RELATIONSHIP BETWEEN BASELINE INFLAMMATION AND PEAK ERYTHROPOIETIN LEVELS IN PEOPLE UNDERGOING CARBON MONOXIDE INHALATION AND HOT WATER IMMERSION

by

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Title: Relationship between Baseline Inflammation and Peak Erythropoietin Levels in People Undergoing Carbon Monoxide Inhalation and Hot Water Immersion

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Erythropoietin (EPO) is a hormone produced by the kidneys that is responsible for stimulating red blood cell (RBC) production. The stimulus for EPO production is a reduction in oxygen delivery to the kidneys, which can occur by reducing either the rate of blood flow or the oxygen content of the blood being delivered to the kidneys. However, EPO is not the only protein that can regulate RBCs. High levels of circulating inflammatory proteins can negatively impact RBC mass, and one of the pathways by which this can occur is by inhibiting EPO production. Recently, carbon monoxide (CO) inhalation and heat have been used as ways to reduce renal oxygen delivery, yet no studies have examined these treatments in combination nor the effects of inflammation on the EPO response in humans. The purpose of this study was twofold: 1) to determine whether combining CO inhalation and heat via hot water immersion has an additive effect on circulating EPO concentration, and 2) to examine the association between baseline inflammatory protein concentrations and EPO concentrations. It was hypothesized that 1) CO and heat will have an additive effect on circulating EPO concentrations, and 2) higher baseline levels of circulating inflammation will result in reduced EPO concentration in response to heat and CO stimuli. By inducing a hypoxic response through the interventions of CO inhalation, hot water immersion, and combined CO inhalation and heat, the mechanism(s) by

which EPO is released can be better understood. Research on this topic also has important implications in treating high circulating inflammation in chronic diseases and female athletes.

Male and female subjects underwent three treatments: inhalation of CO, hot-water immersion (HWI), and a combination of CO inhalation and HWI. On the CO inhalation visits, the volume of CO administered was 1.0mL/kg body weight for men and 0.8mL/kg body weight for women, and subjects breathed that volume twice, each bout lasting 10 minutes. This volume of CO was intended to raise blood carbon monoxide saturation to 10-15% and reduce functional oxygen saturation to 85-90% to simulate a moderate altitude. On the heat visits, subjects sat in a hot tub heated to 40˚C for 45 minutes. An intravenous (IV) catheter was placed for all study visits to collect venous blood at baseline and every hour after treatment for six hours. Whole blood was spun and the serum stored at -80˚C until analysis. The serum was analyzed for EPO concentration at all time points using an ELISA kit. Baseline inflammation was analyzed using a multi-plex flow cytometry assay (Human Inflammation Panel 1, BioLegend) that measures the following inflammatory proteins: interleukins (IL)-1β, -6, -8, -10, -12p70, -17A, -18, -23, and -33; interferons (IFN)-α2, -γ; tumor necrosis factor (TNF)-α; and monocyte chemoattractant protein (MCP)-1.

All interventions were found to increase concentrations of EPO. Contrary to our hypothesis, there were no additive effects of CO inhalation and hot water immersion. A positive linear relationship was found between peak EPO concentration and baseline IL-18 concentration, although the reasoning for this relationship must be explored further.

Acknowledgements

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Introduction

Erythropoietin (EPO) is a hormone produced by the kidneys that is responsible for stimulating red blood cell (RBC) production. The stimulus for EPO production is a reduction in oxygen delivery to the kidneys, which can occur by reducing either the rate of blood flow or the oxygen content of the blood being delivered to the kidneys. However, EPO is not the only protein that can regulate RBCs. High levels of circulating inflammatory proteins can negatively impact RBC mass, and one of the pathways by which this can occur is by inhibiting EPO production. The main function of RBCs is to deliver oxygen throughout the body via the protein hemoglobin, so a reduction in RBCs decreases oxygen delivery. Recently, carbon monoxide (CO) inhalation and heat have been used as ways to reduce renal oxygen delivery. Because CO has a greater binding affinity for hemoglobin than oxygen, CO reduces the arterial oxygen content and therefore leads to hypoxemia. Heat diverts blood flow away from the kidney to the periphery for heat dissipation and body cooling, thereby reducing blood flow and oxygen delivery to the kidney. However, no studies have examined the effect of these interventions in combination on the concentration of circulating EPO, and no studies have examined the effects of inflammatory proteins on circulating EPO levels in humans. The purpose of this study was twofold: 1) to determine whether combining CO inhalation and heat via hot water immersion had an additive effect on circulating EPO concentration, and 2) to examine the associations between baseline inflammatory protein concentrations and EPO concentrations following the three challenges. It was hypothesized that 1) CO and heat would have an additive effect on circulating EPO concentrations, and 2) higher baseline levels of circulating inflammation would result in a reduced EPO concentration in response to heat and CO stimuli. Research on this topic has important implications in treating high inflammation in chronic diseases and female athletes.

Background

Regulation of EPO

The main function of RBCs is to deliver oxygen throughout the body. Most of the oxygen is delivered bound to hemoglobin. An RBC life span is approximately 120 days, and healthy adults produce a turnover of about 200 billion blood cells a day (Bhoopalan, Huang, and Weiss 2020). To support the high cell turnover rate, low levels of EPO are expressed to maintain RBC mass in healthy humans. Additionally, EPO can then increase significantly in response to various stimuli to produce more RBCs (Jelkmann 2011).

When oxygen delivery is reduced systemically, hypoxia inducible factor (HIF) pathways are stimulated. HIFs are transcription factors that modulate the cellular response to low oxygen and are composed of an alpha and beta subunit. In normoxia, higher oxygen availability leads to proteasomal degradation of the HIF-α subunit and therefore transcriptional inhibition of HIF. In hypoxia, the lack of oxygen allows the HIF-α subunit to dimerize with the HIF-β subunit and move to the nucleus. Once dimerized, HIFs can bind to hypoxia-response elements (HREs) and other factors to promote transcription of HIF-dependent genes. EPO is one gene downstream of the HIF pathway, and is dependent on HIF-2α (Malkov, Lee, and Taylor 2021). HIF-2α is also crucial for iron uptake and utilization, which is important for RBC production (Haase 2013).

