12-2013

Changes in Vascular Reactivity of Mesenteric Arteries Following Hyperbaric Oxygen Treatment

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Changes in vascular reactivity of mesenteric arteries following hyperbaric oxygen treatment

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A Thesis Submitted to the Graduate Faculty of

GRAND VALLEY STATE UNIVERSITY

In

Partial Fulfillment of the Requirements

For the Degree of

Masters in Health Science

Biomedical Science

December 2013

Acknowledgments

I would like to take this opportunity to thank my committee, Frank Sylvester, Dawn Richiert, and Doug Graham. Without their help I would not have been able to get as far academically as I am today. I would also like to thank De Vries Meats for being so gracious with the facilities and allowing us to procure organs from them. Thank you to the statistical consulting department for helping analyze the mountains of data I collected. Finally, thank you to my family and friends for all their support.

Abstract

The objective of this study was to evaluate the changes in vascular reactivity of mesenteric arteries resulting from hyperbaric oxygen treatment. It was hypothesized that hyperbaric oxygen treatment alters vascular reactivity in mesenteric arteries due to enhanced production of ATP resulting in significantly larger responses to vasoactive stimuli. All arteries were dissected from porcine mesenteries and placed in Krebs-Henseleit solution. Arteries were initially mounted in isolated organ baths and passively loaded with tensions ranging from 1 to 25 grams at odd intervals to ascertain the optimal passive tension for studying mesenteric arteries. Following a 1-hour equilibration in Krebs-Henseleit solution, arteries were treated with potassium chloride (a nonreceptor-mediated vasoconstrictor; KCl; 15 – 60 mM) to assess vascular reactivity. Following determination of the optimal passive tension, additional arteries were dissected and tested for viability with KCl. Viable arteries were then subjected to a 2-hour hyperbaric treatment in 100% oxygen, 100% nitrogen, or ambient air at 1.00 or 1.75 ATA. Immediately following treatments, arteries were again mounted in isolated organ baths and passively loaded with 7 grams of tension. Following a 1-hour equilibration in Krebs-Henseleit solution, arteries were treated with KCl (15 – 60 mM). Arteries were then treated with increasing concentrations of phenylephrine (a receptor-mediated vasoconstrictor; 10^{-7} – 10^{-4} M) followed by increasing concentrations of sodium nitroprusside (a potent vasodilator; 10^{-7} – 10^{-4} M) to measure changes in vascular reactivity. Additional arteries were subjected to the 2-hour hyperbaric regiment involving 5 different gas/ pressure exposures. Arteries were then processed for determination of protein concentration and measurement of ATP concentration using commercially

available assay kits. When compared to the 1 ATA room air control, KCl-induced constriction was significantly increased for the hyperbaric oxygen exposure. Treatment with hyperbaric oxygen also augmented vascular responses to phenylephrine and sodium nitroprusside relative to nitrogen, but not ambient air. There was no significant difference in ATP concentrations found among treatments. The results from these studies provide insight into the vascular effects of hyperbaric oxygen treatment.

Table of Contents

Acknowledgments 3

Abstract 4

List of Tables 7

List of Figures 7

I. Introduction 8

II. Methods 11

Animals & Reagents 11

Dissection 11

Optimal Tension 12

Hyperbaric Treatment 13

Vascular Reactivity 13

ATP Analysis 14

Protein Concentration Assay 14

ATP Concentration Assay 15

III. Results 16

Optimal Tension 16

Vascular Reactivity 18

ATP Analysis 24

IV. Discussion 26

References 33

List of Tables

Table 1. Mean vasoconstrictor responses to KCl in porcine mesenteric arteries 18

List of Figures

Figure 1. KCl induced optimal tension 17

Figure 2. KCl induced constriction following HBO treatment 20

Figure 3. PHE induced constriction following HBO treatment 21

Figure 4. SNP induced dilation following HBO treatment 22

Figure 5. Weight comparison of arterial rings 23

Figure 6. Outer diameter comparison of arterial rings 24

Figure 7. ATP concentration analysis 25

Figure 8. Protein determination curve 26

Figure 9. Hypothesis summary 31

Abbreviations

ANOVA Analysis of variance

ATA Atmosphere absolute

ATP Adenosine triphosphate

HBO Hyperbaric oxygen

IRI Ischemic reperfusion injury

KCl Potassium chloride

NADH Nicotinamide adenine dinucleotide + hydrogen

PHE Phenylephrine

ROS Reactive oxygen species

SNP Sodium nitroprusside

Introduction

Ischemic reperfusion injury (IRI) is quickly becoming a leading problem in today's hospitals. As the average age in the United States population increases, the chances of IRI also increase due to the higher associated cardiovascular risks. Hypertension, obesity, hyperlipidemia, and diabetes all contribute to a higher association of morbidity and mortality in the clinical setting. IRI is the complex phenomenon that occurs following treatment which restores blood flow to the initial site of injury.[28,32] Clinically there is already damage incurred by the initial ischemic event, which can be catastrophic. It is then compounded by further injury that occurs when blood flow is reintroduced. This is particularly destructive in the mesentery because ischemia with subsequent reperfusion can lead to acute respiratory distress syndrome.[22,32]

The lack of oxygen and nutrients during the initial ischemic event decreases the production of ATP.[9,13] Lack of ATP causes a decrease in intracellular pH because breakdown of lactic acid is arrested. Insufficient ATP will also cause failure of the sodium-potassium ATPase pump, which in turn alters cellular membrane potential via a loss of potassium and gain of sodium. Additionally, the distribution of intracellular ions is altered with an overload of intracellular calcium being observed.[6] The change in ion concentrations leads to many deleterious consequences. The cell begins to swell, eventually leading to its death. Further, an increase in reactive oxygen species (ROS), due to the elevated levels of intracellular calcium, leads to oxidative stress and cell death.[29,37] Although the ischemia is the primary source of injury, reperfusion poses an equal, if not greater, clinical problem. During the period of reperfusion, there is an exacerbated and accelerated injury to the previously hypoxic tissues. A burst in ROS generation causes

damage in tissue surrounding the ischemic event that was previously unaffected.[31] The rise in ROS may be in part from the reintroduced oxygen after the restoration of blood flow. Ischemia may also cause a loss of normal antioxidant defense mechanisms.[25, 33] Without these, there is further damage to tissues that would otherwise function normally. Corresponding with the reintroduction of blood is an influx of leukocytes and plasma proteins which coincides with activation of the compliment system.[19] The subsequent inflammation results in new tissue damage.

The vasculature then preforms a significant role in ischemia and reperfusion. Decrease in oxygen to vessels affects the endothelium by altering production of soluble mediators that act on vessel tone.[7,9,17] By normally controlling homeostatic vasodilation and constriction the integrity of the vasculature is upheld. Consequently, the protective barrier function of the vascular endothelium is decreased by the deteriorating condition of the cells.[24] An increase in growth factors is also associated with the lack of oxygen.[27] During reperfusion, the endothelial cells initiate expression of adhesion molecules for leukocytes and neutrophils which leads to further damage of the tissue.[5, 38]

Interestingly, the location of the initial ischemic event can affect the progression of reperfusion syndrome. In particular, if the ischemic event occurs in intestinal regions of the body, an even more threatening progression occurs. In the mesentery, manifestations of both the ischemia and reperfusion injury lead to breakdown of the vasculature and intestinal mucosa.[10, 36] The resulting consequence is the escape of the intestinal flora. Once systemic, the bacteria can colonize the lungs and start a pathologic inflammatory response that leads to acute respiratory distress syndrome, a potentially life threatening disorder.

