lalande et al. (2010) determined that total blood volume was lower in eight men with type 2 diabetes when matched for age, weight, and physical activity levels to non-diabetic individuals (5). Despite normal hematocrit levels, total blood volume was reduced by 20%, suggesting a possible reduction in hemoglobin mass. Conversely, total blood volume, measured by the radioisotope method was reduced by 3% among 19 patients with poorly controlled diabetes in comparison to body size predicted values. However, patients with well controlled diabetes failed to demonstrate any reduction (86). Yet, a meta-analysis of 14 studies incorporating patients with type 1 and 2 diabetes reported a reduction in blood volume ranging from 3 to 23% with assessment methods predominately using Evans Blue dye and few using the carbon monoxide rebreathing technique (87). Of the works that utilized carbon monoxide rebreathing, Koponen et al. (2013) described an 8% reduction in hemoglobin mass and calculated blood volume in men with type 1 diabetes without complication matched for age, anthropometrics and level of physical activity to healthy non-diabetic individuals (4). Conversely, Rissanen et al. (2015) found a 9% decline in blood volume but no reported change in hemoglobin mass among young patients with type 1 diabetes. The authors suggest the decline was associated in part from poor hyperglycemic control that impairs ventricular relaxation and

compliance (88). Yet, in a separate investigation, patients with uncomplicated type 2 diabetes displayed a nonsignificant decline in blood volume in comparison to healthy non-diabetic controls (10%, p=0.09). Despite using the carbon monoxide rebreathing technique, hemoglobin mass was not presented (89). As such, it remains unknown as to whether a hemoglobin mass reduction or central limitations affecting stroke volume such as a hyperglycemia impaired ventricular relaxation or compliance that contributes to the ~20% decline in blood volume and maximal oxygen consumption among patients with well controlled type 2 diabetes.

MOLECULAR AND PHARMACOLOGICAL CONSIDERATIONS TO ERYTHROPOIETIN PRODUCTION IN PATIENTS WITH TYPE 2 DIABETES

Despite the evidence supporting a maximal oxygen consumption and blood volume decline, possible mechanisms that may contribute to a reduction in hemoglobin mass in patients with type 2 diabetes, have yet to elucidated. Reasonably, the involvement of EPO synthesis may play a critical role in understanding the upstream implications on hemoglobin mass. As such, Mojiminiyi et al. found 14% of 161 screened patients with type 2 diabetes expressing normal renal and hematological parameters, had a mean EPO of 8.1mU/mL, which was significantly lower (i.e., 19.1mU/mL) than non-diabetic controls (90). However, Craig et al. reported maintained EPO concentrations and an appropriate EPO synthesis response to lower hemoglobin levels in patients with type 2 diabetes without nephropathy (91). Despite an appropriate EPO response, there was no increase to reticulocytes counts. The authors suggest that elevated levels of inflammation associated with pro-inflammatory cytokines (92) may suppress erythrocyte maturation (93). Additionally, other plausible considerations resulting in impaired EPO synthesis or

effectiveness in patients with type 2 diabetes are elevated levels of systemic inflammation (94, 95), a decline EPO receptor quantity (60), hypoxia inducible transcription factor dysregulation (96, 97), diminished bone marrow responsiveness (98), pharmacological modulation (99-101), and/or the deleterious effects from advanced glycation end products (AGEs) (98). Specifically, AGEs triggered by chronic hyperglycemia and oxidative stress have been demonstrated to impair erythrocyte senescence (98), reduce erythrocyte deformability, facilitate incomplete erythrocyte phagocytosis, and trigger a decline in erythrocyte survival (102, 103). Secondarily, the increased renal oxygen consumption that accompanies chronic hyperglycemia and increases flux through the Na^+-glucose cotransporter creates renal tubular hypoxia. As a result, HIFs can become stabilized under normoxic conditions. Furthermore, this inappropriate hypoxic response stimulates an acute inflammatory reaction that encourages macrophage infiltration resulting with increased renal fibrosis (96, 97) and potentially leading to EPO synthesis impairment, even at early disease stages with limited symptomology (104). Conversely, a carbohydrate response element triggered by a separate hyperglycemic pathway in the hypoxia inducible factor promotor region, upregulates HIF-1α and 2α transcription, irrespective of oxygen levels (105). As such, first line pharmacologic treatment for patients with type 2 diabetes includes medications such as Metformin that protects renal tissue by reducing renal oxygen consumption and the resultant renal hypoxia. Specifically, Metformin has been indicated to directly impair HIF-1α accumulation (101). While there remains no measurement of EPO concentrations in response to metformin alone, a combination of metformin and another first line pharmacologic treatment, sulfonylureas (glimepiride), has been demonstrated to reduce red blood cell count and hemoglobin in patients with type 2 diabetes over time (99). Conversely, sodium glucose cotransporter 2 inhibitors that have recently become more popular for the management of type 2 diabetes effectively act to

stabilize HIF-2 and have been demonstrated to increase EPO concentrations and erythrocyte counts (100). As such, it remains important to consider the effects of routine pharmacology in connection to possible hemoglobin mass and EPO dysregulation assessments. However, medication use was excluded from the previous studies reporting a low hemoglobin mass with diabetes (4, 5), suggesting that diabetes independently attenuates signaling for EPO production.

HYPOXIA USE AND POTENTIAL IN FOR USE IN DIABETIC POPULATIONS

While no study has investigated the acute or chronic erythropoietic outcomes in response to intermittent hypoxia in patients with type 2 diabetes, Bosman et al. (2002) determined patients with type 1 diabetes with EPO-deficient anemia could raise EPO levels to a similar degree as healthy subjects 2 hours into a 6-hour hypoxia exposure (11.6-12.6% fraction of inspired oxygen, equivalent to 4000m). While no significant difference between the rate of EPO synthesis occurred, a nonsignificant trend of a slowed response was observed. As such it remains unanswered as to the effects of intermittent hypoxia, which may increase HIF accumulation over exposure time contrary to the effects from continuous hypoxia (22, 39, 40), could have at increasing EPO concentrations and the effects an increase may have on hemoglobin mass.

Objectives and hypotheses

STUDY 1:

The objective of Study 1 was to identify the shortest intermittent hypoxia protocol necessary to increase EPO concentration. The EPO response to the following four protocols

was determined: 1) spontaneous change under normoxia; 2) five 4-minute cycles of intermittent hypoxia; 3) eight 4-minute cycles of intermittent hypoxia, and; 4) 120 minutes of continuous hypoxia. It was hypothesized that both intermittent hypoxia protocols would significantly increase EPO concentrations in healthy individuals.

STUDY 2:

The objective of Study 2 was to determine whether the same intermittent hypoxia protocol from Study 1 also triggers an increase in EPO concentration in older adults. A secondary objective was to determine whether a single session of intermittent hypoxia leads to an increase in hemoglobin mass in older adults. It was hypothesized that a single exposure to intermittent hypoxia would increase EPO concentrations, which would lead to an increased hemoglobin mass in this population.

STUDY 3:

The objective of this Study 3 was to determine the effect of intermittent hypoxia on EPO concentration and hemoglobin mass in patients with type 2 diabetes. It was hypothesized that a single session of intermittent hypoxia will increase EPO concentrations and lead to an increase in hemoglobin mass in patients with type 2 diabetes.

CHAPTER III. STUDY #1: SHORT EXPOSURE TO INTERMITTENT HYPOXIA INCREASES ERYTHROPOIETIN LEVELS IN HEALTHY INDIVIDUALS

Abstract

Few minutes of hypoxic exposure stabilizes hypoxia-inducible factor-1α, resulting in erythropoietin (EPO) gene transcription and production. The objective of this study was to identify the shortest intermittent hypoxia protocol necessary to increase serum EPO levels in healthy individuals. In a first experiment, spontaneous EPO changes under normoxia (NORM) and the EPO response to five 4-minute cycles of intermittent hypoxia (IH5) were determined in six individuals. In a second experiment, the EPO response to eight 4-minute cycles of intermittent hypoxia (IH8) and 120 minutes of continuous hypoxia (CONT) was determined in six individuals. All hypoxic protocols were performed at a targeted arterial oxygen saturation of 80%. There was no significant change in EPO levels in response to normoxia or in response to five cycles of intermittent hypoxia (NORM: 9.5±1.8 to 10.5±1.8, IH5: 11.4±2.3 to 13.4±2.1 mU/ml, main effect for time $p=0.35$). There was an increase in EPO levels in response to eight cycles of intermittent hypoxia and 120 minutes of continuous hypoxia, with peak levels observed 4.5 hours after the onset of hypoxia (IH8: 11.2±2.0 to 16.7±2.2, CONT: 11.1±3.8 to 19.4±3.8 mU/ml, main effect for time $p<0.01$). Eight cycles of intermittent hypoxia increased EPO levels to a similar extent as 120 minutes of continuous hypoxia (main effect for condition $p=0.36$). Eight 4-minute cycles of intermittent hypoxia represent the shortest protocol to increase serum EPO levels in healthy individuals.

Introduction

Low partial pressure of oxygen triggers the release of erythropoietin (EPO), a glycoprotein that stimulates red blood cell production in order to increase oxygen-carrying capacity (10, 57). Exposure to hypoxia stabilizes hypoxia-inducible factor-1α (HIF-1α) within few minutes, which results in EPO gene transcription and production (10). Continuous exposure to hypoxia lasting between 84 to 120 minutes consistently increases serum EPO levels (11, 24-27, 106, 107). However, exposure to several short successive bouts of hypoxia also stabilizes HIF-1α and triggers similar increases in EPO levels. Indeed, a 4-hour intermittent hypoxia protocol consisting of 2.5 minutes of hypoxia alternating with 1.5 minute normoxia significantly increased EPO levels (11). Moreover, several sessions of intermittent hypoxia, consisting of 3-5 hypoxic cycles lasting 3-5 minutes interspersed with 3-minute normoxic cycles at an arterial oxygen saturation of approximately 80% increased red blood cell count in elderly men with and without coronary artery disease, suggesting that each session of intermittent hypoxia triggers the release of EPO (14). These findings suggest that intermittent hypoxia represents a time-efficient approach to induce increases in EPO levels. The objective of the present study was to identify the shortest intermittent hypoxia protocol necessary to increase EPO levels. The EPO response to the following four protocols was determined: 1) spontaneous change under normoxia; 2) five 4-minute cycles of intermittent hypoxia; 3) eight 4-minute cycles of intermittent hypoxia, and; 4) 120 minutes of continuous hypoxia. All hypoxic protocols were performed at a targeted arterial oxygen saturation of 80%. It was hypothesized that both intermittent hypoxia protocols would significantly increase EPO levels in healthy individuals.