Renal EPO production increases in response to a reduction in oxygen delivery specifically to the kidneys. This can occur via reduced blood flow or reduced oxygen content of blood (equation 1). The production of EPO occurs in the renal cortex (Haase 2013). Once released from the kidney, EPO is responsible for stimulating erythropoiesis in red bone marrow. More specifically, EPO acts on committed erythroid progenitor cells known as colony forming unit-erythroid (CFU-E) and burst forming unit-erythroid (BFU-E) to stimulate cell proliferation

and erythroid fate (Bhoopalan, Huang, and Weiss 2020; Koury and Bondurant 1991). Erythropoiesis leads to increased RBC mass so that oxygen delivery to tissues increases. This increase in the capacity of the blood to carry oxygen eventually allows systemic oxygen levels to return to normal. From here, EPO production can be inhibited via a negative feedback loop (Bhoopalan, Huang, and Weiss 2020; Koury and Bondurant 1991).

Many studies reveal high altitude hypoxia induced an increase in EPO expression. Chronic altitude studies with an elevation threshold of ≥2,100-2,500 meters result in significant increases in EPO, with peaks seen within 48 hours after onset (Faura et al. 1969; Ge et al. 2002). In addition, acute hypoxia studies show that EPO peaks 4-6 hours after the period of hypoxic breathing (Berglund et al. 2002; Wojan et al. 2021).

Mechanisms of CO and Heat

Oxygen delivery to an organ or tissue is defined as the regional volume of oxygen supplied per minute. It is dependent on regional blood flow (Q) and the arterial oxygen content (CaO_2) (equation 1). CaO_2 is determined by the hemoglobin (Hb) saturation, Hb saturated with O_2 (SaO2), and the amount of O_2 that each gram of Hb can carry (1.34 ml) (equation 2). An additional small amount of the arterial oxygen content is determined by the amount of oxygen dissolved in plasma. Changes in any of the variables in equations 1 and 2 can affect O2 delivery.

1. O_2 ***Delivery = Q x Ca*** O_2
2. ***Ca*** O_2 ***= [(1.34 ml O2/ g Hb)([Hb]/ 100 ml blood)(Sa*** O_2***)] + [(0.003 ml*** O_2***/ mm Hg/ 100 ml blood)(Pa*** O_2***)]***

For this study, CO and heat were chosen as interventions because of their ability to target different components of the oxygen delivery equation (equation 1). Because CO binds with a greater affinity to Hb than O_2 it therefore outcompetes O_2 for Hb binding and reduces arterial

oxygen content. This results in reduced oxyhemoglobin (Hb O_2) and increased carboxyhemoglobin (HbCO). Because Hb has an affinity for CO 200-300 times greater than Oxygen (O_2), Sa O_2 will decrease and oxygen delivery is impaired (Bolla and Cadet 2007).

An additional important consideration with the use of CO is the duration of hypoxic stimulus. Hypoxia, regardless of whether it is normobaric (breathing a reduced fraction of inspired oxygen), or hypobaric (at altitude), will reduce Ca O_2. However, altitude and breathing low oxygen restrict the period of hypoxia to the duration at high altitude or the duration of breathing low oxygen (Subudhi et al. 2014). To the contrary, CO inhalation alone at normobaric pressures will stimulate hypoxia or hypoxemia and remain in the blood for up to 4-5 hours beyond the period of CO breathing (Zavorsky et al. 2012).

Heat changes regional oxygen delivery by redirecting blood flow to different vascular beds. The kidney receives up to 20% of cardiac output at rest, but when heat diverts blood to the skin and skeletal muscles during physical stress, this percentage decreases (Rowell, 1993).

Despite these interventions largely targeting different components of oxygen delivery, it is possible that CO alters kidney blood flow and heat alters arterial oxygen content. Heat can decrease arterial oxygen content by right shifting the O_2-Hb dissociation curve. As temperature increases, hemoglobin's affinity for oxygen decreases and promotes its release at the tissues. As blood temperature increases, the partial pressure of oxygen at which 50% of the Hb is bound to O_2 (p50) decreases and O_2 is released from Hb (Bolla and Cadet 2007). Despite knowing how heat alters renal blood flow, it is unknown if CO can affect renal blood flow.

Despite numerous studies showing the EPO response to altitude or acute hypoxic breathing (Berglund et al. 2002; Faura et al. 1969), fewer studies have examined the effect of chronic low-dose CO inhalation on circulating EPO concentration. A study by Wang et al.

(2019) found a significant increase in EPO levels 4 hours post CO inhalation of a single 2-minute bout (1.0 mL·kg−1 body mass), 5 times per week prior to training. Another chronic CO inhalation study examined an increase in serum EPO after about one week of initial exposure in moderately active male subjects who inhaled a small dose of CO (0.8-1.0 mL·kg−1 body mass) five times per day for three weeks. These studies were intended to mimic altitude exposure and analyzed the effects of chronic inhalation as opposed to intermittent inhalation (Schmidt et al. 2020; Wang et al. 2019).

No studies have analyzed the effects of heat alone on circulating EPO levels, but we do know that it can decrease renal blood flow (Chapman et al. 2021). Heat stressors alone and in combination with interventions such as CO inhalation must be explored further to better understand their roles on EPO release.

Immune System

The immune system functions to defend the body against infection through a series of vascular, cellular, and chemical responses and consists of the innate and adaptive immune systems. The hematopoietic stem cell gives rise to a myeloid cell line, which is part of our innate immune system, and a lymphoid cell line, which is part of our adaptive immune system. The innate immune response is the body's first line of defense, acting nonspecifically to pathogens and activated by chemical properties of an antigen. It is composed of various immune cells, including granulocytes (neutrophils, basophils, and eosinophils) and phagocytes (macrophages, monocytes, and dendritic cells). The adaptive immune response is more complex and responds to specific antigens, and it is composed of T cells, B cells, and natural killer cells. All immune cells in both the innate and adaptive immune systems release proteins called cytokines. Cytokines are small proteins that help activate and induce immune responses (Owen et al. 2013).

