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# Hyperbaric Oxygen Therapy Alters Vascular Reactivity Independent of Elevations in ATP

Benjamin Hake<sup>1</sup>, Cole Waggener<sup>1</sup>, Benjamin Eovaldi<sup>2</sup>, Claude Zanetti<sup>3</sup> and Francis Sylvester<sup>1</sup>

Hyperbaric oxygen (HBO) is a pharmacologic therapy used to treat a variety of medical conditions. The underlying mechanisms of HBO's effect on vascular reactivity are poorly understood. The purpose of this study was to record the acute effects of HBO on vascular reactivity and determine the potential role of ATP in mediating these effects. Porcine mesenteric arteries were dissected and mounted in isolated organ baths to record changes in tension in response to potassium chloride (KCl, 15-60 mM), phenylephrine (PHE,  $10^{-7}$ - $10^{-4}$  M), and sodium nitroprusside (SNP,  $10^{-7}$ - $10^{-4}$  M) following a 2-hour exposure to HBO (1.75 ATA). HBO augmented responses to KCl and PHE compared to control arteries exposed to room air or nitrogen at 1.75 ATA as well as to room air at 1 atm. When compared to the 1 ATA room air control, KCl-induced constriction was significantly increased for the HBO exposure. Treatment with HBO also augmented vascular responses to PHE and SNP relative to nitrogen, but not ambient air. We hypothesized that HBO increased ATP production in vascular smooth muscle leading to enhanced vascular reactivity. Consequently, ATP levels were measured in mesenteric arteries but no significant differences in ATP levels were observed regardless of hyperbaric treatment. Direct measurements of ATP's effects on porcine vasculature with and without hyperbaric treatment resulted in no significant findings. These results suggest that HBO alters vascular reactivity independent of elevations in ATP.

**Keywords:** Hyperbaric oxygen; vascular reactivity; artery

## 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 higher levels 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.<sup>26, 30</sup> 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.<sup>20, 30</sup>

The lack of oxygen and nutrients during the initial ischemic event decreases the production of ATP.<sup>9, 13</sup> Lack of ATP causes a decrease in intracellular pH due to lactic acid accumulation. Insufficient ATP will also impair 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 elevation of intracellular calcium being observed.<sup>6</sup> 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.<sup>27, 35</sup>

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.<sup>29</sup> 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.<sup>23, 31</sup> 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 complement system.<sup>18</sup> The subsequent inflammation results in new tissue damage.

The vasculature then performs 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.<sup>7, 9, 16</sup> By normally controlling homeostatic vasodilation and constriction the integrity of the vasculature is maintained. Consequently, the protective barrier function of the vascular endothelium is decreased by the deteriorating condition of the cells.<sup>22</sup> An increase in growth factors is also associated with the lack of oxygen.<sup>25</sup> During reperfusion, the endothelial cells initiate expression of adhesion molecules for leukocytes and neutrophils which leads to further damage of the tissue.<sup>5, 36</sup>

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.<sup>10, 34</sup> 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.<sup>3, 17, 19</sup> 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

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or above, neurotoxicity is observed. 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.<sup>8, 39</sup> 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.<sup>14</sup> HBO therapy has not only been shown to aid in vascularization in ischemic tissue but directly enhance fibroblast replication and collagen synthesis in ischemia.<sup>26</sup> Some evidence suggest HBO stimulates blood cell progenitors, which aid in the recovery of damaged tissue, possibly utilizing a nitric oxide dependent pathway.<sup>37</sup>

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.

## Results

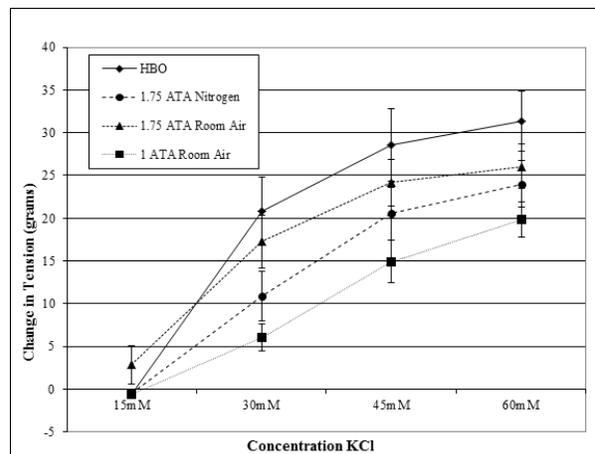
### Optimal Tension

*In vivo*, arteries have a continual amount of tension in their walls which can also be described as tone. 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 were 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 then analyzed for significance by ANOVA. 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. 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.