Methods

A total of seven healthy, recreationally active adults participated in the study. Participants were excluded from the study if they had uncontrolled hypertension, were smokers, pregnant, had a history of cardiovascular disease, diabetes or lung disease, or were taking medication affecting the cardiovascular system. All participants provided informed written consent to participate in the study, which was approved by the Institutional Review Board of the University of Texas at Austin. The study consisted of two separate experiments. In Experiment 1, the EPO response to five 4-minute cycles of intermittent hypoxia (IH5) was compared to the spontaneous change in EPO levels under normoxic conditions (NORM) in order to account for diurnal variations. In Experiment 2, the EPO response to eight 4-minute cycles of intermittent hypoxia (IH8) was compared to the EPO response to 120 minutes of continuous hypoxia (CONT), a protocol that consistently increases EPO levels. All protocols within one experiment were separated by at least one day. Experiment 1 and 2 were performed approximately three months apart. Data analysis for Experiment 1 was conducted before performing Experiment 2, which resulted in slight modifications to the study protocol for Experiment 2.

HYPOXIC PROTOCOLS

Participants inhaled hypoxic air through a mask connected to a two-way rebreathing valve, which was connected to a five-liter non-diffusing gas bag (Hans Rudolph, Inc, Shawnee, KS, USA USA). The non-diffusing bag was itself connected to a gas tank of compressed air. Air was made hypoxic by titrating nitrogen into the breathing circuit. The flow of nitrogen was controlled to achieve an arterial oxygen saturation of 80%. Due to the high individual variability in hypoxic ventilatory responses, a fixed fraction of inspired

oxygen can result in a wide range of arterial oxygen saturation across individuals. In addition, arterial oxygen saturation increases over the course of five cycles of intermittent hypoxia at a fixed oxygen level (46). Thus, exposure to hypoxia was not performed at a fixed oxygen level but at a targeted arterial oxygen saturation in order to induce the same level of hypoxemia for each cycle of intermittent hypoxia and throughout the continuous hypoxia protocol in all participants. An arterial oxygen saturation of 80% was chosen based on our previous observation that an arterial oxygen saturation of 90% corresponding to a fraction of inspired oxygen of 0.12 ± 0.01 was not sufficient to significantly increase EPO levels in young healthy individuals (42). Each 4-minute hypoxic bout started once the participant reached an arterial oxygen saturation of 83%. For the IH5 protocol, each 4-minute hypoxic bout was followed by 4 minutes of normoxia. In order to shorten the total duration of the protocol, normoxic bouts of the IH8 protocol ended once arterial oxygen saturation reached baseline levels, which took approximately two minutes.

ERYTHROPOIETIN LEVELS

In previous studies, EPO levels consistently peaked 4 to 4.5 hours following the onset of a continuous hypoxic exposure (11, 25-27, 107). In Experiment 1, venous blood samples were therefore collected before, 2.5 and 4.5 hours after the beginning of the IH5 and NORM protocols. However, a delayed EPO response was observed following intermittent hypoxia, with EPO levels peaking 6 hours following the start of the exposure (11). In Experiment 2, venous blood samples were therefore collected before, 4.5 and 6 hours after the beginning of the IH8 and CONT protocols. Blood was centrifuged, serum aliquoted and stored at -80°C for subsequent analyses. Erythropoietin levels were determined using an enzyme-linked immunosorbent assay (Abcam, Cambridge, UK). The

coefficient of variations for the erythropoietin assay were 6.8 and 11.0% for Experiment 1 and 2, respectively.

PULMONARY GAS EXCHANGE AND HEMODYNAMICS

Breath-by-breath measures of pulmonary gas exchange were collected and analyzed every 10 seconds throughout all hypoxic protocols using a metabolic cart calibrated with room air and standardized gas (Ultima Cardio2, MGC Diagnostics, St. Paul, MN, USA). The pneumotachometer was mounted between the mask and the non-rebreathing valve of the breathing circuit. An arterial waveform obtained by finger plethysmography from the middle finger of the left hand was continuously recorded during all hypoxic protocols (NOVA, Finapres Medical Systems, Amsterdam, Netherlands). Brachial arterial blood pressure, heart rate, stroke volume, and cardiac output were derived from the arterial waveform, a method validated against invasive measures (108)**.** Arterial oxygen saturation was monitored by pulse oximetry throughout all hypoxic protocols. All data were recorded in LabChart for later analysis (Powerlab, ADI Instruments Inc., Colorado Springs, CO, USA).

DATA AND STATISTICAL ANALYSES

Participants' characteristics from both experiments were compared using a Student's t-test. In Experiment 1, a two-way repeated measures analysis of variance was used to evaluate the effect of condition (NORM and IH5) and time (Baseline, 2.5 and 4.5 hours) on EPO levels. In Experiment 2, a two-way repeated measures analysis of variance

was used to evaluate the effect of condition (IH8 and CONT) and time (Baseline, 4.5 and 6 hours) on EPO levels.

For the IH5 and IH8 protocols, 4-minute average values for pulmonary gas exchange and hemodynamic variables were calculated for each hypoxic bout. For the CONT protocol, 5-minute average values for pulmonary gas exchange and hemodynamic variables were calculated every 30 minutes (minutes 26-30, 56-60, 86-90, and 116-120). Baseline values for each physiological variable consisted of the 1-minute average preceding the start of the hypoxic protocol. A one-way repeated measured analysis of variance was used to evaluate the effect of each hypoxic bouts on all physiological variables. When main effects were significant, post hoc analyses were performed using Tukey's test. Unless specified, all values are presented as means ± standard deviations. Significance was set at $p \leq 0.05$.

Results

Six individuals (three women) participated in Experiment 1, and six individuals (three women) participated in Experiment 2. Five of these six individuals participated in both studies. Age, weight, height, systolic and diastolic blood pressure, heart rate and physical activity levels were similar between experiments (Table 1).

ERYTHROPOIETIN LEVELS

There was no significant spontaneous change in EPO levels under normoxic conditions and there was no significant increase in EPO levels in response to five cycles of intermittent hypoxia (Figure 1 and 2). Greater EPO levels were observed during IH5 in

comparison to NORM (main effect for condition, $p = 0.046$) (Figure 1). There was a significant increase in EPO levels in response to eight cycles of intermittent hypoxia and 120 minutes of continuous hypoxia, with peak levels observed 4.5 hours following the start of the hypoxic exposure for both protocols (main effect for time, $p < 0.01$) (Figure 1 and 2). Serum EPO levels increased to a similar extent in response to both protocols, with observed increases of 65 ± 65% and 85 ± 76% in response to eight cycles of intermittent hypoxia and 120 minutes of continuous hypoxia, respectively.

PULMONARY GAS EXCHANGE AND HEMODYNAMICS

Experiment 1. By design, intermittent hypoxia induced an average arterial oxygen saturation of 79 ± 1% (Figure 3), which translated to a fraction of inspired oxygen of 10.3 ± 0.7% (Figure 4) (main effect for hypoxia, $p < 0.001$ for both variables). Intermittent hypoxia did not significantly affect minute ventilation (Figure 4). Exposure to intermittent hypoxia did not affect blood pressure but triggered an increase in heart rate (main effect for hypoxia, $p = 0.01$), which resulted in an increased cardiac output (main effect for hypoxia, $p < 0.01$) (Figure 3). Specifically, heart rate during the first hypoxic cycle was greater than at baseline and during the last hypoxic cycle, and cardiac output was greater than baseline during the first two hypoxic cycles.

Experiment 2. Similar to Experiment 1, the arterial oxygen saturation of 80 ± 1% (Figure 3) achieved during intermittent hypoxia was equivalent to a fraction of inspired oxygen of 10.4 ± 0.2% (Figure 4) (main effect for hypoxia, $p < 0.001$ for both variables). Intermittent hypoxia did not significantly affect minute ventilation (Figure 4). Exposure to eight cycles of intermittent hypoxia did not affect blood pressure or cardiac output (Figure

3) but increased heart rate (main effect for hypoxia, $p = 0.02$), with a greater heart rate observed during the first two hypoxic cycles in comparison to baseline.

Two hours of continuous hypoxia at an arterial oxygen saturation of 81 ± 2% (Figure 3) resulted in a fraction of inspired oxygen of 11.9 ± 0.5 (Figure 4) (main effect for hypoxia, $p < 0.001$ for both variables). Exposure to continuous hypoxia increased systolic and diastolic blood pressures (main effect for hypoxia of $p = 0.03$ and $p = 0.04$, respectively) but did not affect heart rate or cardiac output (Figure 3). In comparison to baseline, minute ventilation was reduced during minutes 56 to 60 and 86 to 90 of continuous hypoxia (main effect for hypoxia, $p = 0.02$) (Figure 4). The reduced ventilation was caused by a significantly reduced respiratory rate at minutes 86 to 90 when compared to baseline (main effect for hypoxia, $p = 0.03$; baseline: 12.4 ± 3.0 vs. min 86-90: 11.7 ± 2.5 breaths/min).

Discussion

The objective of the present study was to identify the shortest intermittent hypoxia protocol needed to stimulate an increase in serum EPO levels in healthy individuals. The primary finding was that eight cycles of intermittent hypoxia increased EPO levels to a similar extent as 120 minutes of continuous hypoxia. Conversely, five cycles of intermittent hypoxia were not sufficient to increase EPO levels in healthy individuals. The longer total hypoxic duration or the additional hypoxic bouts may be responsible for the increase in EPO levels observed following eight, but not five, cycles of intermittent hypoxia.

Two hours of continuous hypoxia at a fraction of inspired oxygen ranging from 10 to 12.5% consistently increase EPO levels by 28-52%, with peak values observed four

hours after the onset of the hypoxic exposure (11, 26, 106, 107). Accordingly, the present two hours of continuous hypoxia at a fraction of inspired oxygen of 11.9 ± 0.5% triggered an average 85% increase in EPO levels observed 4.5 hours after the onset of hypoxia. Only one study previously determined whether intermittent hypoxia leads to a similar increase in EPO levels. A 4-hour intermittent hypoxia protocol consisting of 2.5 minutes of hypoxia at a fraction of inspired oxygen of 10.5% alternating with 1.5 minute of normoxia, representing a total hypoxic duration of approximately 108 minutes, resulted in a 52% increase in EPO levels (11). In the present study, the shorter intermittent hypoxia protocol consisting of eight 4-minute bouts of hypoxia at a fraction of inspired oxygen of 10.4 ± 0.2 %, translating to a total of 32 minutes under an arterial oxygen saturation of 83%, resulted in a similar 65% average increase in serum EPO levels. These findings suggest that brief successive bouts of hypoxia can stabilize HIF-1α and lead to EPO gene transcription and production to the same extent as two hours of continuous hypoxia. These results are also consistent with the observation that an intermittent hypoxia protocol, consisting of six 5-minute hypoxic bouts interspersed with 5-minute normoxic bouts, and two hours of continuous hypoxia induced comparable stabilization of HIF-1α in mice (12). Interestingly, it has been reported that one hour of continuous hypoxia at a fraction of inspired oxygen of 10.5 % does not induce an increase in EPO levels (11). Thus, the intermittence of the stimulus appears to have an important effect on the release of EPO. However, five cycles of intermittent hypoxia at a fraction of inspired oxygen of 10.3 ± 0.7%, representing a total duration of 20 min under an arterial oxygen saturation of 83%, was not sufficient to trigger a significant increase in EPO levels. Therefore, at a fraction of inspired oxygen of 10.5%, a total hypoxic duration above 20 minutes or more than five hypoxic bouts are needed to induce increases in serum EPO levels.