Cytokines and chemokines are very important in the inflammatory response. Cytokines typically act either in a pro-inflammatory or anti-inflammatory way, while chemokines are secreted proteins that induce the cell migration of leukocytes. Cytokines can be divided into interferons and interleukins. Interferons activate macrophages and neutrophils and induce NK cell activity. Interleukins are chemotactic, induce fever and fibroblast proliferation, promote leukocyte binding, and enhance maturation of T cells and B cells (Owen et al. 2013).

The following cytokines will be analyzed for this thesis: interleukins (IL)-1β, 6, 8, 10, 12p70, 17A, 18, 23, and 33; interferons (IFN)-α2, γ; tumor necrosis factor (TNF)-α; and monocyte chemoattractant protein (MCP)-1. IL-1β, IL-18, and IL-33 are part of the interleukin (IL)-1 family, which are responsible for inducing a pro-inflammatory response (Arend, Palmer, and Gabay 2008; Owen et al. 2013). IL-6, IL-12p70, IL-23 are part of the hematopoietic family. Interestingly, EPO is classified in the hematopoietic family as well. IL-6 is primarily involved in stimulation of B cells and can be considered both pro- and anti-inflammatory, while IL-12p70 and IL-23 are known to induce the generation of T helper cells, which promote inflammation and can eventually down regulate the immune response when the antigen is cleared (van der Heijden, Bot, and Kuiper 2019). IL-6 can also help suppress a downstream inflammatory cascade by inhibiting TNF-α and inducing IL-10 expression. TNF-α and IL-17A are pro-inflammatory (Gaffen 2009). IL-8 is essential to the recruitment of neutrophils. Interestingly, EPO can also act as an anti-inflammatory cytokine (Nairz et al. 2012).

EPO – Inflammation Crosstalk

Inflammatory cytokine expression increases in response to an immune challenge but can also be due to non-pathogenic stimuli, such as the presence of benign foreign particles, physical debris, hypoxia, or tissue death (Ansar and Ghosh 2016). If cytokine expression remains

elevated chronically, it can alter many other physiological systems and sometimes result in pathologies, such as anemia of inflammation. Anemia of inflammation is insufficient RBCs due to an overactive immune system (Adamson 2008). One of the ways in which the immune system can inhibit RBC production is due to impaired EPO production (Adamson 2008). Jelkmann et al. (2011) showed that anemic patients who present with low oxygen content still have low EPO concentration, even though low oxygen content should be stimulating the release of EPO. It is hypothesized that baseline levels of inflammatory cytokines have a role in causing this suppressed EPO release despite low oxygen levels. This is supported by studies reporting that inflammation impairs EPO release in response to hypoxia. Specifically, IL-1β and TNF-α were found to inhibit hypoxia-induced EPO expression (Faquin, Schneider, and Goldberg 1992; Jelkmann 1998). Interestingly, IL-6 promoted hypoxia-induced EPO production (Faquin, Schneider, and Goldberg 1992), emphasizing that the actions of specific cytokines may act differently on these pathways.

Methods

Subjects

A total of 12 recreationally active non-smoking subjects (6 male and 6 female) between the ages of 19-32 years with no history of cardiovascular limitations underwent three treatments: inhalation of Carbon-Monoxide (CO), hot-water immersion (HWI), and a combination of CO inhalation and HWI. Each treatment was done in a randomized order with at least one week between interventions to avoid any compounding influence of the previous intervention. In addition, subjects were asked to abstain from drug consumption, alcohol, caffeine, exercise, and whole-body heating at least 24 hours prior to study visits. Table 1. presents all anthropomorphic data.

Study design

Upon arrival for all study days, subjects were shown to a private restroom to provide a small urine sample, which was used to test for urine specific gravity using a refractometer to ensure sufficient hydration. If the urine specific gravity was less than 1.020, they were asked to consume 5 mL of water per 1 kg bodyweight. For females, this same urine sample was also used for a pregnancy test. After confirming hydration (and negative pregnancy test for women), an IV was placed to collect a small venous blood sample at baseline. This baseline blood sample was used to analyze for EPO and cytokine concentrations. After the IV catheter placement and baseline blood draw, subjects completed a baseline resting period followed by the assigned intervention. Baseline measurements and measurements during the intervention period included blood pressure and heart rate, as well as core temperature on days involving HWI. Renal artery velocity was also collected using renal ultrasonography. Following the intervention, an

additional venous blood sample was taken every hour for 6 hours post-intervention for analysis of EPO concentration.

Carbon Monoxide (CO) Inhalation Protocol

Subjects breathed a small volume of CO for two 10-minute periods through a rebreathe circuit. The volume of CO administered was 1.0mL/kg body weight for men and 0.8mL/kg body weight for women. The reason the volume of CO was different in men and women is due to the differences in RBC mass in men versus women. Testosterone stimulates erythropoiesis (Bachman et al. 2014). Women have lower levels of testosterone than men, and female sex hormones are inhibitory towards the maturation of RBCs (Blobel and Orkin 1996). This volume of CO was intended to raise blood carbon monoxide saturation to 10-15% and reduce oxygen saturation to 85-90%. The rebreathe circuit was pre-filled with 100% oxygen, and oxygen was added to the circuit as needed. A CO_2 absorbent (soda lime) was added to the circuit to prevent hypercapnia. During CO inhalation, subjects had renal artery velocity measured with ultrasound and blood pressure measurements taken.

Hot Water Immersion (HWI) Protocol

For HWI, subjects sat in a hot tub heated to 40˚C for 45 minutes. During this time, core temperature was monitored. For core temperature measurements, a rectal thermistor or ingestible telemetric pill was used. If the subject used the telemetric pill, they were instructed to take the pill 10 hours prior to this study visit. If the thermistor was used, the subject self-inserted the thermistor. Subjects had renal artery velocity measured with ultrasound, blood pressure measurements taken, and heart rate monitored while in the hot tub as well. Pre- and post-intervention nude weight was measured.

Combined CO and HWI protocol

On combination treatment days, subjects underwent CO inhalation first and immediately completed HWI. Procedures were identical to those performed on the single intervention days.