Hyperbaric oxygen (HBO) therapy has been shown to be an effective treatment option with disorders related to IRI.[3, 18, 21] HBO is a treatment in which the patient breathes 100% oxygen at pressures greater than atmospheric. HBO treatment is done within a range of pressures from 1.00 to 3.00 atmosphere absolute (ATA). At pressure levels of 3.00 ATA or above, neurotoxicity is observed.[16] Clinically, a wide variety of applications are currently in use. HBO therapy is used to treat a variety of ailments, including decompression sickness, thermal burns, exceptional blood loss, crush injuries, compromised skin grafts, and carbon monoxide poisoning.[8, 41] Currently, the majority of literature involving HBO therapy and IRI has been focused on treatments of myocardial infarction and cerebral vascular accident. At present, HBO treatment is thought to work by increasing the partial pressures of oxygen in tissue, thereby making more oxygen available.[14] HBO therapy has not only been shown to aid in vascularization in ischemic tissue but directly enhance fibroblast replication and collagen synthesis in ischemia.[16] Some evidence suggest HBO stimulates blood cell progenitors, which aid in the recovery of damaged tissue, possibly utilizing a nitric oxide dependent pathway.[39]

There is presently a lack of substantial understanding of the effects of HBO on mesenteric arteries. Thus this study investigated the effects of hyperbaric treatment on mesenteric arteries in an attempt to increase our understanding of this increasingly utilized clinical treatment. Specifically, we examined the direct effects of HBO on vascular reactivity and ATP levels in porcine mesenteric arteries. We hypothesize that HBO treatment causes an increase in ATP production, thus enhancing vascular reactivity. Increased vascular reactivity could then confer some protection against reperfusion injury.

Methods

Animals & Reagents

All specimens were obtained from De Vries Meats Inc. of Coopersville, Michigan. Animals are procured from local farms, with inclusion of both female and castrated males, approximately 5 months old. A portion of fresh porcine mesentery often containing segments of duodenum and pancreas was placed on ice for transportation following exsanguination and evisceration.

Dissection

In the laboratory, arteries were dissected from the mesenteric tissue based on size and accessibility. Arteries chosen were approximately three to five millimeters in outer diameter and were at least ten millimeters in length. The specific branch from the porcine mesenteric network is unknown but believed to be either the right gastroepiploic artery or pancreaticduodenal artery due to relative size and position. Dissected arteries were cleaned of adherent connective tissue and either used immediately or placed in HEPES buffer solution for overnight storage at approximately 4°C. HEPES buffer solution was composed of 10 mM HEPES, 151 mM NaCl, 4.7 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 7.8 mM glucose, with the pH adjusted to 7.4. Cleaned arteries, either freshly dissected or following overnight storage, were placed in Krebs-Henseleit buffer solution and rings of approximately three to five millimeter in length were cut out and left in solution. Krebs-Henseleit buffer solution has a composition of 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose. All reagents are from Sigma-Aldrich (St. Louis, MO).

Optimal Tension

To obtain more reliable data for vascular reactivity experiments, determination of optimal tension was necessary. There is currently no definitive data on an optimal tension for porcine mesenteric arteries. Dissected arterial rings were mounted in individual baths on two steel hooks for isometric force measurement. Force transducers were attached to each hook to measure changes in tension. Each transducer was calibrated to 0 grams and 40 grams using standard masses. Measurements were recorded using Lab Scribe 2 software from iWorx Systems (Dover, NH). Each isolated organ bath contained 25 mL of freshly made Krebs-Henseleit buffer solution with temperature maintained at 37 ^{o}C. 95% O_2 and 5% CO_2 gas was bubbled into each bath. Arterial rings were stretched at randomly assigned passive tensions (1 – 25 grams at each odd increment) for a 1-hour equilibration period. Arterial rings were randomly assigned using Excel by Microsoft (Redmond, WA). Each bath was drained, refilled, and tensions readjusted to respective magnitudes every ten minutes. Following equilibration, arteries were treated with a cumulative dose of potassium chloride, 30 then 60 mM to assess reactivity. Changes in tension were recorded continuously at each dose. At the completion of the procedure, each artery was removed from the bath, weighed and outer diameter measurements were taken. Arteries were then discarded. Data were plotted using Excel. Statistical analysis was completed with Sigma Stats by Systat Software (San Jose, CA).

Hyperbaric Treatment

Dissected arterial rings were placed in a petri dish of Krebs-Henseleit buffer solution and positioned in a continuous-flow hyperbaric chamber. The hyperbaric chamber is custom built for Dr. C. Zanetti of Swedish Covenant Hospital in Chicago, IL. The chamber is a

metal cylinder (approximately 29 cm inner-diameter with a depth of 8 cm) with a solid plate that seals the on the top with four bolts. For treatment, arterial rings were subjected to a 2-hour hyperbaric treatment of either 100% oxygen at 1.75 ATA, 100% nitrogen at 1.75 ATA, or room air at 1.00 or 1.75 ATA.

Vascular Reactivity

Immediately following hyperbaric treatment, arterial rings were mounted in isolated organ baths containing 25mL of Krebs-Henseleit buffer solution. Baths were maintained at 37°C with 95% O_2 and 5% CO_2 gas bubbled into each bath. The arterial rings were stretched to a passive tension of 7 grams and subjected to a 1-hour equilibration period in Krebs-Henseleit solution that was changed and tension readjusted to 7 grams every ten minutes. Following equilibration, arteries were treated with cumulative doses of potassium chloride (15, 30, 40, 60 mM) to induce vasoconstriction and assess viability. Arteries exhibiting an increase in tension by at least 5 grams from passive tension were used in further experimentation. Viable arteries were rinsed 3 times with fresh Krebs-Henseleit solution over a 15 minute period before being treated with phenylephrine to induce vasoconstriction and sodium nitroprusside to induce vasodilation. Arteries were then treated with increasing concentrations of phenylephrine (10^{-7}, $3x10^{-7}$, 10^{-6}, $3x10^{-6}$, 10^{-5}, $3x10^{-5}$, 10^{-4} M) followed by increasing concentrations of sodium nitroprusside (10^{-7}, $3x10^{-7}$, 10^{-6},$3x10^{-6}$, 10^{-5}, $3x10^{-5}$, 10^{-4} M). Changes in tension were recorded continuously at each dose. The cumulative changes in tension represent each artery's ability to function properly. At the completion of the procedure, each artery was removed from the bath, weighed and outer diameter measurements were taken as a way to normalize the results for comparison. Results were plotted on Excel, statistical analysis was completed with

SPSS software by IBM SPSS Statistics (Armonk, NY). Weight and outer diameter measurements were analyzed using Sigma Stats and plotted using Excel. Phenylephrine was purchased from Spectrum Chemicals (Gardena, CA). Sodium nitroprusside was from Sigma-Aldrich (St. Louis, MO).

ATP Analysis

Sample Preparation

Arteries were dissected as previously described, except an individual 15 mm arterial section was cut into rings of three to five millimeters and all rings were kept and placed in Krebs-Henseleit buffer solution. Outer diameter measurements were taken for each ring prior to being cut into rings. Arteries were then subjected to hyperbaric treatment as previously described. Following treatment, arteries were removed from the Krebs-Henseleit solution, placed in 2.0 mL cryo.s snap tubes (Greiner Bio-one, Frickenhausen, Germany) and immediately frozen in liquid nitrogen. Frozen samples were stored at -80º C for later processing. Frozen arteries were homogenized cold, using a mortar and pestle in 1.5 mL of 2 M $HClO_4$. The perchloric acid was purchased from Sigma-Aldrich (St Louis, MO). Approximately 1.25 mL of homogenate was transferred to new 1.5 mL economy micro snap tubes (VWR, Chicago, IL). Samples were centrifuged at 0 º C for 3 minutes at 12,000 rpm. The supernatant was removed and refrozen in liquid nitrogen for further analysis.

Protein Concentration Assay

Protein concentration determination was carried out as per the instructions by Thermo Scientific for the Coomassie Plus (Bradford) Assay Kit (item #23236). Bovine serum albumin (Thermo Scientific, Rockford, IL) was used to generate a standard curve. For

preparation of the standards, 2 M $HClO_4$ was used as the diluent. Samples were thawed but kept on ice during preparation. All other reagents were allowed to come to room temperature. When preparing and appropriating samples or standards into respective microplate wells, care was taken to briefly vortex each for approximately 10 seconds before allocution. 10 µL of sample and standard were placed in each well. All samples and standards were analyzed in triplicate. The plate was then placed on a plate rotator for 10 minutes to mix and aid in reduction of air bubbles formed in wells. Remaining air bubbles were burst using a sterile pipette tip. Plates were then mixed for 30 seconds immediately prior to plate reading at 595 nm (BioRad, iMark microplate reader, Hercules, CA). Results were plotted and analyzed in Excel.