We previously reported that a single session of intermittent hypoxia consisting of five 4-minute hypoxic bouts at a targeted arterial oxygen saturation of 90% was not sufficient to increase EPO levels in young healthy individuals (42). An arterial oxygen saturation of approximately 80% was administered during repeated sessions of intermittent hypoxia that increased red blood cell count in elderly men with and without coronary artery disease (14). Thus, protocols of five and eight cycles of intermittent hypoxia at a targeted oxygen saturation of 80% were performed to determine the contribution of hypoxic duration on the release of EPO. Eight, but not five, cycles of intermittent hypoxia at an arterial oxygen saturation of 80% induced the release of EPO, establishing the minimal hypoxic duration needed to trigger a release of EPO in healthy individuals. Future studies are required to determine whether a more severe hypoxic exposure would trigger an increase in EPO levels following five cycles of intermittent hypoxia.

Intermittent hypoxia at an arterial oxygen saturation of 80% did not affect minute ventilation. These findings are in accordance with our previous observations that intermittent hypoxia protocols of three or five 4-minute bouts of poikilocapnic hypoxia at an average arterial oxygen saturation of 87 and 89%, respectively, did not affect pulmonary gas exchange in young healthy individuals (42, 43). However, the observed lack of change in ventilation contradicts previous findings that intermittent hypoxia protocols of five 6-minute hypoxic bouts at oxygen levels of 10% or at a targeted arterial oxygen saturation of 80% as well as an intermittent hypoxia protocol of seven 5-min hypoxic bouts at an arterial oxygen saturation of 70-80% increased ventilation (36, 45, 46). The lack of significant effect on ventilation observed in the present study may result from the shorter duration of the hypoxic cycles. While others (36) reported a lack of increase in minute ventilation during one hour of continuous hypoxia at an arterial oxygen saturation of 70-80%, the observed reduction in minute ventilation during minutes 56-60 and 86-90 of the continuous

hypoxia protocol was unexpected. However, this statistically significant reduction in minute ventilation represents a small 2 L/min difference caused by a slightly greater respiratory rate at baseline. The lack of increase in minute ventilation during continuous hypoxia was possibly due to a progressive reduction in the fraction of inspired oxygen when titrating nitrogen in the breathing circuit.

Exposure to continuous hypoxia increased systolic and diastolic blood pressure when compared to baseline values. These findings are in accordance with previous observations that one hour of continuous hypoxia at an arterial oxygen saturation of 70-80% increased systolic and diastolic blood pressure (15), and that 20 minutes of continuous hypoxia at an arterial oxygen saturation of 80% increased sympathetic activity, systolic blood pressure, and mean arterial pressure (31, 33). On the other hand, both intermittent hypoxia protocols did not significantly affect blood pressure, which is in agreement with our previous findings that intermittent hypoxia consisting of three or five 4-min hypoxic cycles at a targeted arterial oxygen saturation of 90% did not have any effect on blood pressure (42, 43). Moreover, repetitive bouts of normobaric, poikilocapnic hypoxia consisting of 5-6 minutes at a fraction of inspired oxygen of 0.10 or at an arterial oxygen saturation of 80% did not affect blood pressure in young healthy individuals (45, 46, 109). Hypercapnia, but not hypocapnia, induces a sympathoexcitation that persists following a 20-minute continuous hypoxic exposure (110). Therefore, the isocapnia or hypocapnia observed during short hypoxic bouts likely contributes to the lack of increase in blood pressure observed with intermittent hypoxia.

A reduced partial pressure of oxygen stimulates erythropoiesis. In the present study, exposure to hypoxia was performed at a targeted arterial oxygen saturation to induce the same level of hypoxemia across cycles and across participants. However, this targeted saturation potentially corresponds to varying partial pressures of oxygen across

individuals. Measures of partial pressures of oxygen through arterial blood gases would allow true uniformity of the stimulus across individuals. Three of the four women participating in the study were using hormonal contraceptives, and there was no control for menstrual cycle phase when performing experiments. However, the menstrual cycle phase does not seem to affect the erythropoietic response to hypoxia as similar increases in EPO levels were reported upon ascent to altitude in women in the luteal or follicular phase of the menstrual cycle (111). In conclusion, eight 4-minute bouts of intermittent hypoxia, or 32 minutes under an arterial oxygen saturation of 83%, represent the shortest hypoxic exposure to significantly increase EPO levels in healthy individuals. A rise in EPO levels eventually leads to the creation of reticulocytes that mature into red blood cells within 5-6 days (8). A single 90-minute session of continuous hypoxia increased EPO levels by 39% and led to an increased number of immature red blood cells in untrained young healthy men (27). Thus, future studies should determine whether a single session of intermittent hypoxia can significantly increase red blood cells volume and hemoglobin mass in different populations ranging from endurance-trained athletes to clinical populations with impaired oxygen-carrying capacity such as patients with type 2 diabetes (5, 112).

Table 1. Participants' characteristics

Variables	Experiment 1	Experiment 2
Age (years)	28 ± 7	28 ± 7
Weight (kg)	78.1 ± 19.3	81.3 ± 17.4
Height (cm)	176 ± 11	178 ± 7
Systolic blood pressure (mmHg)	121 ± 12	122 ± 15
Diastolic blood pressure (mmHg)	77 ± 11	77 ± 10
Heart rate (bpm)	62 ± 19	70 ± 12
Physical activity levels (hours/week)	4.9 ± 4.2	6.7 ± 3.7

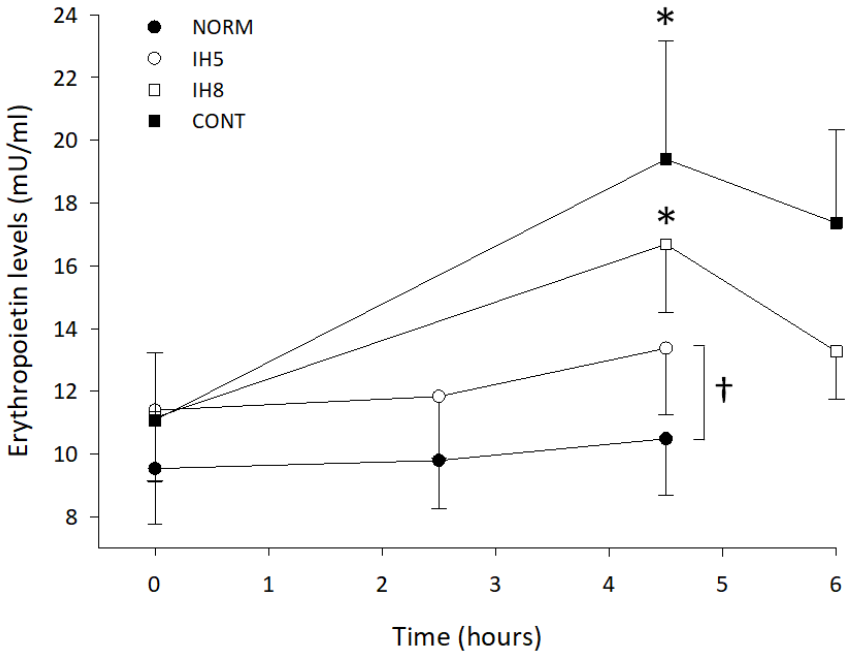

Figure 1. Erythropoietin response to spontaneous change under normoxia (NORM), five cycles of intermittent hypoxia (IH5), eight cycles of intermittent hypoxia (IH8) and 120 minutes of continuous hypoxia (CONT). Values are presented as means ± standard error of the mean. * $p < 0.05$ between baseline and 4.5 hours, † main effect for condition. (Wojan et al., 2021)

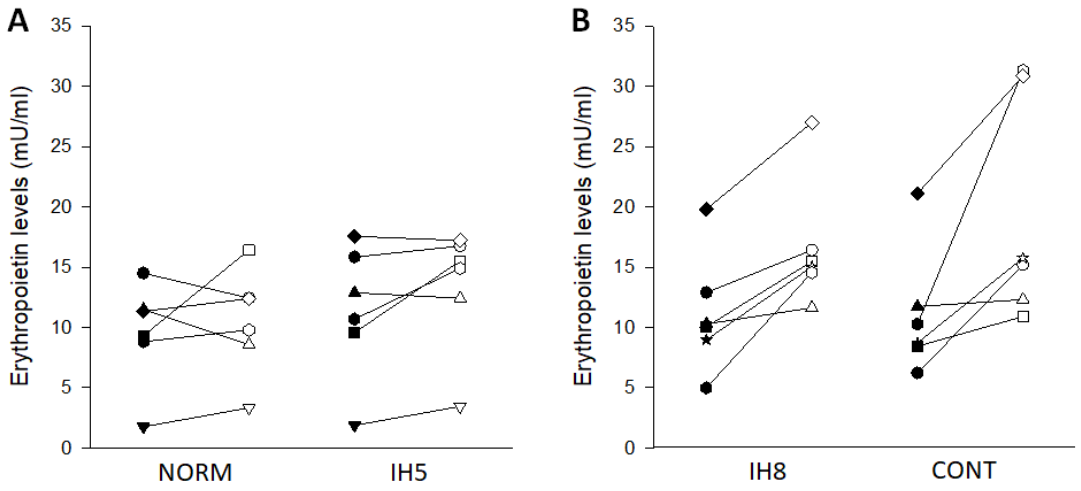

Figure 2. Individual EPO responses to (A) spontaneous change under normoxia (NORM) and five cycles of intermittent hypoxia (IH5), and (B) eight cycles of intermittent hypoxia (IH8) and 120 minutes of continuous hypoxia (CONT). (Wojan et al., 2021)