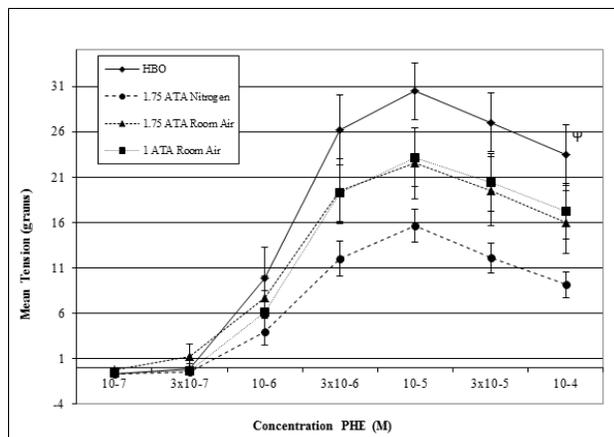
### Vascular Reactivity

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

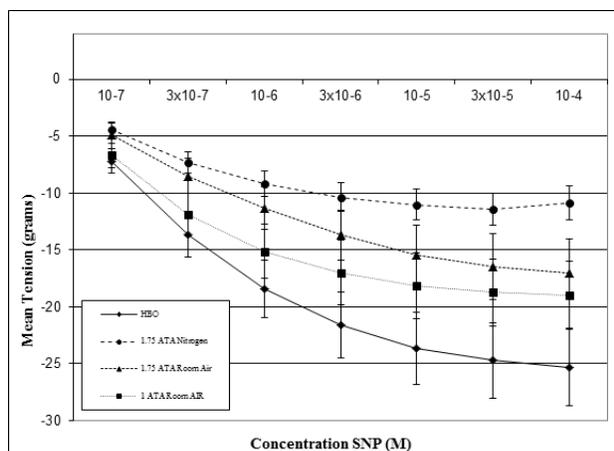
vascular reactivity for each arterial ring was defined as the change in tension from the initial passive tension set by the experimenter to the new recorded tension observed after administration of the stimulus. The vascular reactivity for arterial rings was compared among treatments. The results of 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 1). 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 2). At their largest differences (10<sup>-5</sup> 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 3). For example at a concentration of 10<sup>-5</sup> 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 (ANOVA). 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. 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. Results were stated as mean±SEM.



**Figure 1:** The effects of cumulative increases 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 2:** The effects of PHE on vasoconstriction of porcine arteries following 2 hour exposure to one of various hyperbaric conditions. The changes in arterial tension are differences measured in grams in response to increasing concentrations of PHE. Results are expressed as mean±SEM. † Response significantly different from 100% nitrogen (n=12 p<0.05, regression analysis).

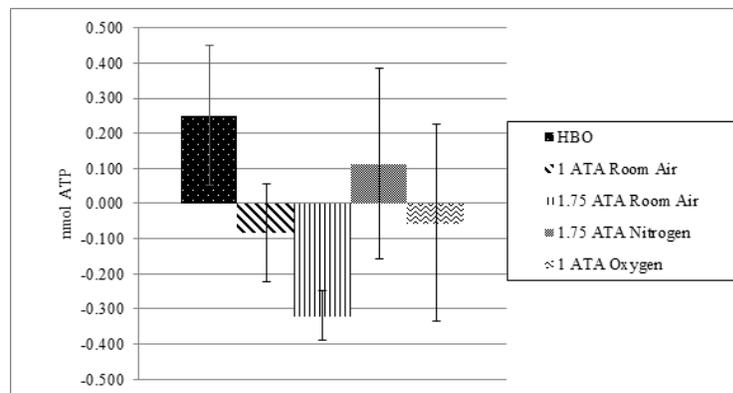


**Figure 3:** The effects of SNP on vasodilation of porcine arteries following precontraction with PHE. Treatments were 2 hour exposures to one of various hyperbaric conditions. The changes in arterial tension are differences measured in grams in response to increasing concentrations of SNP. Results are expressed as mean±SEM. † Response significantly different from all other treatments (n=12 p<0.05, ANOVA).