ATP Concentration Assay

ATP concentration determination was carried out as per the instructions by Bio Vision (San Francisco, CA) for the ATP Colorimetric/ Fluorometric Assay Kit (item #K354-100). Samples were thawed and kept on ice during preparation. When preparing and appropriating samples or standards into respective microplate wells, care was taken to briefly vortex each for approximately 10 seconds before allocution. For each well the sample was transferred and adjusted to 10 µL as necessary with 2 M $HClO_4$. All samples and standards were analyzed in triplicate. Microplates were covered and placed on a plate rotator for 30 minutes to mix and reduce air bubbles. Any remaining air bubbles were burst using a sterile pipette tip. Measurements were taken at 570 nm (BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader, Winooski, VT) with results being plotted and analyzed in Excel.

Results

Optimal Tension

In vivo, arteries have a continual amount of tension in their walls which can also be described as blood pressure. As such, changes in tension within the arterial wall are key determinants of blood pressure. To be able to compare functionality of each arterial ring, the optimal tension *in vitro* for porcine mesenteric arteries was assessed. To determine this optimal passive tension, we placed the arterial rings in organ-tissue baths and subjected them to doses of potassium chloride (KCl). Changes in tension were recorded at different passive tensions to see where the greatest change in tension would occur. The results of this study suggest that there was no significant difference in KCl-induced vasoconstriction among the passive tensions in porcine mesenteric arteries. The results were plotted as a function of their passive tensions at both doses of KCl (figure 1). The largest difference was seen at 17 grams of passive tension, as arterial tension increased 23.03 and 30.96 g in response to 30 and 60 mM KCl (table 1). The next largest difference was observed at 7 grams of passive tension, 26.47 g when 60 mM KCl was administered. The weakest responses were observed at 3, 15, and 25 grams of passive tension. There was no significant difference found between weight and outer diameter measurements. This strengthens the argument that changes in tension were due to changes in passive tensions and not confounded by differences in arterial sections.

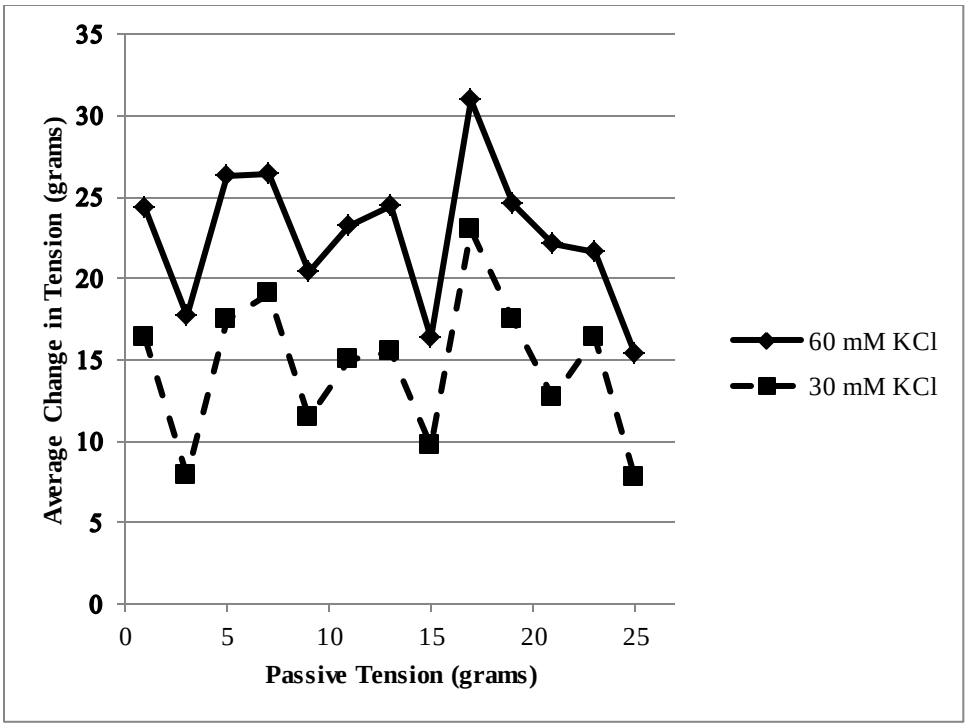

Figure 1. KCl induced vasconstriction in porcince mesenteric arteries. Effects of varying passive tensions (1 to 25 grams) on arterial responsiveness to a cummulative dose of 30 mM and 60 mM KCl. Responsiveness is represented as the mean change in tension of each artery at a given passive tension. Each point represents the mean of 5-9 experiments.

Passive Tension (grams)	30 mM	60 mM	n
1	16.39	24.36	5
3	7.85	17.70	6
5	17.51	26.32	6
7	19.04	26.47	9
9	11.44	20.45	7
11	15.03	23.18	6
13	15.50	24.49	6
15	9.76	16.38	6
17	23.03	30.96	5
19	17.49	24.55	6
21	12.72	22.11	6
23	16.41	21.66	6
25	7.77	15.40	6

Table 1. Mean vasoconstrictor responses to KCl in porcine mesenteric arteries. Results of 30 and 60 mM KCl-induced vasoconstriction at varying passive tensions to determine an optimal tension for mesenteric arteries are listed. Passive tension was measured in grams. A cumulative dose of 30 then 60 mM KCl was given to induce vasoconstriction. Responses are the mean changes in tension of each group.

Vascular Reactivity

Once optimal passive tension was decided, the artery's vascular reactivity, i.e. its ability to change its diameter in response to a stimulus, was assessed in response to the various gas/pressure treatments. Arterial rings were placed in an organ-tissue bath and vasoconstriction followed by dilation was induced experimentally to determine their reactivity. The vascular reactivity for each arterial ring was defined as the change in

tension from the initial passive tension set by the experimenter to the new recorded tension observed after administration of the stimulus. The vascular reactivity for arterial rings was compared among treatments. The results of this experiment show that vascular reactivity was significantly increased in HBO (31.35±3.48 g) when compared to the ambient air control (19.81±2.06 g) in response to 60 mM KCl (figure 2). Interestingly, the hyperbaric nitrogen treatment (24.02±2.69 g) performed better than ambient air control in response to 60 mM KCl. Treatment with HBO augmented vascular responses to phenylephrine (PHE) relative to nitrogen but not ambient air (figure 3). At their largest differences (10^{-5} M), HBO had a reactivity of 30.47±3.12 g as compared to hyperbaric nitrogen at 15.66±1.83 g. The hyperbaric room air (22.51±3.94 g) and ambient room air (23.18±3.19 g) were not significantly different and similar in mean reactivity. Regression analysis showed a significant difference in mean changes in tension for sodium nitroprusside (SNP)-induced dilation for all other treatments compared to HBO (figure 4). For example at a concentration of 10^{-5} M (SNP), HBO (-23.66±3.19 g) had the greatest response when compared to all other treatments. Nitrogen (-11.03±1.36 g) was the least responsive while hyperbaric room air (-15.46±2.6 g) was less responsive than ambient room air (-18.14±2.87 g). Each arterial ring was weighed and outer diameter was measured for subsequent comparison to ensure that the observed changes in vascular reactivity were due to the different gas/pressure treatments and not differences in arterial ring size. There were no significant observed differences in outer diameter or weight among treatments. The outer diameter measurements of HBO, hyperbaric nitrogen, hyperbaric room air and ambient room air were 4.37±0.28mm, 4.82±0.28 mm, 4.32±0.17 mm and 4.05±0.21 mm respectively (figure 5). The weight measurements of HBO,

hyperbaric nitrogen, hyperbaric room air and ambient room air were 0.030±.002 g, 0.030±.002 g, 0.029±.002 g and 0.027±.001 g respectively (figure 6). Results were stated as mean plus or minus standard error of the mean.