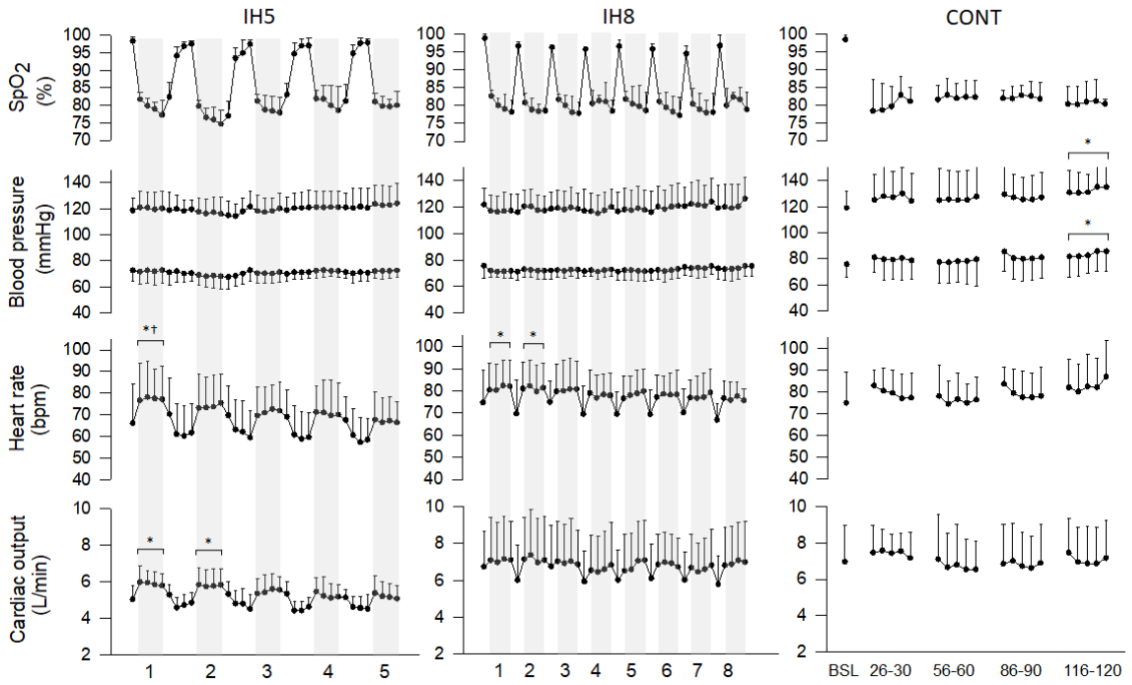

Figure 3. Five cycles of intermittent hypoxia (IH5): 1-minute averages for baseline and each hypoxic and normoxic bouts. Eight cycles of intermittent hypoxia (IH8): 1-minute averages for baseline, each hypoxic bouts and the highest 10-second average based on arterial oxygen saturation during each normoxic bout. Two hours of continuous hypoxia (CONT): 1-minute averages for baseline and minutes 26-30, 56-60, 86-90 and 116-120. Main effect for hypoxia ($p < 0.001$) for arterial oxygen saturation (SpO2) all conditions. Main effect for hypoxia for heart rate for IH5 ($p = 0.01$) and IH8 ($p = 0.02$). Main effect for hypoxia for cardiac output ($p < 0.01$) for IH5. Main effect for hypoxia for systolic and diastolic blood pressures ($p = 0.03$ and $p = 0.04$, respectively). * $p < 0.05$ different from baseline. (Wojan et al., 2021)

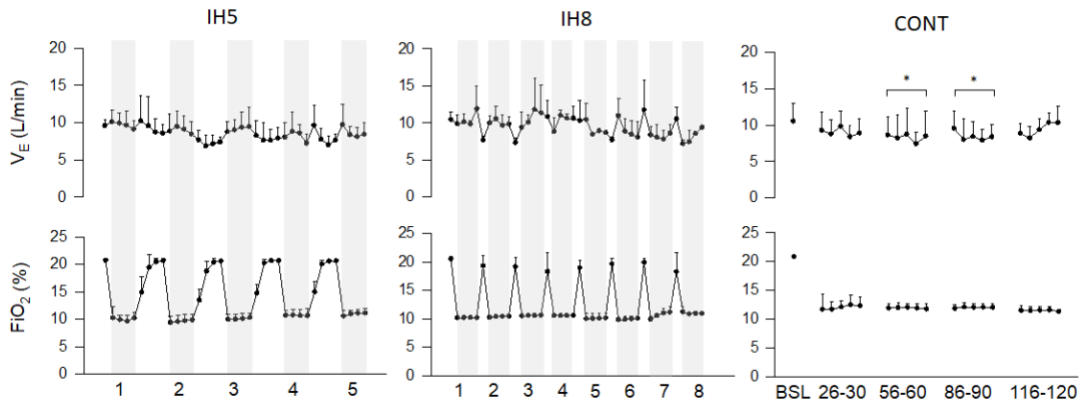

Figure 4. Five cycles of intermittent hypoxia (IH5): 1-minute averages for baseline and each hypoxic and normoxic bouts. Eight cycles of intermittent hypoxia (IH8): 1-minute averages for baseline, each hypoxic bouts and the highest 10-second average based on arterial oxygen saturation during each normoxic bout. Two hours of continuous hypoxia (CONT): 1-minute averages for baseline and minutes 26-30, 56-60, 86-90 and 116-120. Main effect for hypoxia ($p < 0.001$) for fraction of inspired oxygen (FiO2) all conditions. Main effect for hypoxia ($p = 0.02$) for ventilation (VE) for CONT. * $p < 0.05$ different from baseline. (Wojan et al., 2021)

CHAPTER IV. STUDY #2: INTERMITTENT HYPOXIA INCREASES ERYTHROPOIETIN LEVELS IN OLDER INDIVIDUALS

Abstract

Few minutes of hypoxia exposure stabilizes hypoxia-inducible factors, resulting in erythropoietin (EPO) gene transcription and production. A brief intermittent hypoxia exposure increased EPO levels in young individuals, suggesting that a single session of intermittent hypoxia has the potential to increase oxygen-carrying capacity. The objective of this study was to determine the effect of a single session of intermittent hypoxia on EPO levels and hemoglobin mass in older individuals. Twenty-two participants (12 women, age: 53±7 years) were randomly assigned to an intermittent hypoxia group (IH, n=11) or an intermittent normoxia group (IN, n=11). Intermittent hypoxia consisted of eight 4-min cycles at a targeted oxygen saturation of 80% interspersed with normoxic cycles to resaturation. Air was made hypoxic by titrating nitrogen into a breathing circuit. Intermittent normoxia consisted of the same protocol, but nitrogen was not added to the breathing circuit. EPO levels were measured before and 4.5 hours after the beginning of each protocol. Hemoglobin mass was assessed via carbon monoxide rebreathing before and seven days following intermittent hypoxia or normoxia exposure. Intermittent hypoxia lowered oxygen saturation to 82±3%, corresponding to a fraction of inspired oxygen of 10.9±1.0%. There was a greater increase in EPO levels following intermittent hypoxia than intermittent normoxia (IH: 3.2±2.2 vs. IN: 0.7±0.8 mU/ml, $p<0.01$). There was no change in hemoglobin mass in response to intermittent hypoxia. In conclusion, a single session of eight 4-min cycles of hypoxia increased EPO levels in older individuals. Additional sessions of intermittent hypoxia are potentially needed to increase hemoglobin mass.

Introduction

Maximal oxygen consumption, the ability of the cardiovascular system to transport and use oxygen during maximal exercise, predicts mortality to the same or potentially greater extent as traditional risk factors such as smoking, hypertension, high cholesterol, and type 2 diabetes (1). Moreover, maximal oxygen consumption progressively decreases with advancing age (48). Oxygen transport is achieved through the binding of oxygen to hemoglobin contained in red blood cells, therefore, both hemoglobin mass and red blood cell volume strongly correlate with maximal oxygen consumption (2). A reduced red blood cell volume, mainly due to a lower fat free mass and decreased physical activity levels, has been reported in older men and women (3, 6). In these studies, red blood cell volume was calculated from plasma volume and hematocrit levels, which are greatly influenced by hydration status (52). When directly assessed, red blood cell count was also reported to decrease with aging in men (54). Therefore, an intervention that increases hemoglobin mass and red blood cell volume could ultimately improve maximal oxygen consumption in an older population characterized by reduced fat free mass and physical activity levels.

Erythropoietin (EPO) stimulates red blood cell production in response to hypoxia (10). Indeed, a rise in serum EPO levels results in the creation of reticulocytes that mature into red blood cells within 7 days (9). With aging, circulating EPO levels have been reported to be either higher (64-68, 113), lower (114) or not different (69, 70) from the EPO levels observed in young individuals. However, the impact of aging on the EPO response to a hypoxic stimulus remains equivocal. We previously reported that short, intermittent exposure to mild hypoxia triggers an increase in serum EPO levels in young adults (115). Thus, the objective of this study was to determine whether the same intermittent hypoxia protocol also triggers an increase in EPO levels in older adults. A secondary objective was to determine whether a single session of intermittent hypoxia

leads to an increase in hemoglobin mass in older adults. It was hypothesized that a single exposure to intermittent hypoxia would increase EPO levels, which would lead to an increased hemoglobin mass in this population.

Methods

PARTICIPANTS AND STUDY DESIGN

Both men and women were recruited to participate in the study. Participants provided written informed consent for participating in the study, which was approved by the Institutional Review Board of the University of Texas at Austin (IRB study number 2017090015). Participants were excluded from the study if they had uncontrolled hypertension or were taking more than one antihypertensive medication, were smokers, were pregnant, or had a history of cardiovascular disease, diabetes, or lung disease. Twenty-two participants were randomly assigned to an intermittent hypoxia group (IH, n = 11, six women) or a placebo intermittent normoxia group (IN, n = 11, six women). The study consisted of three visits over a period of eight days. Measures of hemoglobin mass were performed on Visits 1 and 3. The EPO response to intermittent hypoxia or intermittent normoxia was assessed on Visit 2, which always took place in the morning (start time ranging between 7:00 and 10:45 am). Visit 3 took place seven days following Visit 2. Since menstrual blood loss has no impact on hemoglobin mass (116), visits were scheduled during any phase of the menstrual cycle in the three premenopausal women participating in the study. All participants were asked to avoid alcohol and intense physical activity on the day preceding all visits.

INTERMITTENT HYPOXIA AND INTERMITTENT NORMOXIA

The intermittent hypoxia protocol consisted of eight 4-min hypoxic cycles at a targeted oxygen saturation of 80% interspersed with normoxic cycles to resaturation (115). Participants inhaled hypoxic air through a mask connected to a two-way rebreathing valve that was itself connected to a five-liter non-diffusing bag (Hans Rudolph, Inc, Shawnee, KS, USA). The non-diffusing bag was connected to a gas tank of compressed air and a gas tank of nitrogen. Air was made hypoxic by titrating nitrogen into the breathing circuit to achieve an oxygen saturation of 80%. Each 4-min hypoxic cycle began once the participant reached an oxygen saturation of 83%. Intermittent normoxia consisted of the same protocol but nitrogen was not introduced in the breathing circuit.