Blood Processing & Analysis Procedure

Whole venous blood was allowed to clot at room temperature for at least 30 minutes. After, it was spun at 1500 G for 10 minutes to separate the serum from the RBCs and platelets. Serum was pipetted into cryotubes and stored at -80˚C until analysis. All serum samples were thawed fully on ice prior to analysis.

The serum was analyzed for EPO at all time points and all study days using a Human Erythropoietin ELISA Kit (Sandwich Enzyme-Linked Immunosorbent Assay, BioLegend LEGEND MAX) with a sensitivity of 0.25 mIU/mL and standard range of 2.0-125 mIU/mL. Samples were not diluted prior to analysis. All samples were run in duplicate. Absorbance was read on the plate reader at 450 nm and 570 nm.

Baseline inflammation from all three study days was analyzed using a Multi-plex flow cytometry assay (Human Inflammation Panel 1 (13-plex), BioLegend LEGENDplex) that measures the following inflammatory proteins: interleukins (IL)-1β, -6, -8, -10, -12p70, -17A, -18, -23, and -33; interferons (IFN)-α2, -γ; tumor necrosis factor (TNF)-α; monocyte chemoattractant protein (MCP)-1. The Multi-plex flow cytometry assay uses two beads (Beads A and Beads B) coated with antibodies that are specific to each cytokine and can be further distinguished through specific internal fluorescent intensities. Samples were diluted twofold prior to analysis. All samples were run in duplicate. Table 2. provides information about the sensitivity and standard ranges for each individual analyte.

Statistical Analysis

To determine whether each intervention resulted in a significant increase in EPO over time, a one-way ANOVA was performed with a Tukey's post hoc analysis. To determine whether EPO concentration significantly increased from baseline to peak, a paired t-test was performed. A two-way ANOVA was performed to determine whether there were differences between the three interventions on EPO concentration over time. A simple linear regression model was used to determine if there was a significant relationship between each baseline cytokine concentration and peak EPO concentration for each intervention.

Results

Erythropoietin Response to CO, Heat, and Combined CO and Heat

There was a significant effect of time on EPO concentration for all three interventions (carbon monoxide p = 0.0108, heat p = 0.0259, and combined CO and heat p = 0.0249) (Fig 1A, 2A, 3A). EPO concentration significantly increased from baseline to peak in response to CO (p=0.0214) (Fig 1B), heat (p=0.0134) (Fig 2B), and combined CO and heat (p=0.0203) (Fig 3B).

Comparison of EPO response to each intervention

There was no interaction between time (baseline through 6hr) and intervention (CO, heat, and combined CO and heat) (p = 0.1675). There was a main effect of time (p = 0.0265) but no main effect of the intervention (p= 0.3730) on EPO concentration (Fig 4A). Similarly, there was no interaction between time (baseline and peak) and the intervention (CO, heat, and combined CO and heat) (p = 0.7728). There was a main effect of time (p = 0.0215) but no main effect of the intervention (p= 0.9067) on EPO concentration (Fig 4B).

Inflammation and EPO Relationships

There was a significant and positive relationship between IL-18 and peak EPO concentration for combined CO and heat intervention (p=0.0479, R^2=0.3677) (Fig 5). There were no other significant relationships between other cytokines and EPO concentration in any other interventions (Table 3).

Discussion and Conclusion

Overall we found that circulating EPO concentration increased significantly with each intervention: CO inhalation, heat, and combined CO and heat. Despite CO and heat providing an additive stimulus to reduce kidney oxygen delivery, EPO concentrations were not significantly higher after the combined interventions compared to the single interventions.

Higher baseline levels of IL-18, a proinflammatory cytokine, was associated with higher levels of peak EPO concentrations in subjects undergoing the combined CO and heat intervention. Approximately 37% of the variability observed in the peak EPO concentration is explained by baseline IL-18 concentration.

Effects of Interventions on EPO

These results indicate that each intervention (CO, heat, and combined CO and heat) decreased oxygen to the kidney sufficiently to induce a significant increase in EPO concentration. High altitude is known to increase EPO (Abbrecht and Littell 1972; Faura et al. 1969; Ge et al. 2002; Pham, Parikh, and Heinrich 2021; Płoszczyca, Langfort, and Czuba 2018), and CO inhalation is known to simulate a similar response to also increase EPO (Berglund et al. 2002; Wang et al. 2019), which our data support. However, unlike high altitude, CO decreases oxygen delivery to the kidney by directly outcompeting O_2 binding on Hb. Furthermore, we are the first to show that EPO concentration increases acutely in response to hot water immersion. The proposed mechanism for the increased EPO is through a reduction in renal blood flow (Chapman et al. 2021; Rowell 1993).

Although CO and heat both increase serum EPO levels, combining CO and heat had no additive effects on EPO concentration ($p>0.05$). No studies have specifically examined CO and heat, but these results are supported by many studies that reveal non-additive effects of

traditional hypoxia and heat, and even antagonistic effects, on exercise performance (Bradbury et al. 2019; Charkoudian et al. 2019; Lloyd and Havenith 2016; Mccleave et al. 2017; Nybo, Rønnestad, and Lundby 2022). Despite an increase in Hb mass, there was no improvement in performance after a combined stimulus (Mccleave et al. 2017). Furthermore, the lack of performance benefits of a chronic additive stimulus is most likely due to an acute mechanism or feedback mechanism that prevents the overproduction of EPO that would otherwise illicit a significant in Hb mass and performance.

Inflammation and EPO

There are many studies that analyze the blunted and enhanced inflammatory responses to hypoxia in both animals and humans (Baze, Hunter, and Hayes 2011; Facco et al. 2005; Feuerecker et al. 2019; Kvamme et al. 2013), but few have analyzed the effects inflammation may have on hypoxic responses. More specifically, few studies analyze the effects specific inflammatory proteins have on hypoxia induced EPO release, and none of these studies are in humans (Faquin, Schneider, and Goldberg 1992; Jelkmann 1998).