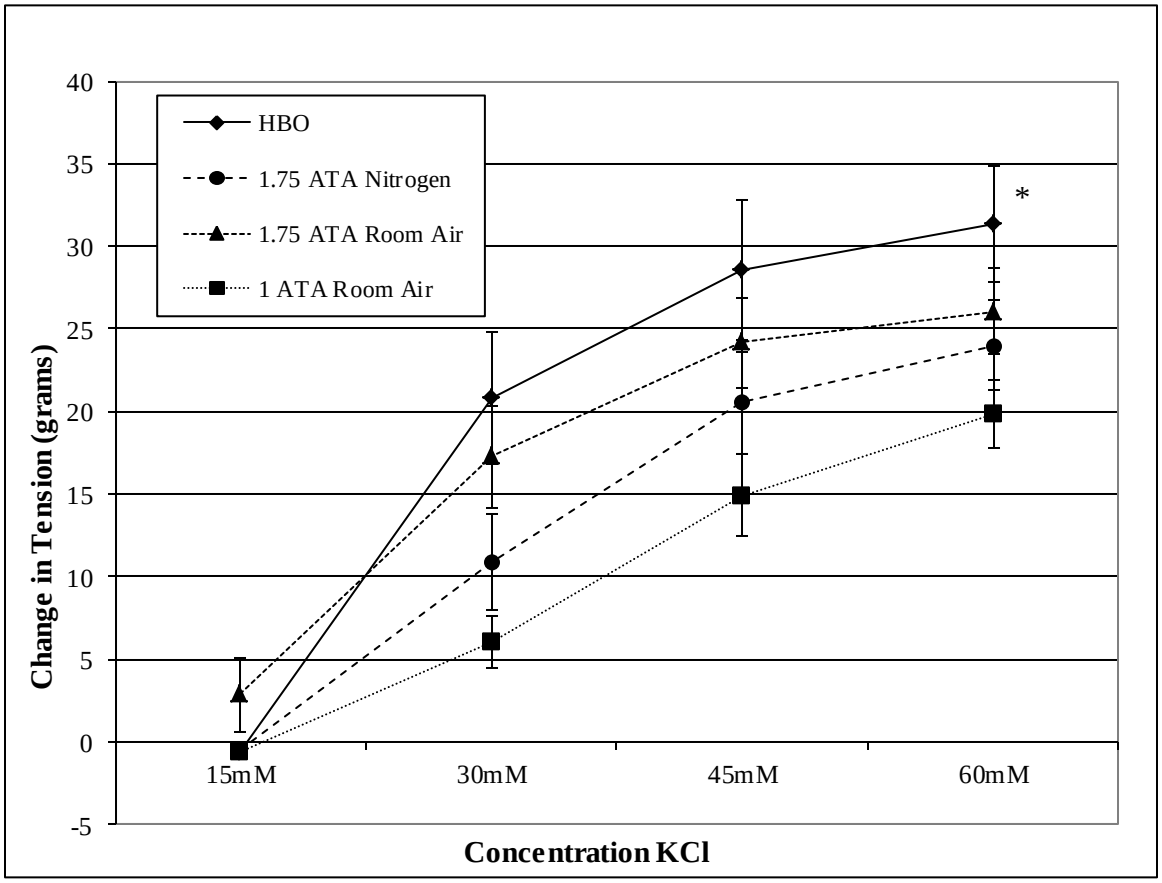

Figure 2. The effects of cumulatively increasing concentrations of KCl on arterial tension following 2 hour exposure to one of various hyperbaric conditions. Results are expressed as mean±SEM. * Response significantly different from ambient air at 1 ATA (n=12, $p<0.05$, ANOVA).

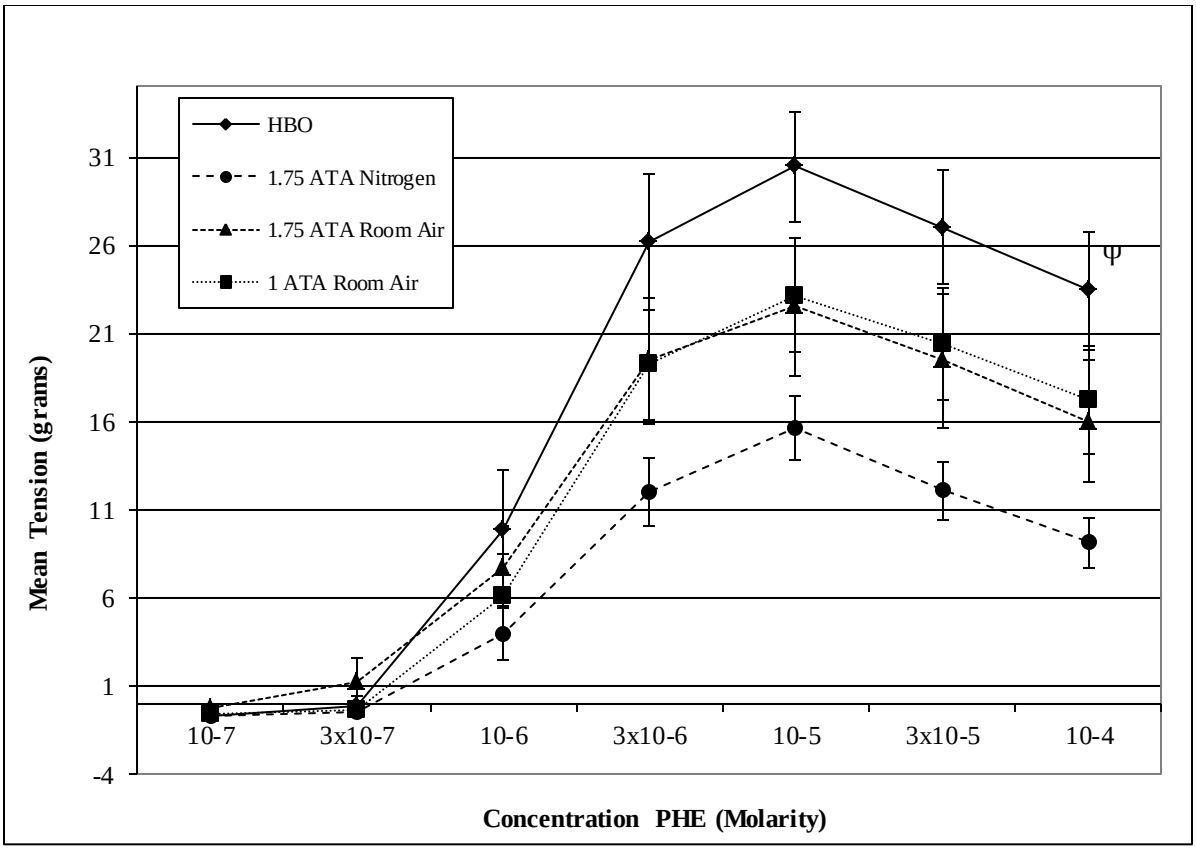

Figure 3. The effects of PHE on vasoconstriction of porcine arteries following 2 hour exposure to one of various hyperbaric conditions (n=12). The changes in arterial tension are differences measured in grams in response to increasing concentrations of PHE. Results are expressed as mean±SEM. $^{\psi}$ Response significantly different from 100% nitrogen ($p<0.05$, Regression Analysis).