ERYTHROPOIETIN LEVELS

Venous blood samples were collected before and 4.5 hours after the beginning of intermittent hypoxia and intermittent normoxia. Blood was centrifuged, serum aliquoted, and stored at -80°C for subsequent analyses. Erythropoietin levels were determined using an enzyme-linked immunosorbent assay (Abcam, Cambridge, UK). The average coefficient of variation for the erythropoietin assays was 7.2%.

HEMATOLOGICAL VARIABLES

Hemoglobin mass was determined using a modified version of the optimized carbon monoxide rebreathing technique (53, 117). A venous blood draw was obtained to determine baseline carboxyhemoglobin, hematocrit, and hemoglobin levels (ABL 80 FLEX OSM, Radiometer, Copenhagen, Denmark). Participants rebreathed a bolus of carbon monoxide from a low-volume closed-circuit system containing air over a period of

two minutes. Carboxyhemoglobin levels were measured again 10 minutes following the start of the carbon monoxide rebreathing. Hemoglobin mass, red blood cell volume, plasma volume, and total blood volume were calculated from the change in carboxyhemoglobin levels induced by carbon monoxide rebreathing (118). In our laboratory, the coefficient of variation for hemoglobin mass, based on duplicate measures performed on consecutive days in five individuals is 2.6%.

PULMONARY GAS EXCHANGE AND HEMODYNAMICS

On Visit 1, average heart rate and blood pressure were calculated from two measures obtained following five minutes of supine rest (Omron Healthcare, Inc., Lake Forest, IL, USA). Breath-by-breath measures of pulmonary gas exchange were collected using a metabolic cart calibrated with standardized gas (Ultima Cardio2, MGC Diagnostics, St. Paul, MN, USA), and averaged every 10 seconds throughout intermittent hypoxia and intermittent normoxia. The pneumotachometer was mounted between the mask and the non-rebreathing valve of the breathing circuit. An arterial waveform obtained via finger plethysmography and oxygen saturation obtained via pulse oximetry were continuously recorded throughout both protocols (NOVA, Finapres Medical Systems, Amsterdam, Netherlands). Brachial arterial blood pressure, heart rate, stroke volume, cardiac output and total peripheral resistance were derived from the arterial waveform. All data were recorded in LabChart for later analysis (Powerlab, ADI Instruments Inc., Colorado Springs, CO, USA).

DATA AND STATISTICAL ANALYSES

Participants' characteristics were compared using a Student's t-test. A two-way analysis of variance was used to evaluate the effect of condition (intermittent hypoxia vs. intermittent normoxia) and time (pre- vs. post-intervention) on EPO levels and hematological variables. When main effects or interactions were significant, post hoc analyses were performed using Tukey's test. Average values for each hemodynamic and pulmonary gas exchange were calculated from each 4-min hypoxic cycle of the intermittent hypoxia protocol and for each 4-min normoxic cycle of the intermittent normoxia protocol. A Student's t-test was used to evaluate the effect of condition on pulmonary gas exchange and hemodynamic variables. Pearson's correlation was used to determine the relation between oxygen saturation, fraction of inspired oxygen and changes in EPO levels. A post-hoc power analysis using EPO levels before and after intermittent hypoxia and intermittent normoxia in our 22 participants resulted in a power of 0.61. Significance was set at $p \leq 0.05$. Unless stated, all values are presented as means ± standard deviations.

Results

Age, weight, height, body mass index, hematocrit levels, hemoglobin concentration, blood pressure, heart rate, and physical activity levels were not different between groups (Table 2). According to the World Health Organization's criteria, none of the participants had anemia as defined as hemoglobin levels <12.0 g/dL in women and <13.0 g/dL in men. Visit 3 took place seven days following Visit 2 for all but one participant where Visit 3 took place eight days following Visit 2. EPO levels were greater during intermittent hypoxia than intermittent normoxia (main effect for condition, $p = 0.02$), with greater post-intervention levels observed with intermittent hypoxia than

intermittent normoxia (Figure 5). EPO levels tended to increase following exposure to intermittent hypoxia and intermittent normoxia (main effect for time, $p = 0.08$). The change in EPO levels was greater following intermittent hypoxia than intermittent normoxia (IH: 3.2 ± 2.2 vs. IN: 0.7 ± 0.8 mU/ml, $p < 0.01$). There was a correlation between changes in EPO levels and oxygen saturation ($r = -0.63$, $p < 0.01$) and fraction of inspired oxygen ($r = -0.75$, $p < 0.01$). There was no change in any of the hematological variables in response to either intermittent hypoxia or intermittent normoxia (Table 3). Figure 6 shows the average fraction and inspired oxygen and oxygen saturation responses to eight cycles of intermittent hypoxia. Intermittent hypoxia resulted in lower oxygen saturation levels compared to intermittent normoxia (Table 4) but did not affect any other hemodynamic variables. Intermittent hypoxia induced a lower fraction of inspired oxygen and end-tidal oxygen in comparison to intermittent normoxia (Table 5). There was no difference in respiratory rate and end-tidal CO_2 between conditions while a greater tidal volume resulted in a greater minute ventilation during intermittent hypoxia in comparison to intermittent normoxia.

Discussion

The purpose of the present study was to determine whether a single session of intermittent hypoxia increases EPO levels in older adults. Eight 4-min hypoxic cycles at an oxygen saturation of 82 ± 3 % corresponding to a fraction of inspired oxygen of 10.9 ± 1.0% induced a 31% increase in EPO levels in older adults. In contrast, intermittent normoxia induced a 9% increase in EPO levels, consistent with the reported 15% diurnal variation in EPO levels from mid-morning to late afternoon (107). A secondary objective of the present study was to determine whether this single session of intermittent hypoxia

leads to an increase in hemoglobin mass. Contrary to our hypothesis, one session of intermittent hypoxia did not induce a rise in hemoglobin mass.

Exposure to hypoxia stabilizes hypoxia-inducible factors (HIFs) within few minutes, increasing EPO gene transcription and production (10). However, aging seems to negatively impact the EPO production in response to hypoxia. Indeed, exposure to three hours of continuous hypoxia at a targeted oxygen saturation of 80% increased EPO levels in both young and older adults, however, EPO levels were approximately three times greater in young vs. older adults (73). It was therefore suggested that an age-dependent defect in HIF-1 action may reduce EPO gene expression in response to hypoxia (75). In the present study, eight 4-min hypoxic cycles, corresponding to a total hypoxic duration of 32 minutes at a targeted oxygen saturation of 80%, increased EPO levels in older adults. Similarly, this rise in EPO levels was half the rise in EPO levels previously observed in response to the same intermittent hypoxia protocol in younger adults (115). Nonetheless, the present findings further confirm that a short, intermittent hypoxic stimulus triggers EPO production, challenging the long-standing belief that continuous hypoxic exposures ranging between 84 and 120 minutes are necessary to trigger an increase in EPO levels (11, 24-27, 107). The present findings are supported by the observation that exposure to 30 minutes of intermittent hypoxia or two hours of continuous hypoxia induced comparable activation of the HIF pathway as defined by stabilization of HIF-1α protein (12). Thus, intermittent hypoxia represents an optimal approach to elicit EPO production.

A rise in EPO levels leads to the creation of reticulocytes that eventually mature into red blood cells (9). An increase in EPO levels induced by a single 90-min session of continuous hypoxia previously resulted in an increased number of reticulocytes two days following the hypoxic exposure (27). Despite the observed increase in EPO levels in the present study, eight 4-min hypoxic cycles did not result in an increased hemoglobin mass.

It is therefore hypothesized that additional sessions of intermittent hypoxia are necessary to induce an increase in red blood cell volume and, thereby, hemoglobin mass. Although EPO levels were not assessed, five consecutive days of a similar intermittent hypoxia protocol, consisting of 4-6 min hypoxic bouts at a mean oxygen saturation of 85% for a total hypoxic duration of approximately 70 minutes, increased red blood cell count in young (119) and older adults (15). Moreover, 15 sessions of intermittent hypoxia increased red blood cell count and hemoglobin mass in elderly men with and without coronary artery disease, and in individuals at risk for or with mild chronic obstructive pulmonary disease (13, 14).

The lower average fraction of inspired oxygen of 10.9% accompanying intermittent hypoxia resulted in a greater tidal volume and minute ventilation in comparison to intermittent normoxia. These results are in contrast with our previous findings that three 4-min hypoxic cycles at a fraction of inspired oxygen of 11.4% and eight 4-min hypoxic cycles at a fraction of inspired oxygen of 10.4% did not affect minute ventilation (44, 115). However, the present findings are in agreement with others who showed that five 6-min hypoxic cycles at a fraction of inspired oxygen of 10% increase ventilation (45, 46) through an increase in tidal volume (46). There was no difference in blood pressure or heart rate between intermittent hypoxia and intermittent normoxia in the present study. These results are consistent with previous findings that repetitive bouts of normobaric, poikilocapnic hypoxia, consisting of 4-6 min at a fraction of inspired oxygen of 10% or at an oxygen saturation of 80%, did not affect arterial blood pressure in young and older individuals (44-46, 109, 115). Thus, intermittent hypoxia consisting of eight 4-min hypoxic cycles at a targeted oxygen saturation of 80% has minimal impact on hemodynamics and pulmonary gas exchange in older adults.

PERSPECTIVE AND SIGNIFICANCE

A single session of intermittent hypoxia elicited a rise in EPO levels in older men and women. Future studies are needed to determine the minimum number of intermittent hypoxia sessions necessary to induce significant increases in red blood cell volume and hemoglobin mass in this population. Thus, intermittent hypoxia represents a novel intervention to mitigate the decline in oxygen-carrying capacity associated with the reduced maximal oxygen consumption observed with aging.

Table 2. Participants' characteristics

Variables	IH	IN
Age (years)	53 ± 8	54 ± 7
Height (cm)	176 ± 10	172 ± 11
Weight (kg)	73.2 ± 10.5	73.4 ± 20.0
Body mass index (kg/m^2)	23.7 ± 2.7	24.6 ± 5.6
Systolic blood pressure (mmHg)	116 ± 13	120 ± 10
Diastolic blood pressure (mmHg)	75 ± 9	77 ± 6
Heart rate (bpm)	60 ± 10	59 ± 11
Hemoglobin ($g \bullet dL^{-1}$)	13.9 ± 1.3	14.0 ± 1.0
Hematocrit (%)	43 ± 4	43 ± 3
Physical activity (hours/week)	7.6 ± 9.0	5.1 ± 4.0
Medication use, n		
ACE inhibitor	1	0
Angiotensin II blocker	1	0
Beta blockers	0	1
Statin	2	2
Levothyroxine	0	3
Estrogen/progesterone	0	3

IH: Intermittent hypoxia; IN: Intermittent normoxia; ACE: angiotensin converting enzyme.