Not only was IL-6 was found to stimulate hypoxia-induced EPO production, but IL-1 and TNF-a were found to have inhibitory effects on EPO production *in vitro*. The response to the release of these cytokines were additive, in which each IL-1α, IL-1β, TNF-α, and IL-6 were analyzed individually and in combination, with hypoxia-induced EPO production increasing when IL-6, IL-1 and TNF-a were analyzed together (Faquin, Schneider, and Goldberg 1992; Jelkmann 1998). Despite the relationship between IL-6, IL-1, and TNF-α and EPO in animals *in vitro*, no significant relationship between baseline levels of these cytokines and levels of EPO were found in the current study in humans. Only baseline IL-18 concentration was found to have a positive relationship with peak levels of EPO (Arend, Palmer, and Gabay 2008; Owen et al.

2013). Interestingly, IL-18 is part of the same cytokine pro-inflammatory cytokine family as IL-1, but IL-1 was found to have a negative relationship with EPO release, where our study showed that IL-18 had a positive relationship with EPO concentration (Faquin, Schneider, and Goldberg 1992).

IL-18 is a pro-inflammatory protein important in innate and adaptive immune responses. Although IL-18 is responsible for enhancing the production of IFN-γ, which is known to reduce erythrocyte life span and inhibit erythropoiesis (Li et al. 2020; Libregts et al. 2011; Nakanishi 2018), we found no significant relationship between IFN-γ and EPO.

We hypothesized there would be a negative linear relationship between pro-inflammatory cytokines such as IL-18 and EPO. However, we found a positive linear relationship. The reason for this is unclear. Interestingly, high levels of serum IL-18 were found in chronic obstructive pulmonary disease (COPD) patients (Imaoka et al. 2008), and plasma EPO concentration was reduced in response to hypoxic episodes in those patients who experienced acute elevations in inflammation during their episodes (Sala et al. 2010). These data support our hypothesis in which we would expect decreased EPO concentration with increased IL-18, but current results reveal a positive relationship. Further research is needed to understand this relationship in otherwise healthy humans.

Baseline IL-18 concentration and peak EPO concentration had a significant and positive linear relationship only on the combined CO and heat intervention day, leading to the speculation that baseline levels of IL-18 promoted EPO production due to a possible interaction between CO and heat only present during combined treatment. The reason for the lack of this association on the single treatment study days is unclear.

Limitations

This study included a small number of subjects who were all moderately active. A larger sample size would allow for more data points to be analyzed. In addition to a small sample size to analyze, cytokine concentrations are typically below limits of detection in non-pathologic conditions, and it further limits analysis. In addition, only levels of baseline inflammation were examined in relation to EPO levels. In the future, inflammation should be studied at numerous time points throughout the intervention, which can be informative in further understanding the variability in EPO and cytokine levels in response to these interventions. Additionally, an interventional study could be run, in which subjects are given a pro or anti-inflammatory supliment, such as Advil or Tylenol, prior to undergoing treatment. This would alter levels of baseline inflammation and can then be compared to EPO concentrations in the same way.

Overall Significance

It is important to understand the underlying sources of variability in EPO in both healthy and individuals with various diseases. The current study is key in furthering our understanding of the kidneys' response to differing forms of hypoxia. Anemia of chronic disease (ACD), or anemia of inflammation, is characterized by low levels of RBCs and therefore low Hb. Cases of chronic inflammation brought on by factors such as disease, infection, severe trauma, kidney failure, or even cancer prevents or inhibits the development of healthy RBCs. Finding useful interventions such as CO inhalation, heat, or even a combined CO and heat treatment to increase RBC mass may be crucial in finding ways to reverse the effects of ACD and other diseases, but we must first understand the mechanisms by which these interventions increase RBC mass and determine what might blunt or enhance the response to these interventions.

Additionally, understanding the role inflammation has in hypoxia induced EPO release is important to understanding how to improve aerobic exercise performance. Both CO and heat have been found to increase exercise performance (Lorenzo et al. 2010; Nybo, Rønnestad, and Lundby 2022; Smith-Ryan et al. 2016; Wang et al. 2019), but understanding the mechanism(s) by which this happens is crucial to its application in specific sports and specific populations. Females have a lower RBC mass and therefore lower levels of oxygen delivery. This leads females to be more susceptible to anemia, while also being more prone to having higher levels of inflammation due to having a stronger pathogenic immune response (Klein and Flanagan 2016; Murphy 2014; Spitzer 1999). Understanding the effects inflammation may have on the kidneys' response to release EPO may be beneficial to finding specific cytokines to target in order to allow females to benefit to the same degree as men to these interventions and therefore combat the lower RBC mass in females.

More recently, an additional significant application of this work is finding ways to target EPO transcription through the HIF pathways which can be beneficial in "hypoxic conditioning" to reduce illness severity and improve function of patients with serious cases of COVID-19 (Serebrovska et al. 2020). Thus, these and other novel approaches to survive and even thrive under conditions of hypoxia may allow for the development of novel therapeutic approaches to treating pathologies where oxygen transport is the underlying problem.