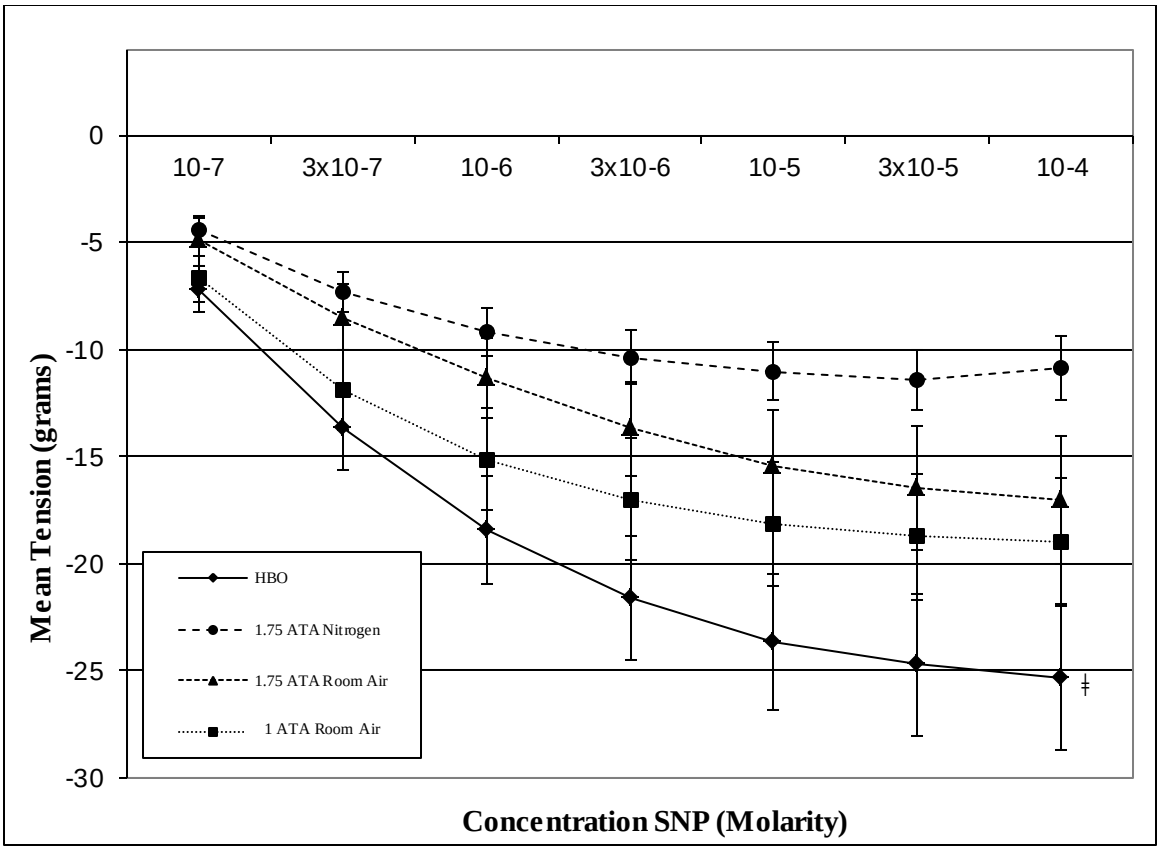

Figure 4. The effects of SNP on vasodilation of porcine arteries following preconstriction with PHE. Treatments were 2 hour exposures to one of various hyperbaric conditions (n=12). The changes in arterial tension are differences measured in grams in response to increasing concentrations of SNP. Results are expressed as mean±SEM. ǂ Response significantly different from all other treatments ($p<0.05$, ANOVA).

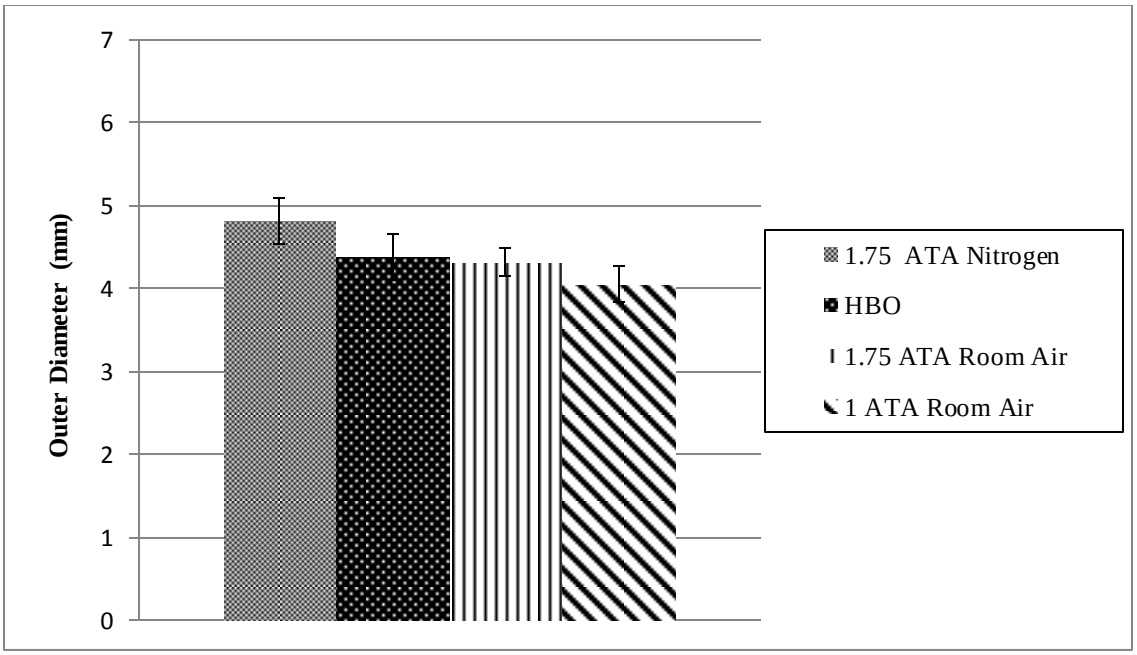

Figure 5. Outer diameter comparision of porcine arterial rings per treatment. Bars represent the average outer diameter per treatment. The error bars represent standard errors of the mean (n=12). There was no significant difference found in outer diameters among treatments (ANOVA).

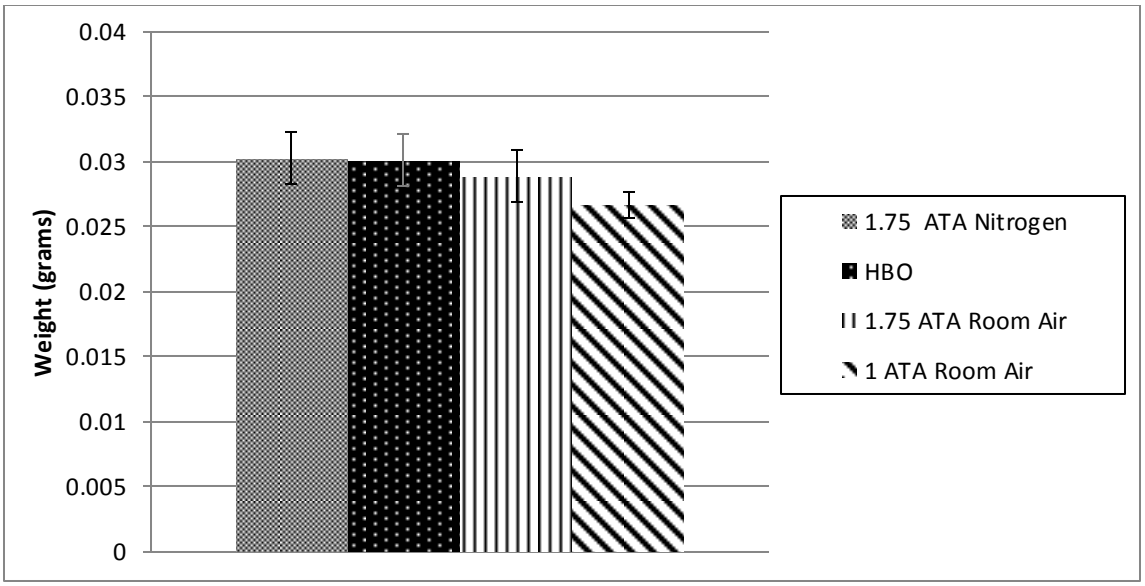

Figure 6. Weight comparision of porcine arterial rings per treatment. Bars represent the average weight per treatment. The error bars represent standard errors of the mean (n=12). There was no significant difference found in weights among treatments (ANOVA).

ATP Analysis

The previous experiment suggests that HBO increases porcine mesentery vascular reactivity. Given the ability of HBO to increase oxygen to the tissue, it could be possible that this increased oxygen availability causes increased ATP production. Increased ATP could account for the increases in reactivity observed. However, when ATP was measured following HBO treatment, it was found that ATP levels were not significantly increased in mesenteric arteries. HBO treatment (0.2506±0.2010 nmol) did show the largest average ATP concentration per 1 microgram of protein when compared to all other treatments (figure 7). Hyperbaric nitrogen (0.1141±0.2705 nmol) was the next largest average ATP concentration when compared to ambient air (-0.0824±0.1471 nmol)

and hyperbaric room air(-0.3193±0.0652 nmol) showing the lowest average concentration. After consideration, it was determined that 100% oxygen at 1.0 ATA (-0.0556±0.2779 nmol) would make another suitable control and was therefore added to the ATP assay for comparison. The 100% oxygen at 1 ATA had a similar mean ATP concentration when compared to all other treatments and was therefore not significantly different. All protein concentration analyses were within the standard curve (figure 8). This demonstrated that each arterial ring contained approximately the same amount of protein. Determination of protein also allowed us to load equal amounts of protein for precise ATP comparison.