#### ATP Analysis

The previous experiments suggest 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 4). Hyperbaric

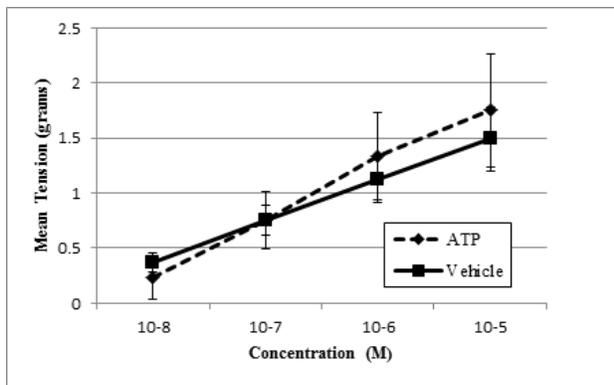
nitrogen (0.1141±0.2705 nmol) was the next largest average ATP concentration when compared to ambient air and hyperbaric room air showing the lowest average concentration. After consideration, it was determined that 100% oxygen at 1.0 ATA would make another suitable control and was therefore added to 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 (data not shown). This demonstrated that each arterial ring contained approximately the same amount of protein.



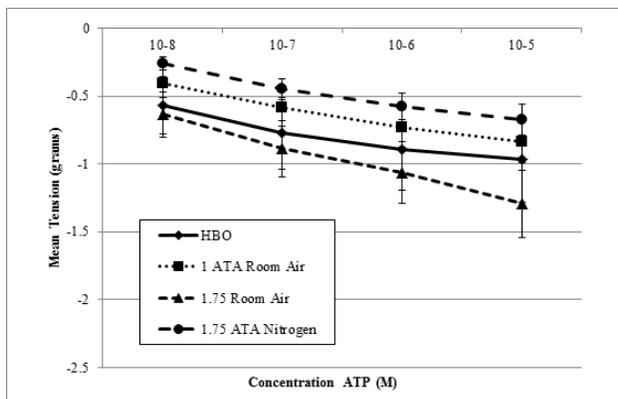
**Figure 4:** Comparison of ATP concentrations and pressure/gas treatments. Bars represent average nmol ATP per 1 microgram of protein from porcine mesenteric arteries following 2 hour exposure to one pressure/gas treatment. Colorimetric concentration was measured at 570 nm with quantities being in the nanomolar range. Results are expressed as mean±SEM (n=6). No significant difference was found among treatments (ANOVA).

#### Direct effect of ATP on Vascular Rings

Vascular rings were pretreated with a single 30 mM dose of KCl to induce vasoconstriction. After the response to KCl plateaued, ATP or vehicle was added in increasing concentrations. As shown in Figure 5, the mean change in force for both ATP and vehicle, although increased, was not significant. The total mean change in force after the 20 minute period was less than two grams. This experiment was repeated without preconstruction with KCl and, once again, there was no significant response to exogenous ATP (data not shown). Vascular rings were exposed to a two-hour gas/pressure treatment, with the 100% oxygen treatment representing the HBO therapy, prior to the administration of the ATP or vehicle. As shown in figure 6, all four treatments resulted in a decrease in the mean change in force which indicates vasodilation is occurring. However, the total mean change in force after the 20 minute period for all treatments is less than 1.5 grams with no observed significant difference among treatments.



**Figure 5:** Vascular effect of ATP or vehicle on porcine arteries precontracted with 30 mM KCl. Values are expressed as mean±SEM (n=5-9). No significant difference was found among treatments (ANOVA).



**Figure 6:** Vascular effect of ATP or vehicle on porcine arteries after one of four two-hour gas/pressure treatments. Values are expressed as mean±SEM (n=7-8). No significant difference was found among treatments (ANOVA).

## Discussion

This study demonstrated that HBO therapy improved the vascular reactivity of mesenteric arteries in response to KCl, PHE, and SNP. However, the hypothesis that improved vascular reactivity is mediated by changes in ATP concentration in the mesenteric arteries was not supported by this study. The ATP concentrations of the mesenteric arteries did not significantly increase under exposure to HBO conditions (Figure 4).

Prior to the conduction of the vascular reactivity studies, an attempt was made to experimentally determine the optimal tension of mesenteric arteries studied *in vitro*. In this study there was no optimal tension observed. The length-tension relationship of muscle is an important consideration in the study of muscle contraction in the laboratory. The length-tension relationship of vascular smooth muscle is dynamic. As observed in our study, 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 Bednarek et al. (2011) utilizing arterial smooth

muscle.<sup>2</sup> The length-tension relationship was dynamic in rat femoral arteries and exhibited no clear optimal passive tension.