Figures

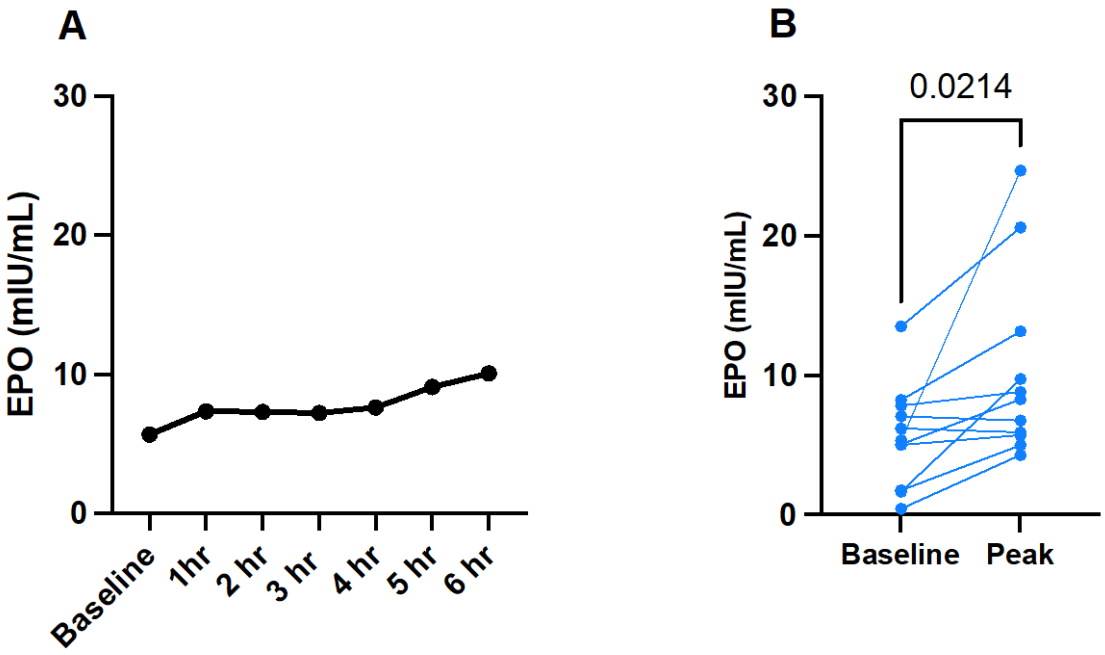

Figure 1. Carbon Monoxide (CO) Inhalation: Erythropoietin (EPO) Over Time

Erythropoietin (EPO) response to CO inhalation over time (Baseline, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr) **(A)** and from baseline to peak **(B)**. EPO is represented as concentration in milli-international units per milliliter and time is represented in hours post-treatment with "Baseline" representing pre-treatment. For both graphs, every blue line represents one subjects EPO concentration over time and from baseline to peak. The black line in Figure 1A represents the average EPO concentration over time. EPO significantly increased over time ($P < 0.05$) and from baseline to peak ($P < 0.05$).

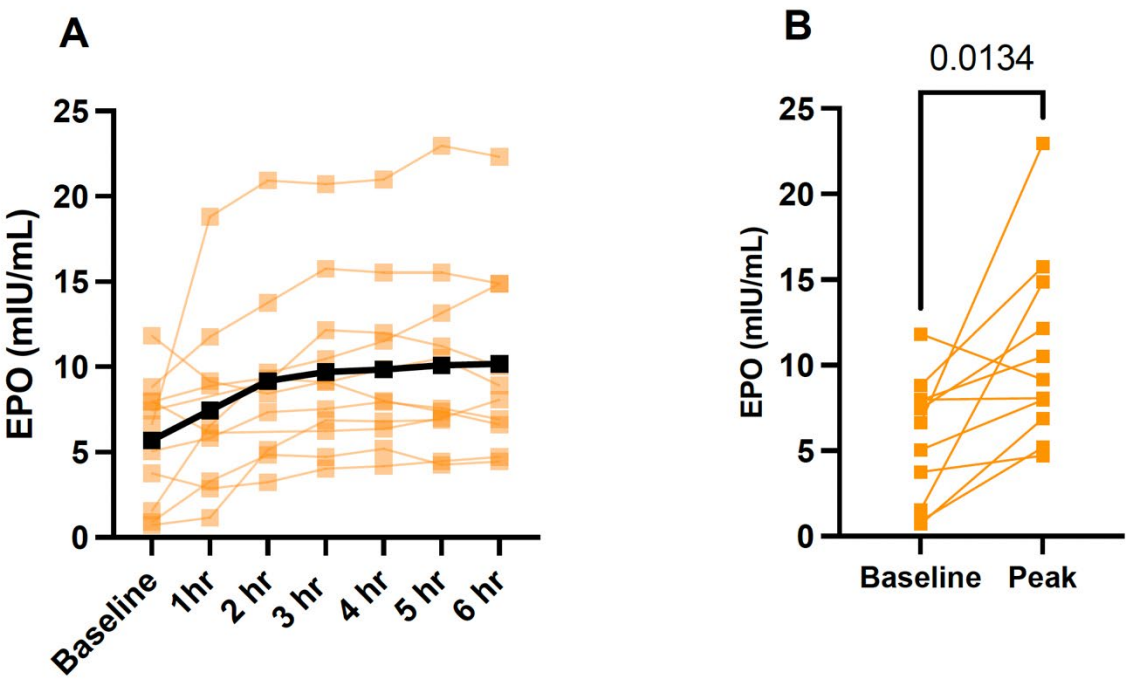

Figure 2. Heat - Hot Water Immersion (HWI): Erythropoietin (EPO) Over Time

Erythropoietin (EPO) response to heat over time (Baseline, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr) **(A)** and from baseline to peak **(B)**. EPO is represented as concentration in milli-international units per milliliter and time is represented in hours post-treatment with "Baseline" representing pre-treatment. For both graphs, every orange line represents one subjects EPO concentration over time and from baseline to peak. The black line in Figure 2A represents the average EPO concentration over time. EPO significantly increased over time ($P < 0.05$) and from baseline to peak ($P < 0.05$).