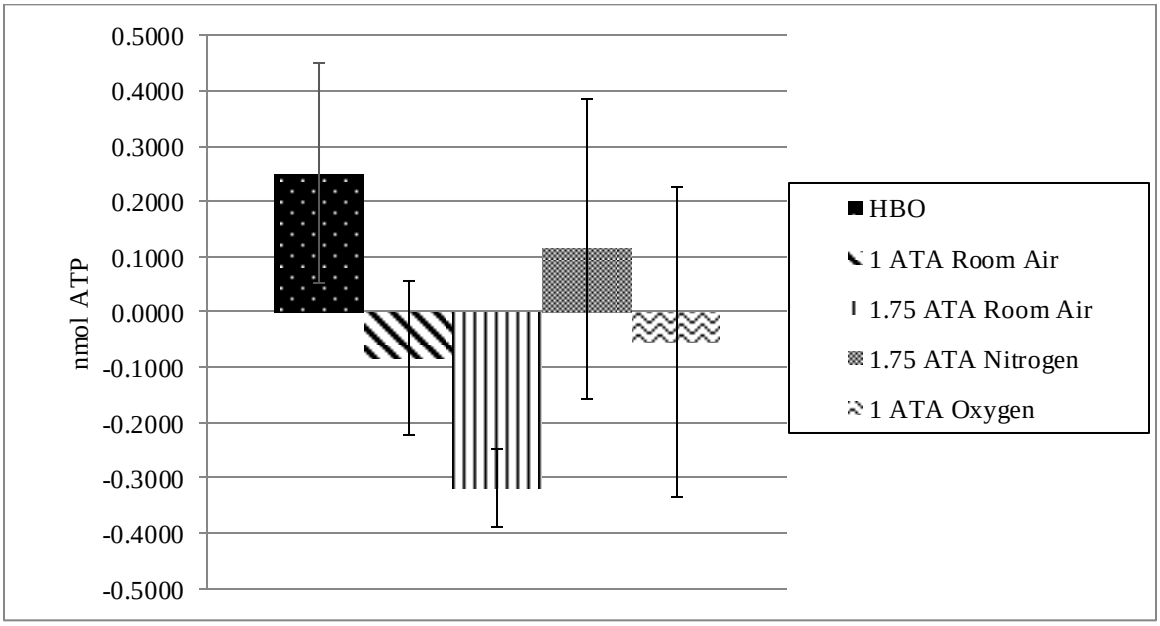

Figure 7. Correlation between ATP concentrations and pressure/gas treatments. Bars represent average nmol ATP per 1 microgram of protein from porcine mestenteric arteries following 2 hour exposure to one pressure/gas treatment. Colorometric concentration was measured at 570 nm with quantities being in the nanomole concentration. Results are expressed as mean±SEM (n=6). No significant difference was found among treatments (ANOVA).

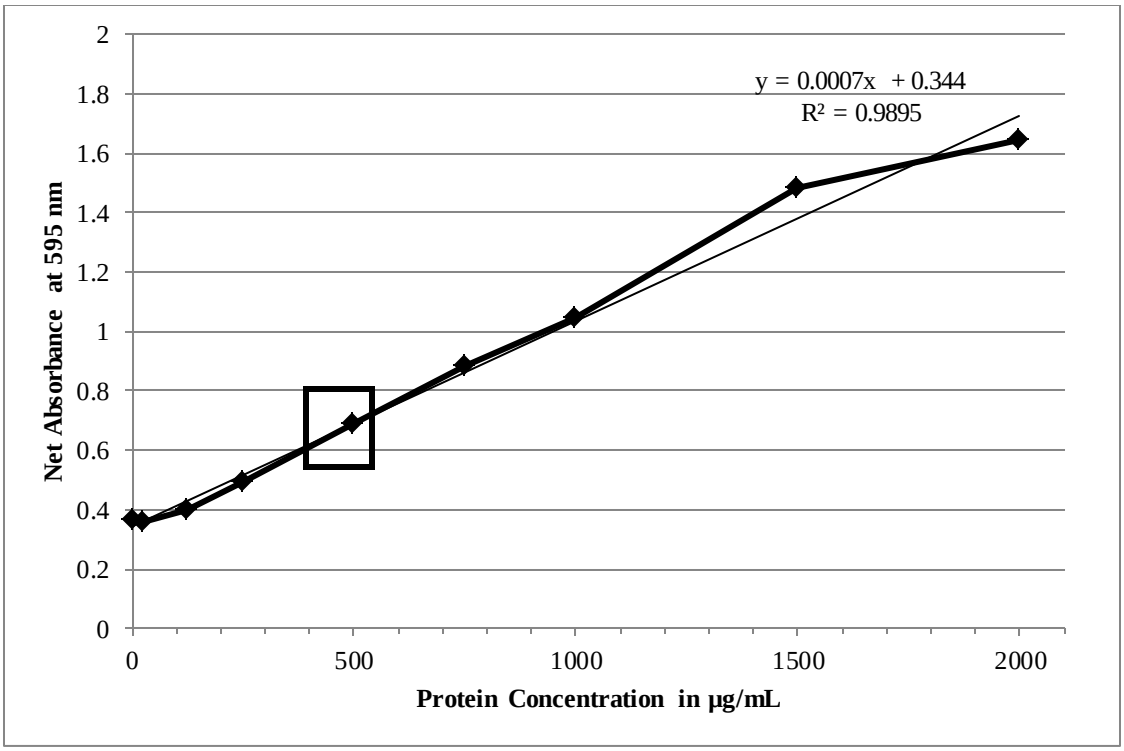

Figure 8. Protein concentration curve of standards of serum albumin for comparative determination of protein content of mesenteric arteries. All experimental samples fell within the box provided. Concentrations were calculated with the trend lines extrapolated by Excel.

Discussion

This study showed HBO treatment has an advantageous effect on the vascular reactivity of mesenteric arteries as observed with KCl induced constriction, PHE induced constriction, and SNP induced dilation. However, this change in vascular reactivity is independent of ATP concentrations in the mesenteric arteries since ATP concentration did not increase significantly under HBO conditions (Figure 7). Prior to the vascular

reactivity studies, an attempt was made to experimental find the optimal tension of mesenteric arteries. In this study there was no optimal tension observed (figure 1).

The length-tension relationship in muscle is used as a measureable and reproducible way to study muscle contraction and function in the laboratory. Manipulating the passive tension is analogous to changing a muscle's length-tension relationship and therefore, reactivity in natural physiological states. In the specific case of smooth muscle, this length-tension relationship is dynamic. As demonstrated in figure 1, arterial smooth muscle was able to produce forceful contractions at multiple passive tensions ranging from 1 to 25 grams. This same relationship is found in a study by Ratz et al. (2011) utilizing arterial smooth muscle. The length-tension relationship was dynamic in rat femoral arteries and exhibited no clear optimal passive tension.[2] By contrast, skeletal muscle which has a more structured organization of its actin and myosin filaments, exhibits a more predictable length tension relationship.[20] The diverse physiological roles required of smooth muscle may account for the necessity of a dynamic length-tension relationship, thereby allowing for optimal constriction under different physiological conditions.

The capability of an artery to constrict and dilate, that is, its vascular reactivity is the basis for its functional ability. Chemical and pharmacological agents are used to induce vasoconstriction and vasodilation; specifically, constriction by KCl and PHE or dilation by SNP. Findings from other studies regarding HBO and its effects on vascular reactivity are scarce and conflicting. Although, hyperbaric oxygen therapy's beneficial clinical effects are well recognized, little is known about hyperbaric oxygen's direct effects on the vasculature. In one study, hyperbaric oxygen treatment of rat aortic rings caused

vasoconstriction independent of any drug and decreases maximal constriction due to norepinephrine.[18] In support of my work, one study showed both increased constriction and dilation due to hyperbaric treatment when compared to their control model. Sprague-Dawley male rats were subjected to two, one hour treatments of HBO at 2 ATA. This study showed that the HBO groups had a significantly increased contraction to norepinephrine and endothelin-1, and dilation to acetylcholine when compared to the non HBO treated group.[21] There is scarce literature demonstrating the effects of HBO on KCl induced vasoconstriction in any arterial network. However, KCl is widely used in many arterial ring studies as a potent vasoconstrictor. As shown in this study, exposure to KCl caused vasoconstriction of mesenteric arteries and when treated with HBO resulted in an increased constrictive response to KCl (figure 2). PHE is another commonly used vasoconstrictor in vascular reactivity studies. Once again there is a lack of literature regarding its effects after HBO treatment. Increased concentrations of PHE resulted in increased arterial vasoconstrictions. Effects of PHE were further increased with the exposure to HBO (figure 3). The diverse findings seen in HBO reactivity might be ascribed to oxygen's various functions in the body and complex integrations in signaling pathways.