An artery's functional ability is measured by its capacity to constrict and dilate upon exposure to pharmacological and mechanical stimuli. KCl and PHE were used as pharmacological stimuli capable of inducing vasoconstriction. SNP was used as a vasodilator, pharmacological stimulus. Findings from other studies involving HBO therapy and its effects on vascular reactivity are scarce and conflicting. Although, HBO therapy's beneficial clinical effects are well recognized, little is known about HBO's direct effects on the vasculature. In one study, HBO treatment of rat aortic rings caused vasoconstriction independent of exogenous stimuli and decreased maximal constriction due to norepinephrine.<sup>17</sup> In contrast, Imperatore et al. demonstrated that HBO therapy increased contraction to norepinephrine and endothelin-1 and dilation to acetylcholine when compared to the control group.<sup>19</sup> There is scarce literature demonstrating the effects of HBO on KCl-induced vasoconstriction in any arterial network. However, KCl is widely used in arterial ring studies as a potent vasoconstrictor. As shown in this study, exposure to KCl caused vasoconstriction of mesenteric arteries and this response was enhanced when arteries were given HBO therapy (figure 1). PHE is another commonly used vasoconstrictor in vascular reactivity studies.

PHE, a known vasoconstrictor, caused dose dependent increases in the force of contraction of arteries in the control and HBO groups. The vasoconstrictive effects of PHE were augmented by HBO exposure (figure 2). The general rationale for use of HBO is that it increases the partial pressure and availability of oxygen to tissues and may ultimately lead to an increase in ATP production.<sup>11</sup> Oxygen 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 serves as a modulator of vascular tone via ATP-sensitive potassium channels.<sup>33, 40</sup> 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. However, this study demonstrated no differences in ATP concentrations among treatment groups suggesting that ATP is not responsible for the observed alterations in vascular reactivity. A possible explanation for this occurrence is that during ATP determination, experimental controls did not accurately mimic *in vivo* conditions. 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, simply increasing ATP concentration does not alter vascular reactivity. Future work should be directed towards investigating other possible mechanisms. Alterations in vascular tone may be caused by

modulation of smooth muscle, the endothelium, or a combination thereof. Recently, oxygen levels have been implicated in regulating ATP-sensitive potassium channels, L-type  $\text{Ca}^{2+}$  channels and CYP450-4A enzymes, all of which regulate vascular responses.<sup>27</sup> This is in support of a previous study that shows oxygen tension as a significant regulator of vascular reactivity.<sup>12</sup> Oxygen tension has also been shown to alter endothelial cell respiration.<sup>21</sup> Additionally, increased oxygen pressure has been shown to increase both nitric oxide synthesis and mitochondrial peroxide production.<sup>1, 26</sup> 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.<sup>32</sup> In summary, although ATP may not be responsible for the increased vascular reactivity, there remains a need to better understand the underlying mechanism associated with HBO therapy. HBO therapy has beneficial clinical applications for diabetic wound healing, organ transplant and ischemic reperfusion syndrome. It is therefore, a significant area of research to pursue in hopes of finding better treatments to various pathologies.

## Experimental Procedures

### *Animals & Reagents*

All specimens were obtained from De Vries Meats Inc. of Coopersville, Michigan. Animals were 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 was unknown but believed to be either the right gastroepiploic artery or pancreaticoduodenal 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  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 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 and left in solution. Krebs-Henseleit buffer solution had a composition of 118 mM NaCl, 4.7 mM KCl, 1.2 mM  $\text{MgSO}_4$ , 1.25 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 25 mM  $\text{NaHCO}_3$ , and 11 mM glucose. All reagents were 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 °C. 95%  $\text{O}_2$  and 5%  $\text{CO}_2$  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 KCl, 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. An ANOVA 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 was custom built metal cylinder (approximately 29 cm inner-diameter with a depth of 8 cm). 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%  $\text{O}_2$  and 5%  $\text{CO}_2$  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 KCl (15, 30, 40, 60 mM) to induce vasoconstriction and assess viability. Arteries exhibiting an increase in tension of 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 PHE to induce vasoconstriction and SNP to induce vasodilation. Arteries were then treated with increasing concentrations of PHE ( $10^{-7}$ ,  $3 \times 10^{-7}$ ,  $10^{-6}$ ,  $3 \times 10^{-6}$ ,  $10^{-5}$ ,  $3 \times 10^{-5}$ ,  $10^{-4}$  M) followed by increasing concentrations of SNP ( $10^{-7}$ ,  $3 \times 10^{-7}$ ,  $10^{-6}$ ,  $3 \times 10^{-6}$ ,  $10^{-5}$ ,  $3 \times 10^{-5}$ ,  $10^{-4}$  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). An ANOVA was conducted for the weight and outer diameter measurements. Data were analyzed using Sigma Stats and plotted using Excel. PHE was