Table 3. Hematological variables before and after intermittent hypoxia and intermittent normoxia

	IH		IN	
Variables	Pre	Post	Pre	Post
Hemoglobin mass (g)	752 ± 189	754 ± 189	800 ± 179	801 ± 186
Hemoglobin mass ($g \cdot kg^{-1}$)	10.3 ± 2.2	10.2 ± 2.1	10.9 ± 1.5	10.9 ± 1.8
Red blood cell volume (L)	2.31 ± 0.58	2.31 ± 0.57	2.45 ± 0.55	2.45 ± 0.57
Plasma volume (L)	3.56 ± 0.51	3.68 ± 0.46	3.81 ± 0.77	3.69 ± 0.72
Blood volume (L)	5.87 ± 1.04	5.99 ± 1.00	6.26 ± 1.29	6.15 ± 1.24
Blood volume ($ml \cdot kg^{-1}$)	80.7 ± 13.3	81.5 ± 12.1	86.5 ± 14.5	84.8 ± 15.4

IH: Intermittent hypoxia; IN: Intermittent normoxia

Table 4. Hemodynamics during intermittent hypoxia and intermittent normoxia

Variables	IH	IN
Systolic blood pressure (mmHg)	117 ± 17	120 ± 10
Diastolic blood pressure (mmHg)	74 ± 13	77 ± 7
Cardiac output ($L \bullet min^{-1}$)	5.7 ± 1.5	5.4 ± 1.5
Heart rate (bpm)	68 ± 9	61 ± 11
Stroke volume (ml)	83 ± 17	89 ± 15
Total peripheral resistance ($mmHg \bullet L^{-1} \bullet min^{-1}$)	16.8 ± 6.1	17.7 ± 3.9
Oxygen saturation (%)	82 ± 3 *	98 ± 2

* $p < 0.05$ between intermittent hypoxia (IH) and intermittent normoxia (IN)

Table 5. Pulmonary gas exchange during intermittent hypoxia and intermittent normoxia

Variables	IH	IN
Respiratory rate (breaths•min^{-1})	11 ± 4	14 ± 3
Tidal volume (ml)	915 ± 431 *	491 ± 185
Ventilation (L•min^{-1})	8.9 ± 1.5 *	6.6 ± 1.9
End-tidal CO_2 (mmHg)	35 ± 3	39 ± 3
End-tidal oxygen (mmHg)	44 ± 4 *	101 ± 4
Fraction of inspired oxygen (%)	10.9 ± 1.0 *	21.0 ± 0.3

* $p < 0.05$ between intermittent hypoxia (IH) and intermittent normoxia (IN)

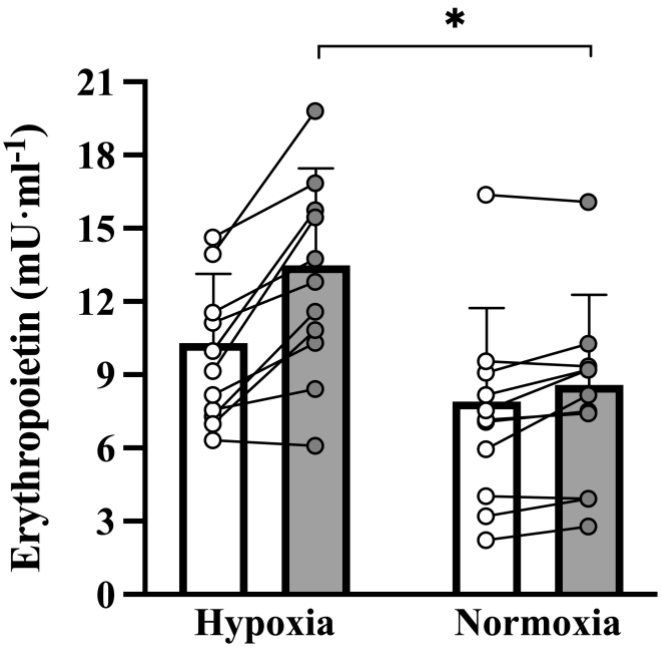

Figure 5. Average and individual erythropoietin levels before (white circles and bars) and after (grey circles and bars) eight cycles of intermittent hypoxia (n = 11, six women) and intermittent normoxia (n = 11, six women). Values are presented as mean ± standard deviation. Main effect for condition, * $p < 0.05$ different from intermittent normoxia.

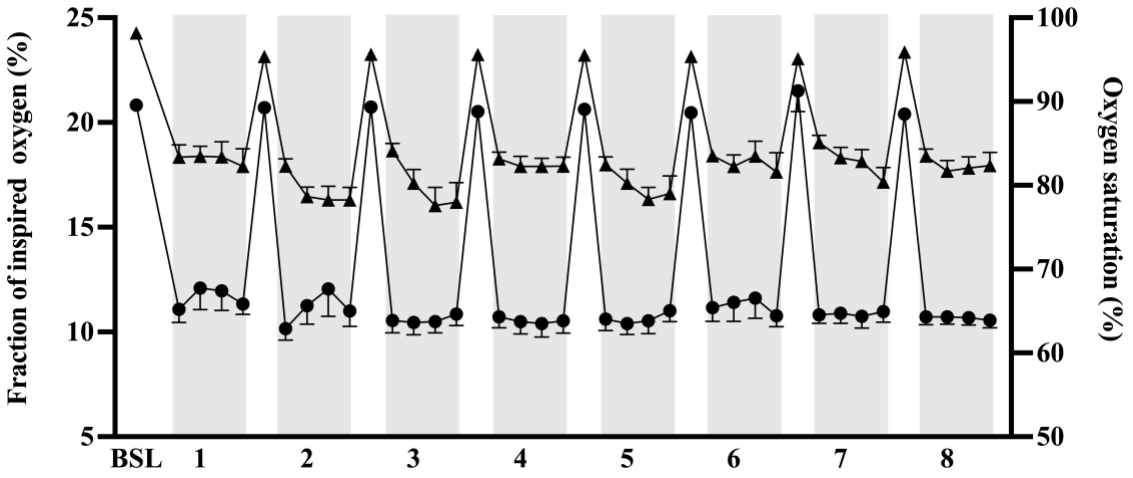

Figure 6. Fraction of inspired oxygen (black circles) and oxygen saturation (black triangles) at baseline (BSL) and in response to eight cycles of hypoxia at a targeted oxygen saturation of 80%. n = 11, six women. Values are presented as mean ± standard error of the mean.

CHAPTER V. STUDY #3: IMPAIRED ERYTHROPOIETIN RESPONSE TO INTERMITTENT HYPOXIA IN PATINETS WITH TYPE 2 DIABETES

Abstract

Patients with type 2 diabetes (T2D) exhibit an approximately 20% decline in maximal oxygen consumption when compared to healthy adults. Hemoglobin mass strongly correlates to maximal oxygen consumption. A reduced total blood volume has been observed in patients with T2D, suggesting that a reduced hemoglobin mass contributes to the decreased maximal oxygen consumption. Intermittent hypoxia, consisting of alternating short bouts of breathing hypoxic and normoxic air, increases erythropoietin (EPO) levels, the hormone regulating red blood cell production. Thus, the objective of this study was to determine the effect of a single session of intermittent hypoxia on EPO levels and hemoglobin mass in patients with T2D. Ten patients with T2D were exposed to an intermittent hypoxia protocol consisting of eight 4-min cycles at a targeted oxygen saturation of 80% interspersed with normoxic cycles to resaturation. EPO levels were measured before and 4.5 hours after the beginning of the protocol. Hemoglobin mass was assessed via carbon monoxide rebreathing before and seven days following intermittent hypoxia. By design, intermittent hypoxia lowered oxygen saturation (97±2 to 81±2%, $p<0.01$). There was no change in EPO levels (11.9±5.3 to 12.1±4.3 mU/ml, $p=0.83$) or hemoglobin mass (864±152 to 850±150 g, $p=0.64$) following exposure to intermittent hypoxia. In conclusion, a single session of intermittent hypoxia did not increase EPO levels or hemoglobin mass in patients with T2D. These findings suggest an impaired EPO response to decreased oxygen levels in patients with T2D, which may contribute to the reduced hemoglobin mass and total blood volume observed in this population.

Introduction

Total hemoglobin mass, the absolute mass of hemoglobin contained in red blood cells, determines oxygen transport capacity and strongly correlates to maximal oxygen consumption (1). Patients with type 2 diabetes exhibit on average a 20% decline in maximal oxygen consumption when compared to healthy adults matched for age, weight, and physical activity levels (78, 80, 82, 84, 85). Interestingly, a 20% reduction in total blood volume along with normal hematocrit levels was also observed in patients with type 2 diabetes, suggesting that a reduced hemoglobin mass contributes to the decreased maximal oxygen consumption observed in this population (5). Accordingly, a reduced hemoglobin mass was correlated to the low maximal aerobic capacity observed in men living with type 1 diabetes (4). Therefore, an intervention that increases hemoglobin mass could lead to an improved maximal oxygen consumption in patients with type 2 diabetes. Alternative interventions to improve maximal oxygen consumption in patients with type 2 diabetes are urgently needed as maximal oxygen consumption predicts mortality to the same or potentially greater extent as traditional risk factors such as smoking, hypertension, high cholesterol, and even type 2 diabetes itself (1). Moreover, small increases in maximal oxygen consumption can lead to a considerable reduction in adverse cardiovascular event rates (1).

Hypoxia stimulates the release of erythropoietin (EPO), the hormone regulating red blood cell production (10). A blunted EPO production may contribute to the low hemoglobin mass reported in diabetes. Patients with type 2 diabetes who have a normal renal function and normal hematological variables have either maintained (91) or lower (90) circulating EPO levels than healthy individuals. However, despite having an EPO deficiency, patients with type 1 diabetes with early nephropathy associated with symptomatic autonomic neuropathy showed an appropriate EPO response to six hours of

moderate hypoxia induced by breathing oxygen levels of approximately 12% (120). We previously showed that one session of intermittent hypoxia, consisting of eight 4-min hypoxic cycles at a targeted oxygen saturation of 80%, increased EPO levels in young (115) and older adults (121). Thus, the purpose of this study was to determine the effect of intermittent hypoxia on EPO levels and hemoglobin mass in patients with type 2 diabetes. It was hypothesized that a single session of intermittent hypoxia will increase EPO levels and lead to an increase in hemoglobin mass in patients with type 2 diabetes.