The chemical energy utilized in cellular respiration involves an electrochemical proton gradient coupled to oxidative phosphorylation. The electron transport chain in eukaryotes is just such a mechanism. NADH dehydrogenase gives two electrons to ubiquinone producing ubiquinol.[16, 40] There is also electron leakage to oxygen at this point. NADH becomes NAD^+ by the reduction of flavin to $FMNH_2$. This allows four protons to translocate to the intermembrane space of the mitochondria. Next, succinate

dehydrogenase procures additional electrons from the succinate itself and transfers them to the quinone pool. In the third step, cytochrome bc_1 allows for the sequestering of electrons that are utilized for production of a proton gradient, via quinone to quinol. Cytochrome c oxidase moves four electrons to molecular oxygen producing water and more protons are moved across the membrane. The proton gradient is then used by F_OF_1 ATP synthase to drive the production of ATP. The general rational for use of HBO is that it increases the partial pressure and availability of oxygen to tissue.[11] The increase in cellular concentration of oxygen leads to an increase availability of oxygen for cytochrome c oxidase to donate electrons to thereby increase ATP production.

Oxygen therefore plays a vital role in ATP production. An increase in both oxygen's partial pressure and concentration could allow for increased oxygen utilization in vascular smooth muscle. Therefore, an increase in oxygen could confer an increase in ATP production. Increased cellular ATP levels would have advantageous effects for both vasoconstriction and dilation. Most notably, ATP plays a role in cross bridge cycling during excitation contraction coupling. The pump used to create a calcium gradient across the sarcoplasm in a smooth muscle cell is also an ATPase pump. Additionally, ATP has been seen to be a direct modulator of vascular tone via ATP-sensitive potassium channels.[35, 42] Given ATP's significant role in smooth muscle regulation, alterations in ATP concentration were examined in attempt to elucidate a mechanism of action for HBO treatment. Unfortunately, no difference in ATP concentration was found among treatment groups when compared to HBO which suggests that ATP is not responsible for the observed alterations in vascular reactivity (figure 9). A possible explanation for this occurrence is that during ATP determination, experimental controls were not as realistic

as they needed to be allowing for the observed variance. For instance, ATP determination was not conducted at normal physiological temperature. Since oxygen is involved in multiple cellular processes such as respiration, signaling and regulation, it is quite possible that increased oxygen causes other unforeseen physiological reactions.

Based on the results of this study and unpublished work from our laboratory, simply increasing ATP concentration does not increase vascular reactivity. Future work should be directed towards investigating a more established pathway. Other mechanisms for alterations in vascular tone may be caused by modulation of smooth muscle directly, endothelium derived factors or a combination thereof. Recently, oxygen levels have been implicated in regulating ATP-sensitive potassium channels, L-type Ca^{2+} channels and CYP450-4A enzymes, all of which regulate vasomotor responses.[29] This is in support of previous studies that show oxygen tension as a significant regulator of vascular reactivity.[12] Oxygen tension has also been shown to alter endothelial cell respiration.[23] Increased oxygen pressure has been shown to increase both nitric oxide synthesis and mitochondrial peroxide production.[1, 28] These can both have effects on vascular tone. Further, the endothelium has been shown to sense oxygen levels and react by releasing autacoids which control vascular reactivity.[34]

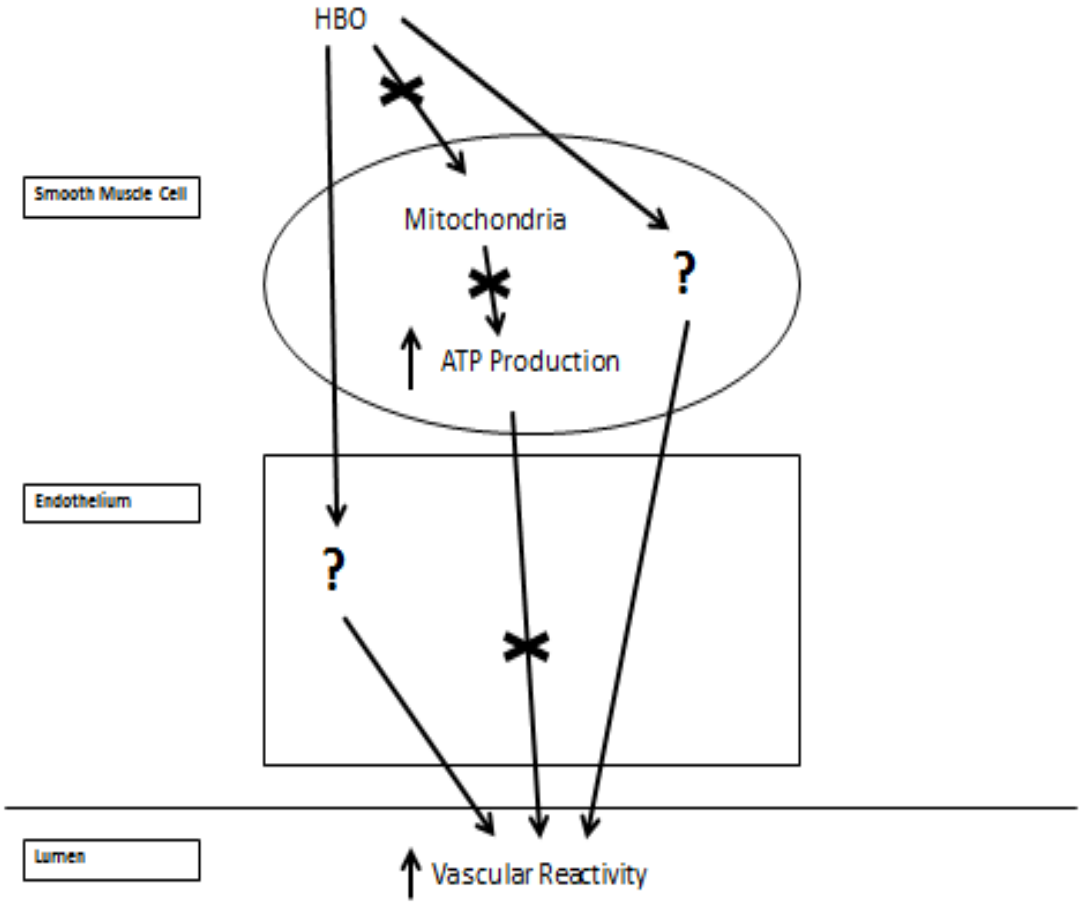

Figure 9. HBO does not increase vascular reactivity due to increased ATP levels in smooth muscle cells. HBO therapy may modulate vascular reactivity in other ways: possibly calcium mobilization, alterations at the transcriptional level or modifications of endothelial mediated responses that cause vasoconstriction or dilation.

In summary, although ATP may not be responsible for the increased vascular reactivity and subsequent advantageous effects seen clinically, there is a need to understand the mechanism to better utilize this treatment. HBO therapy has beneficial clinical applications for diabetic wound healing, organ transplant and ischemic reperfusion

syndrome. It is therefore, a great area of research to pursue in hopes of finding better treatments to various pathologies.

References

1. Baynosa RC, Naig AL, Murphy PS, Fang XH, Stephenson LL, Khiabani KT, Wang WZ, Zamboni WA. (2013) The effect of hyperbaric oxygen on nitric oxide synthase activity and expression in ischemia-reperfusion injury. *Journal of Surgical Research.* 183: 355-61.
2. Bednarek ML, Speich JE, Miner AS, Ratz PH. (2011) Active tension adaptation at a shortened arterial muscle length: inhibition by sytochalasin-D. *American Journal of Physiology Heart and Circulation Physiology.* 300: H1166-H1173.
3. Bertoletto PR, Fagundes DJ, Simones MJ, Oshima CTF, Montero EF, Simoes RS, Fagundes AT. (2007) Effects of hyperbaric oxygen therapy on rat intestinal mucosa apoptosis caused by ischemia-reperfusion injury. *Microsurgery.* 27: 224-227.
4. Buettner, M., Wolkenhauer, D. (2007) Hyperbaric Oxygen Therapy in the Treatment of Open Fractures and Crush Injuries. *Emergency Medicine Clinics of North America.* 25: 177-88
5. Carden DL, Granger DN. (2000) Pathophysiology of ischaemia-reperfusion injury. *Journal of Pathology.* 190: 255-266.
6. Carrozza JP, Bentivegna LA, Williams CP, Kuntz RE, Grossman W, Morgan JP. (1992) Decreased myofilament responsiveness in myocardial stunning follows transient calcium overload during ischemia and reperfusion. *Circulation Research*. 71:1334-1340.