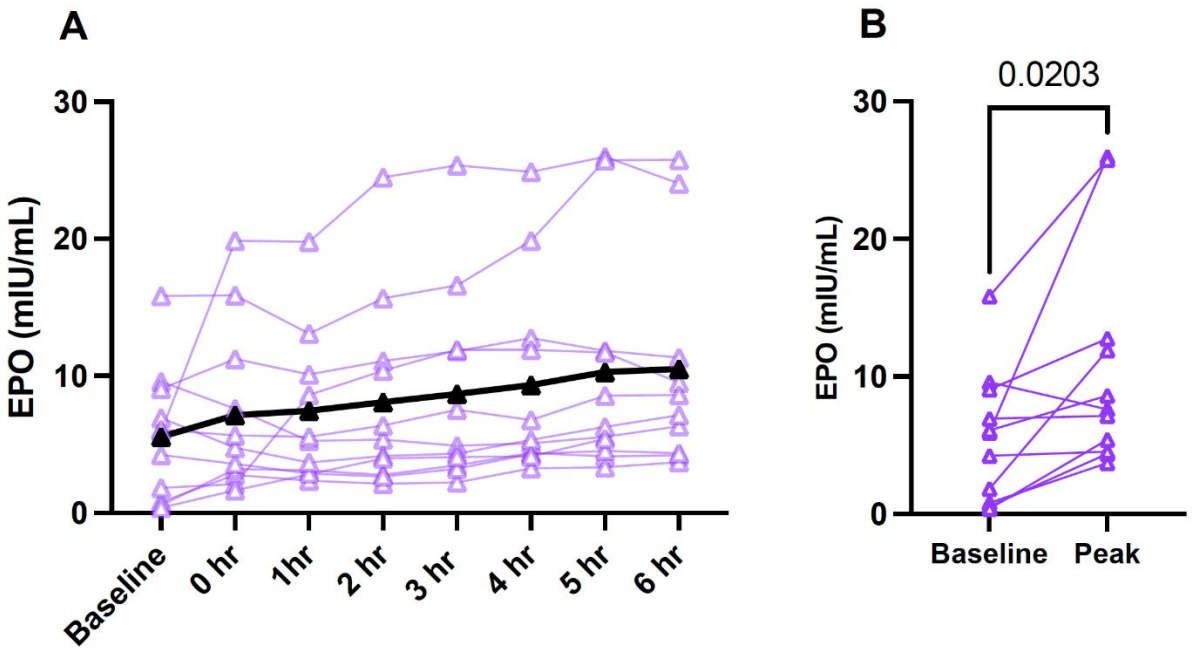

Figure 3. Combined CO and Heat: Erythropoietin (EPO) Over Time

Erythropoietin (EPO) response to combined intervention over time (Baseline, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr) **(A)** and from baseline to peak **(B)**. EPO is represented as concentration in milli-international units per milliliter and time is represented in hours post-treatment with "Baseline" representing pre-treatment. For both graphs, every purple line represents one subjects EPO concentration over time and from baseline to peak. The black line in Figure 3A represents the average EPO concentration over time. EPO significantly increased over time ($P < 0.05$) and from baseline to peak ($P < 0.05$).

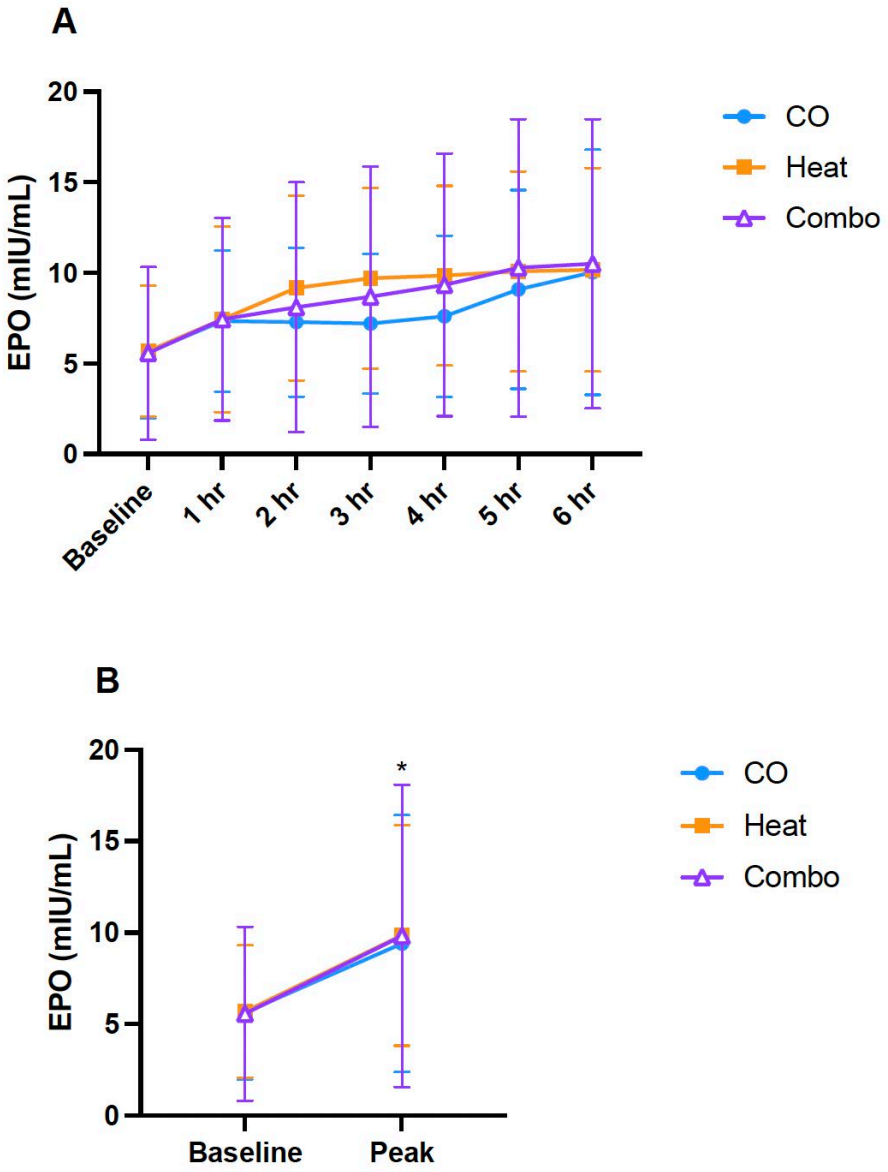

Figure 4. Treatment Comparison: Erythropoietin (EPO) Over Time

Erythropoietin (EPO) response between each treatment (carbon monoxide (CO) inhalation, heat, and combined CO and heat) over time (Baseline, 1hr, 2hr, 3hr, 4hr, 5hr, 6hr) **(A)** and from baseline to peak **(B)**. There was a significant effect of time on EPO concentration over time (P< 0.05) and from baseline to peak (*< 0.05) for each individual treatment. There was no significant effect of intervention on EPO concentration.