Methods

PARTICIPANTS AND STUDY DESIGN

Men and women with type 2 diabetes participated in the study. All individuals provided informed written consent for participating in the study, which was approved by the Institutional Review Board of the University of Texas at Austin (IRB study number 2017090015). Participants were excluded from the study if they had uncontrolled hypertension or were taking more than one antihypertensive medication, were smokers, had a history of cardiovascular disease such as myocardial infarction, prior ischemia, stroke, or lung disease, were taking insulin, or had previously been diagnosed with diabetic complications such as nephropathy, neuropathy, or retinopathy. Participants were assigned to a single-blind, single-arm trial which consisted of three visits over a period of eight days. Hemoglobin mass was measured on Visit 1 and 3, and the EPO response to a session of intermittent hypoxia was assessed on Visit 2. Visit 2 was scheduled the day after Visit 1 and always took place in the morning (start time ranging between 7:30 am and noon). Since a rise in serum EPO levels results in the creation of reticulocytes that

mature into red blood cells within 5–6 days (9), Visit 3 was scheduled seven days following Visit 2. All participants were asked to avoid alcohol and intense physical activity on the day preceding all visits.

INTERMITTENT HYPOXIA PROTOCOL

The intermittent hypoxia protocol consisted of eight 4-min hypoxic cycles at a targeted oxygen saturation of 80% interspersed with normoxic cycles to resaturation (115). Participants inhaled hypoxic air through a mask connected to a two-way rebreathing valve that was connected to a five-liter non-diffusing bag (Hans Rudolph, Inc, Shawnee, KS, USA). The non-diffusing bag was itself connected to a tank of compressed air and a tank of nitrogen. Hypoxia was created by titrating nitrogen into the breathing circuit to achieve an oxygen saturation of 80%. Each 4-min hypoxic cycle began once the participant reached an oxygen saturation of 83%. Normoxic cycles ended once oxygen saturation reached baseline levels.

ERYTHROPOIETIN LEVELS

Venous blood samples were collected before and 4.5 hours after the beginning of intermittent hypoxia (115). Blood was centrifuged, serum aliquoted, and stored at -80°C for subsequent analyses. EPO levels were determined using an enzyme-linked immunosorbent assay (Abcam, Cambridge, UK). The coefficient of variation for the erythropoietin assay was 6.7%.

HEMATOLOGICAL VARIABLES

Hemoglobin mass was determined using a modified version of the optimized carbon monoxide rebreathing technique (53, 117). A venous blood draw was first obtained to determine baseline carboxyhemoglobin, hematocrit, and hemoglobin levels (ABL 80 FLEX OSM, Radiometer, Copenhagen, Denmark) and HbA1c levels (DCA Vantage, Siemens, Tarrytown, NY). Participants then rebreathed an individually calculated volume of carbon monoxide introduced into a low-volume closed-circuit system containing room air for two minutes. Carboxyhemoglobin levels were measured again 10 minutes following the start of the carbon monoxide rebreathing. Hemoglobin mass, red blood cell volume, plasma volume, and total blood volume were calculated from the change in carboxyhemoglobin levels induced by carbon monoxide rebreathing (118). In our laboratory, the coefficient of variation for hemoglobin mass is 2.6%, determined by duplicate measures performed on consecutive days in five individuals.

PULMONARY GAS EXCHANGE AND HEMODYNAMICS

On Visit 1, average heart rate and blood pressure were calculated from two measures obtained after 10 minutes of supine rest (Omron Healthcare, Inc., Lake Forest, IL, USA). Measures of pulmonary gas exchange were collected using a metabolic cart calibrated with standardized gas (Ultima Cardio2, MGC Diagnostics, St. Paul, MN, USA) and averaged every 10 seconds throughout the intermittent hypoxia protocol. The pneumotachometer was mounted between the mask and the non-rebreathing valve of the breathing circuit. Arterial waveforms obtained by finger plethysmography were continuously recorded throughout the intermittent hypoxia protocol (NOVA, Finapres Medical Systems, Amsterdam, Netherlands). Brachial arterial blood pressure, heart rate,

stroke volume, cardiac output and total peripheral resistance were derived from the arterial waveform. Oxygen saturation was monitored via pulse oximetry throughout the intermittent hypoxia protocol (NOVA, Finapres Medical Systems, Amsterdam, Netherlands). All data were recorded in LabChart for later analysis (Powerlab, ADI Instruments Inc., Colorado Springs, CO, USA).

DATA AND STATISTICAL ANALYSES

A paired t-test was used to evaluate the effect of intermittent hypoxia on EPO levels and hematological variables. Baseline values for each pulmonary gas exchange and hemodynamic variable consisted of the 1-min average immediately prior to the start of intermittent hypoxia. Average values for pulmonary gas exchange and hemodynamics variables were calculated using the 4-min average of each hypoxic cycle. Values are presented as means ± standard deviations, unless stated otherwise. Significance was set at $p \leq 0.05$.

Results

Age, height, weight, body mass index, blood pressure, heart rate, HbA1c, hemoglobin concentration, hematocrit levels, physical activity levels, and medication use are presented in Table 6. According to the World Health Organization's criteria, none of the participants had anemia as defined as hemoglobin levels <12.0 g/dL in women and <13.0 g/dL in men. All participants also had hematocrit levels within reference values. EPO levels did not change following intermittent hypoxia (Figure 7). There was also no change in any hematological variables in response to intermittent hypoxia (Table 7).

There was no correlation between HbA1c levels and hemoglobin mass measured prior to intermittent hypoxia ($r = -0.12$, $p = 0.72$). Figure 8 demonstrates the average oxygen saturation and fraction of inspired oxygen responses to eight cycles of hypoxia. Intermittent hypoxia lowered oxygen saturation and increased cardiac output following an increase in heart rate (Table 8). There was no change in stroke volume, blood pressure, and total peripheral resistance with intermittent hypoxia. Intermittent hypoxia reduced fraction of inspired oxygen, end-tidal oxygen, and end-tidal carbon dioxide (Table 9). There was no change in ventilation, tidal volume, and respiratory rate with intermittent hypoxia.

Discussion

The purpose of the present study was to determine whether a single session of intermittent hypoxia increases EPO levels, and consequently triggers an increase in hemoglobin mass, in patients with type 2 diabetes. Contrary to our hypothesis, eight 4-min hypoxic cycles at an oxygen saturation of 81 ± 2% did not induce any change in EPO levels in patients with type 2 diabetes. Accordingly, there was no change in hemoglobin mass following exposure to a single session of intermittent hypoxia.

ERYTHROPOIETIN LEVELS IN RESPONSE TO INTERMITTENT HYPOXIA

Circulating EPO values normally range between 6 and 32 mU/ml (8). In the present study, non-anemic patients with type 2 diabetes showed circulating EPO levels ranging between 6 and 23 mU/ml, indicating normal EPO production. Exposure to hypoxic conditions prevents hypoxia-inducible factors (HIFs) degradation. HIFs directly activate

the expression of a multitude of target genes, including EPO gene transcription and production in the kidneys (8, 10). However, exposure to intermittent hypoxia failed to increase EPO levels in patients with type 2 diabetes. In comparison, the same intermittent hypoxia protocol increased EPO levels by 65% in young adults (115) and by 31% in age-matched older adults (121), suggesting an independent effect of type 2 diabetes on the EPO response to hypoxia. A hyperglycemia-mediated dysregulation in HIF-1 signaling could explain the reduced EPO gene expression in response to hypoxia in patients with type 2 diabetes (96). Contrary to the present findings, Bosman *et al*. (120) observed an appropriate EPO response to continuous hypoxia in five patients with type 1 diabetes, EPO-deficient anemia, early diabetic nephropathy, and symptomatic autonomic neuropathy. Indeed, EPO levels increased to the same extent in patients with type 1 diabetes and healthy individuals in response to six hours of hypoxia at a fraction of inspired oxygen of 12% corresponding to an oxygen saturation of 83% (120). Although the rate of increase in EPO levels was the same between groups, the rise seemed delayed in patients with type 1 diabetes. A delayed EPO response to hypoxia could explain the observed lack of increase following our 32-min intermittent hypoxia protocol in comparison to six hours of continuous hypoxia.

HEMATOLOGICAL RESPONSE TO INTERMITTENT HYPOXIA

As expected, intermittent hypoxia did not change hemoglobin mass following a lack of increase in EPO levels in patients with type 2 diabetes. Nonetheless, the present findings confirm the previously suggested lower hemoglobin mass in patients with type 2 diabetes. Indeed, the previous observation of a 20% lower total blood volume and similar hematocrit levels between patients with type 2 diabetes and age- and weight-matched individuals suggested a lower hemoglobin mass in patients with type 2 diabetes (5). In the

present study, patients with type 2 diabetes had a hemoglobin mass normalized to body weight of 8.2 ± 1.6 g/kg. In comparison, hemoglobin mass normalized to body weight was 10.2 ± 2.1 g/kg in age- but not weight-matched adults (121). Thus, a 20% lower relative hemoglobin mass was observed in patients with type 2 diabetes despite the greater proportion of women, who have lower relative hemoglobin mass than men, in the age-matched group in comparison to the type 2 diabetes group (55% vs. 40%, respectively). These findings are also consistent with the reduced hemoglobin mass previously reported in patients with type 1 diabetes in comparison to age- and weight-matched healthy individuals (4). The authors hypothesized that the low hemoglobin mass observed in patients with type 1 diabetes suggests an attenuated signaling for EPO production in response to low tissue oxygen pressure in peritubular fibroblasts of the renal cortex, which is supported by the lack of EPO production in response to intermittent hypoxia in patients with type 2 diabetes.

PULMONARY GAS EXCHANGE AND HEMODYNAMICS

Eight 4-min cycles of hypoxia at a fraction of inspired oxygen of 11% did not affect minute ventilation in patients with type 2 diabetes. These results are in accordance with our previous findings that the same intermittent hypoxia protocol at a fraction of inspired oxygen of 10% did not affect minute ventilation in young adults (115), and that five 6-min hypoxic cycles at a fraction of inspired oxygen of 13% did not immediately affect minute ventilation in patients with type 2 diabetes (122), but contradicts findings that five to eight hypoxic cycles lasting between 4 to 6 minutes at a fraction of inspired oxygen of 10-11% increased ventilation (45, 46) through an increase in tidal volume (46, 121).

Blood pressure was not affected by intermittent hypoxia in patients with type 2 diabetes. These results are consistent with previous findings showing that repetitive bouts of normobaric, poikilocapnic hypoxia, consisting of 4-6 min at a fraction of inspired oxygen of 10% or at an oxygen saturation of 80%, did not affect blood pressure in young and older individuals (45, 46, 109, 115, 123, 124). In contrast, blood pressure was increased immediately after five 6-min hypoxic cycles at a fraction of inspired oxygen of 13% in patients with type 2 diabetes (122). In accordance with our previous findings, eight 4-min hypoxic cycles increased heart rate (44, 115), which resulted in a slight increase in cardiac output in patients with type 2 diabetes. Thus, intermittent hypoxia at a targeted oxygen saturation of 80% has a minimal impact on hemodynamics in patients with type 2 diabetes.