purchased from Spectrum Chemicals (Gardena, CA). SNP 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  $-80^{\circ}\text{C}$  for later processing. Frozen arteries were homogenized cold, using a mortar and pestle in 1.5 mL of 2 M  $\text{HClO}_4$ . 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  $0^{\circ}\text{C}$  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  $\text{HClO}_4$  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 allocation. 10  $\mu\text{L}$  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 allocation. For each well the sample was transferred and adjusted to 10  $\mu\text{L}$  as necessary with 2 M  $\text{HClO}_4$ . 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.

#### *Direct effect of ATP on Vascular Rings*

Vascular rings were dissected out as previously described. The vascular rings were then placed in the organ bath for 45 minutes to one hour. During this period, the tension on the force transducer was adjusted to seven grams and the Krebs-Henseleit solution was replaced every ten minutes. Following equilibration, the vascular rings were administered increasing concentrations of 15 mM, 30 mM, 45 mM, and 60 mM KCl, at five minute intervals, followed by  $1 \times 10^{-7}\text{M}$ ,  $1 \times 10^{-6}\text{M}$ ,  $1 \times 10^{-5}\text{M}$ , and  $1 \times 10^{-4}\text{M}$  SNP also waiting five minutes between doses. The vessels were equilibrated and tested for viability using KCl to verify vasoconstriction and SNP to verify vasodilation. The organ baths were emptied and the vascular rings were washed three times in Krebs-Henseleit solution every five minutes. The organ baths were refilled with 25 mL of Krebs-Henseleit solution and the vascular rings were exposed to increasing concentrations of  $1 \times 10^{-8}\text{M}$ ,  $1 \times 10^{-7}\text{M}$ ,  $1 \times 10^{-6}\text{M}$ , and  $1 \times 10^{-5}\text{M}$  ATP (Enzo Life Science, Farmingdale, NY) or vehicle alone (deionized water), waiting five minutes between doses. If the vessel was constricted with 30 mM KCl before the addition of ATP, the organ baths were washed three times with Krebs-Henseleit solution for five minutes each and refilled with 25 mL of Krebs-Henseleit solution. The vessels were then given a single dose of 30 M KCl for fifteen minutes to precontract the vessels. The precontracted vessels were treated with  $1 \times 10^{-8}\text{M}$ ,  $1 \times 10^{-7}\text{M}$ ,  $1 \times 10^{-6}\text{M}$ , and  $1 \times 10^{-5}\text{M}$  ATP, waiting five minutes between doses. At the conclusion of the experiment, the vascular rings were dried then weighed, and the outer diameter was measured using a caliper.

Other data was collected using a similar procedure to that previously described but with notable exceptions. Vascular rings were placed in Krebs-Henseleit solution, or, if doing two runs in one day, the vessels of the second run were placed in the long-term storage buffer, HEPES with an adjusted pH of 7.4. Prior to the vascular studies rings were placed in the Krebs-Henseleit solution then into the hyperbaric chamber under one of the four gas/pressure treatments (100%  $\text{O}_2$  at 26psi, 100%  $\text{N}_2$  at 26psi, room air at 26 psi, and room air at atmospheric pressure) for two hours. The vessels stored in HEPES solution were switched into the Krebs-Henseleit solution before being placed in the hyperbaric chamber. Following hyperbaric treatment, the vessels were placed on the force transducers in the organ bath as previously described. The vessels were equilibrated and tested for viability using KCl and SNP. Viable vascular rings were exposed to ATP as in the above procedure; however no precontraction of the vascular rings using KCl was performed. At the conclusion of the experiment, the vascular rings were dried then weighed, and the outer diameter was measured using a caliper.

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