7. Cipolla MJ, Bullinger LV. (2008) Reactivity of brain parenchymal arterioles after ischemia and reperfusion. *Microcirculation.*15: 495–501.
8. Davis, J. (1989) Hyperbaric oxygen therapy. *Intensive Care Medicine.* 4: 55-57.
9. Eltzschig HK, Collard CD. (2004) Vascular ischaemia and reperfusion injury. *British Medical Bulletin.* 70: 71-86.
10. Fink MP, Delude RL. (2005) Epithelial barrier dysfunction: a unifying theme to explain the pathogenesis of multiple organ dysfunction at the cellular level. *Critical Care Clinics.* 21: 117-196.
11. Fisher BR, Palkovic S, Holling M, Wolfer J, Wassman H. (2010) Rational of hyperbaric oxygen in cerebral vascular insult. *Current Vascular Pharmacology.* 8:35-43.
12. Franco-Obregón A, Ureña J, López-Barneo J. (1994) Oxygen-sensitive calcium channels in vascular smooth muscle and their possible role in hypoxic arterial relaxation. *Proceedings of the National Academy of Sciences of the United States of America: Physiology.* 92:4715-4719.
13. Frenguelli BG, Wigmore G, Llaudet E, Dale N. (2007) Temporal and mechanistic dissociation of ATP and adenosine release during ischaemia in the mammalian hippocampus. *Journal of Neurochemistry.* 101: 1400–1413.
14. Gesell LB. (2008) Hyperbaric oxygen therapy indications. The hyperbaric oxygen therapy committee report. Durham, NC: Undersea and Hyperbaric Medical Society.

15. Groot H, Rauen U. (2007) Ichemia-reperfusion injury: processes in pathogenic networks: A Review. *Transplant Proceedings*. 39: 481-484.
16. Guyton AC, Hall Je. (2000) Guyton and Hall textbook of medical physiology 10^{ed}. Philadelphia: Saunders.
17. Hein TW, Ren Y, Potts LB, Yuan Z, Kuo E, Rosa RH, Kuo L. (2012) Acute retinal ischemia inhibits endothelium-dependent nitric oxide–mediated dilation of retinal arterioles via enhanced superoxide production. *Investigative Ophthalmology and Visual Science*. 53: 30–36.
18. Hink J, Thom SR, Simonsen U, Rubin I, Jansen E. (2006) Vascular reactivity and endothelial NOS activity in rat thoracic aorta during and after hyperbaric oxygen exposure. *American Journal of Physiology Heart Circulation Physiology*. 291: 1988-1998.
19. Hughes SF, Hendricks BD, Edwards DR, Bastawrous SS, Roberts GE, Middleton JF. (2007) Mild episodes of tourniquet-induced forearm ischaemia-reperfusion injury results in leukocyte activation and changes in inflammatory and coagulation markers. *Journal of Inflammation*. 4: 12.
20. Huxley AF. (1974) Muscle contraction. *Journal of Physiology*. 243: 1-43.
21. Imperatore F, Cuzzocrea S, Luongo C, Liguori G, Scafuro A, Angelis A. (2004) Hyperbaric oxygen therapy prevents vascular derangement during zymosan-induced multiple-organ-failure syndrome. *Intensive Care Med*. 30: 1175-1181.

22. James IA, Chen CL, Huang G, Zhang HY, Velten M, Besner GE. (2010) HB-EGF protects the lungs after intestinal ischemia/reperfusion injury. *Journal of Surgical Research.* 163: 86-95.

23. Jones CI, Han Z, Presley T, Varadharaj S, Zweier JL, Ilangovan G, Alevriadou BR. (2008) Endothelial cell respiration is affected by the oxygen tension during shear exposure: role of mitochondrial peroxynitrite. *American Journal of Physiology Cell Physiology.* 295: C180-C191.

24. Kong Se, Blennerhassett LR, Heel KA, McCauley RD, Hall JC, (1998) Ischaemia-reperfusion injury to the intestine. *Australian and New Zealand Journal of Surgery.* 68: 554-561.

25. Kutala VK, Khan M, Angelos MG, Kuppusamy P. (2007) Role of oxygen in postischemic myocardial injury. *Antioxidants and Redox Signaling.* 9: 1193-1206.

26. LaVan FB, Hunt TK. (1990) Oxygen and wound healing. *Clinics in Plastic Surgery*. 17:463-472.

27. Marti HJ, Bernaudin M, Bellail A, Schoch H, Euler M, Petit E, Risau W. (2000) Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. *The American Journal of Pathology.* 156: 965-76.

28. Matsunami T, Sato Y, Hasegawa Y, Ariga S, Kashimura H, Sato T, Yukawa M. (2011) Enhancement of reactive oxygen species and induction of apoptosis in streptozotocin-induced diabetic rats under hyperbaric oxygen exposure. *The International Journal of Clinical and Experimental Pathology.* 4: 255-66.

29. Ngo AT, Riemann M, Holstein-Rathlou N, Pedersen CT, Jensen LJ. (2013) Significance of K_{ATP} channels, L-type Ca^{2+} channels and CYP450-4A enzymes in oxygen sensing in mouse cremaster muscle arterioles. *In vivo*. *BioMed Central Physiology*. 13:8.

30. Ngoh GA, Watson LJ, Facundo HT, Jones SP. (2011) Augmented O-GlcNAc signaling attenuates oxidative stress and calcium overload in cardiomyocytes. *Amino Acids*. 40: 895–911.

31. Nosáľová V, Sotníková R, Drábiková K, Fialová S, Košťálová D, Banášová S, Navarová J. (2010) Chemiluminescence response induced by mesenteric ischaemia/reperfusion: effect of antioxidative compounds ex vivo. *Interdisciplinary Toxicology*. 3: 105–108.

32. Park YY. (2009) Ischemia/reperfusion lung injury increases serum ferritin and heme oxygenase-1 in rats. *Korean Journal of Physiology and Pharmacology*. 13:191-197.

33. Perrelli MG, Pagliaro P, Penna C. (2011) Ischemia/reperfusion injury and cardioprotective mechanisms: Role of mitochondria and reactive oxygen species. *World Journal of Cardiology*. 3: 186–200.

34. Pohl U. (1990) Endothelial cells as part of a vascular oxygen-sensing system: Hypoxia-induced release of autacoids. *Experientia*. 46: 1175-1179.

35. Quayle JM, Nelson MT, Standen NB. (1997) ATP-sensitive and inwardly recifying potassium channels in smooth muscle. *Physiological Review*. 77: 1166-1232.

36. Rotstein OD. (2000) Pathogenesis of multiple organ dysfunction syndrome: gut origin, protection, and decontamination. *Surgical Infection*. 1: 217-225.

37. Siegel MP, Kruse SE, Knowels G, Salmon A, Beyer R, Xie H, Remmen HV, Smith SR, Marcinek DJ. (2011) Reduced coupling of oxidative phosphorylation in vivo precedes electron transport chain defects due to mild oxidative stress in mice. *PLoS One*. 11: e26963.

38. Springer TA. (1994) Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell*. 76: 301-314.

39. Thom SR, Bhopale VM, Velazquez OC. (2006) Stem cell mobilization by hyperbaric oxygen. *American Journal of Physiology*. 4: 1378-1386.

40. Voet D, Pratt, CW Voet JG. (2011) Fundamentals of biochemistry: Life at the molecular level 4^{ed}. Toronto: John Wiley & Sons Canada, Limited.

41. Williams, S. (2010) The role of hyperbaric oxygen therapy in trauma. *Trauma*. 12: 13-20.

42. Zhang L, Bonev AD, Mawe GM, Nelson MT. (1994) Protein kinase A mediates activation of ATP-sensitive K^+ currents by CGRP in gallbladder smooth muscle. *American Journal of Physiology*. 267: G494-G499.