LIMITATIONS

This study did not include a control group. However, the present findings were compared to the EPO response to the same intermittent hypoxia protocol performed in a group of age-matched individuals (121, 123). Similarly, hemoglobin mass measured in patients type 2 diabetes was compared to the hemoglobin mass previously measured in age- but not weight-matched individuals. Lean body mass, the anthropometric index that most closely correlates to hemoglobin mass (124), was not assessed in the present study. Using a precise predictive model for lean body mass (125), we calculated hemoglobin mass normalized to lean body mass for patients with type 2 diabetes and for age-matched individuals. Hemoglobin mass normalized to lean body mass was 14.1 ± 1.6 vs. 15.1 ± 1.9 g/kg ($p = 0.11$) in patients with type 2 diabetes and age-matched healthy individuals, respectively, further supporting an effect of type 2 diabetes on hemoglobin mass. Beside

insulin, there was no inclusion restriction on medication used to treat type 2 diabetes and participants did not change their medication over the short time course of the study. However, medication that treats type 2 diabetes such as canagliflozin (100), a sodium-glucose cotransporter 2 inhibitor, mediates EPO production while metformin inhibits renal tubular HIF-1α expression (101). While medication may contribute to the lack of EPO response in response to intermittent hypoxia, it does not solely explain the reduced hemoglobin mass observed with diabetes. Indeed, medication use was excluded from previous studies reporting a low hemoglobin mass with diabetes (4, 5), suggesting that diabetes independently attenuates signaling for EPO production in response to hypoxia.

In conclusion, a single session of intermittent hypoxia failed to trigger EPO production in patients with type 2 diabetes, possibly due to an impaired HIF signaling. The present findings confirm that hemoglobin mass is severely reduced in patients with type 2 diabetes. An impaired EPO response to hypoxia likely contributes to the lower hemoglobin mass, and thereby reduced oxygen-carrying capacity, observed in patients with type 2 diabetes.

Table 6. Participants' characteristics

Variables	
n (women)	10 (4)
Age (years)	53 ± 10
Height (cm)	174 ± 11
Weight (kg)	107.3 ± 19.5
Body mass index (kg/m^2)	36.2 ± 8.5
Systolic blood pressure (mmHg)	126 ± 6
Diastolic blood pressure (mmHg)	87 ± 5
Heart rate (bpm)	73 ± 14
HbA1c (%)	7.2 ± 1.2
Hemoglobin (g/dL)	14.3 ± 1.0
Hematocrit (%)	44 ± 3
Physical activity (hours/week)	1.0 ± 1.9
Diabetes duration (years)	6 ± 5
Medication use, n	
Metformin	7
SGLT2 inhibitors	3
GLP-1-RA	4
DPP-4 inhibitors	1
Sulfonylureas	2
Thiazolidinediones	3
Growth hormone-inhibiting hormone	1
ACE inhibitor	1
Angiotensin II blocker	3
Calcium channel blockers	1
Diuretics	3
Statins	5
Levothyroxine	2

HbA1c: glycated hemoglobin; SGLT-2: sodium-glucose cotransporter-2; GLP1-RA: glucagon-like peptide-1 receptor agonists; DPP-4: dipeptidyl peptidase 4; ACE: angiotensin converting enzyme.

Table 7. Hematological variables before and after intermittent hypoxia

Variables	Pre	Post
Hemoglobin mass (g)	864 ± 152	850 ± 150
Hemoglobin mass (g/kg)	8.2 ± 1.6	8.1 ± 1.9
Red blood cell volume (L)	2.65 ± 0.47	2.61 ± 0.46
Plasma volume (L)	3.97 ± 0.53	4.02 ± 0.57
Blood volume (L)	6.62 ± 0.94	6.63 ± 0.95
Blood volume (ml/kg)	63.4 ± 10.0	63.1 ± 10.5

Table 8. Hemodynamics before and during intermittent hypoxia

Variables	Baseline	IH
Systolic blood pressure (mmHg)	124 ± 9	129 ± 10
Diastolic blood pressure (mmHg)	79 ± 6	81 ± 7
Cardiac output (L/min)	9.1 ± 2.7	9.8 ± 2.8 *
Heart rate (bpm)	78 ± 9	84 ± 10 *
Stroke volume (ml)	116 ± 27	116 ± 29
Total peripheral resistance (mmHg/L/min)	11.3 ± 3.3	10.7 ± 2.9
Oxygen saturation (%)	97 ± 2	81 ± 2 *

* $p < 0.05$ between baseline and intermittent hypoxia (IH)

Table 9. Pulmonary gas exchange before and during intermittent hypoxia

Variables	Baseline	IH
Respiratory rate (breaths/min)	13 ± 4	12 ± 4
Tidal volume (ml)	738 ± 442	799 ± 331
Ventilation (L/min)	7.9 ± 1.7	8.4 ± 1.7
End-tidal carbon dioxide (mmHg)	38 ± 3	34 ± 3 *
End-tidal oxygen (mmHg)	99 ± 5	46 ± 6 *
Fraction of inspired oxygen (%)	20.8 ± 0.1	11.1 ± 1.0 *

* $p < 0.05$ between baseline and intermittent hypoxia (IH)

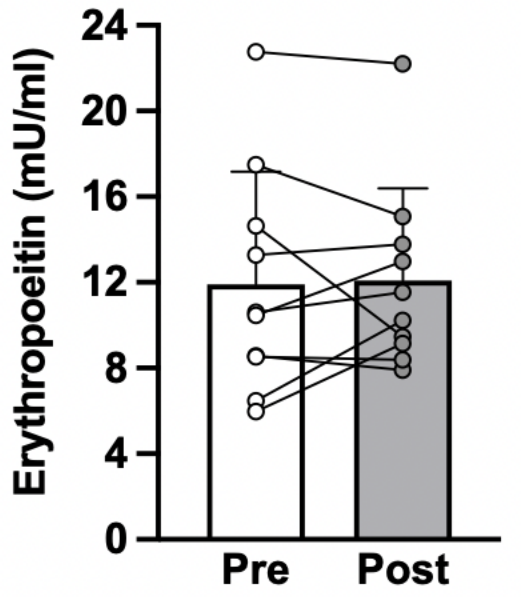

Figure 7. Average and individual erythropoietin levels before (white) and after (grey) eight cycles of hypoxia. n = 10 (4 women). Values are presented as mean ± standard deviation.

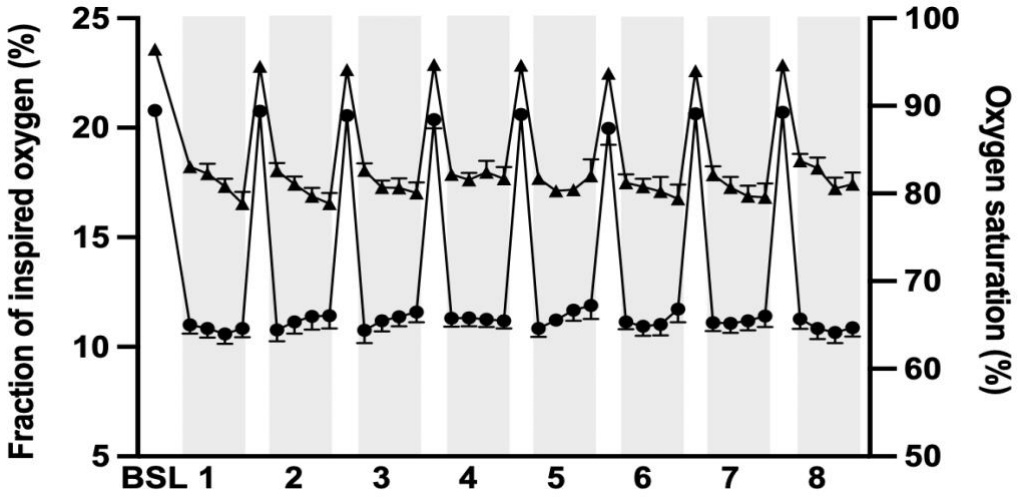

Figure 8. Fraction of inspired oxygen (black circles) and oxygen saturation (black triangles) at baseline (BSL) and in response to eight cycles of hypoxia. n = 10 (4 women). Values are presented as mean ± standard error of the mean.

CHAPTER VI. SUMMARY AND FUTURE DIRECTIONS

This dissertation aimed to define and apply an intermittent hypoxia protocol to increase EPO concentrations and potentially improve hemoglobin mass in young adults, older adults, and patients with type 2 diabetes.

The first study determined the shortest hypoxia protocol to elicit an increase in EPO concentrations in young healthy individuals. Specifically, a 65% increase in EPO concentration from eight 4-minute hypoxic cycles at a targeted oxygen saturation of 80% interspersed with short normoxia periods, totaling 32 hypoxic minutes, was similar to a 2-hour continuous hypoxia protocol. The second study applied the previously identified intermittent hypoxia protocol to a population of older individuals and assessed potential changes to hemoglobin mass levels. While EPO concentrations were increased by 31%, no change to hemoglobin mass levels occurred. The third study replicated the protocol from the second study in a population of patients with type 2 diabetes. Interestingly, EPO levels did not change in response to the intermittent hypoxia protocol, suggesting an impaired EPO response to hypoxia in this population. Unsurprisingly, without a change to EPO concentration, there was no change to hemoglobin mass levels. However, this study is the first to report hemoglobin mass values in a population of type 2 diabetes.

Future studies should investigate the potential mechanisms that may decrease the hypoxia mediated increase to EPO concentrations in older and patients with type 2 diabetes such as inflammatory status. Moreover, an assessment of HIF levels prior to, during, and after intermittent hypoxia exposure could elucidate a true impairment affecting an aging or population of type 2 diabetes. Conversely, alternative intermittent hypoxia protocols that incorporate moderate hyperoxia (30-40% fraction of inspired oxygen) for resaturation cycles, may increase ROS and in turn HIF responses. Secondarily, these hypoxia/hyperoxia

protocols may permit a greater volume of hypoxic stress over a period of time by shortening time to resaturation. Lastly, an investigation that directly assesses hemoglobin mass with age and potentially type 2 diabetes may help to identify cardiorespiratory constraints and a possible mechanism of decline. In conjunction with our current findings, these additional studies could support beneficial health outcomes in diseased populations or lead to potential performance enhancements for athletic endeavors.

CHAPTER VII. REFERENCES

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