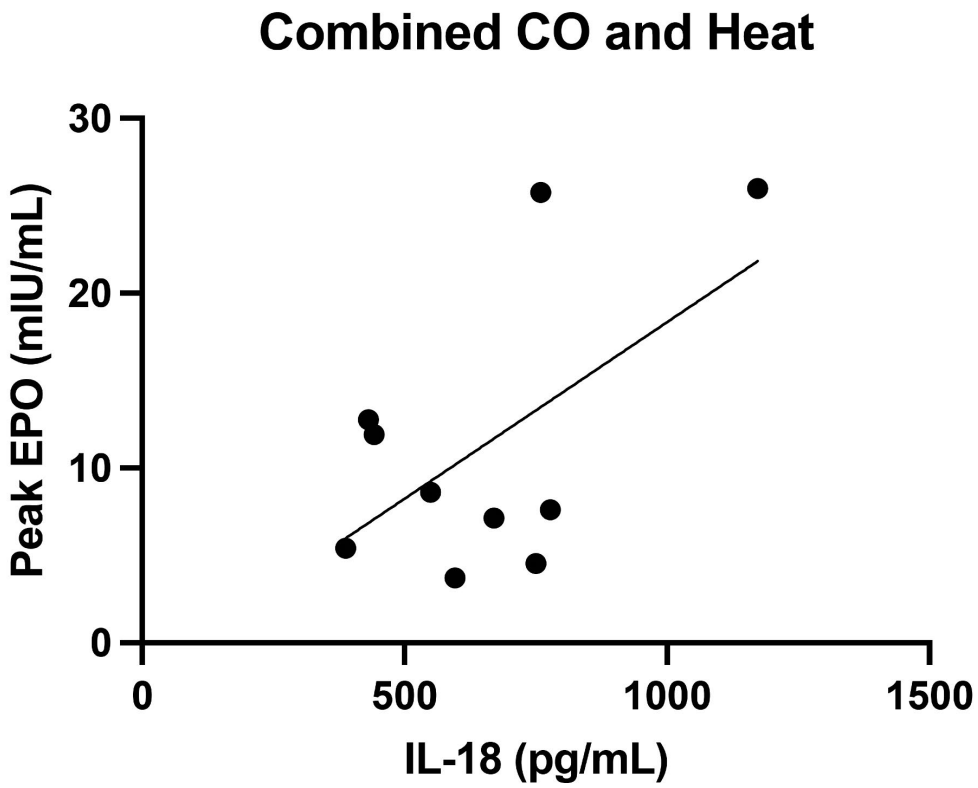

Figure 5. Peak Erythropoietin (EPO) concentration vs Baseline IL-18 concentration

Peak erythropoietin (EPO) and baseline IL-18 concentrations among subjects on combination day (R^2=0.3677, *p<0.05).

Tables

Subjects	Weight (kg)	Height (cm)	Age
Female *(n=6)*	70.3 ± 6.9	164.01 ± 2.7	21 ± 1.6
Male *(n=6)*	70.01 ± 5.7	177.75 ± 3.8	23 ± 3.7
Total *(n=12)*	70.15 ± 4.3	170.88 ± 4.6	22 ± 2.0

Table 1. Subject Anthropomorphic Data

Data is presented as means ± standard deviations.

Analyte	Sensitivity in Serum (pg/mL)	Top Standard Concentration (ng/mL)
IL-1B	1.5 + 0.6	10
IFN-a2	2.1 + 0.2	13
IFN-y	1.3 + 1.0	9
TNF-a	0.9 + 0.8	17
MCP-1	1.1 + 1.2	10
IL-6	1.5 + 0.7	9
IL-8	2.0 + 0.5	12
IL-10	2.0 + 0.5	11
IL-12p70	2.0 + 0.2	14
IL-17A	0.5 + 0.1	2.5
IL-18	1.3 + 0.9	15
IL-23	1.8 + 0.1	12
IL-33	4.4 + 1.5	100

Table 2. Serum Sensitivities and Top Standard Concentrations After Reconstitution (Inflammation Panel)

	CO only			Heat only			Combined		
	Slope	**R^2**	**p-value**	**Slope**	**R^2**	**p-value**	**Slope**	**R^2**	**p-value**
IL-1β	-0.0414	0.0809	0.3966	-0.0143	0.0170	0.7380	-0.0585	0.1104	0.3181
IFN-α2	-0.0424	0.0367	0.5509	-0.0284	0.0253	0.6217	-0.0333	0.0168	0.6880
IFN-γ	-0.0189	0.0227	0.6582	-0.0387	0.1511	0.3888	-0.0414	0.0571	0.5687
TNF-α	-0.0118	0.0344	0.5641	-0.0074	0.0163	0.6926	-0.0249	0.0710	0.4283
MCP-1	0.0056	0.0421	0.5222	-0.0013	0.0025	0.8783	0.0072	0.0381	0.5653
IL-6	-0.0201	0.0090	0.7696	0.0219	0.0109	0.7472	-0.0146	0.0016	0.9076
IL-8	-0.0115	0.0860	0.3815	-0.0139	0.1676	0.2401	-0.0103	0.0335	0.5902
IL-10	-0.0279	0.0302	0.6313	-0.0168	0.0152	0.7346	-0.0261	0.0245	0.6657
IL-12p70	-0.0436	0.0537	0.4928	-0.0290	0.0374	0.5923	-0.0423	0.0316	0.6010
IL-17A	-0.2833	0.0359	0.5555	-0.2332	0.0566	0.4566	-0.4808	0.0985	0.3474
IL-18	0.0083	0.0293	0.5948	0.0147	0.1630	0.1930	0.0227	0.3677	0.0479 *
IL-23	-0.0225	0.0781	0.3791	-0.0194	0.0873	0.3510	-0.0336	0.1339	0.2421
IL-33	-0.0032	0.0671	0.4161	-0.0029	0.0803	0.3722	-0.0049	0.1167	0.3039

Table 3. Regression Results

Baseline cytokine relationships with peak EPO concentrations for each intervention (CO only, Heat only, and Combined) represented by slope, R^2, and p-value. The slope reveals represents the presence of any linear relationship between baseline cytokine and peak EPO concentrations though a regression model. The R^2 reveals how well data fits the regression model. The p-value represents the significance of the relationship. Baseline IL-18 had a positive relationship with peak EPO for Combined intervention days (slope=, *P>0.05). About 37% of the variability in peak EPO concentrations post-Combined treatment was explained by baseline concentrations of IL-18 (R^2=0.3677).

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