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Acute Hyperbaric Oxygen Therapy Alters Vascular Reactivity Independent of ATP

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Abstract

Hyperbaric oxygen therapy (HBO), breathing 100 percent oxygen at greater than one atmosphere, has been prescribed for many different medical reasons. The mechanism for HBO, however, is not entirely understood. Adenosine triphosphate (ATP) is the main energy carrier molecule in the body and its production requires oxygen. By increasing the amount of oxygen carried by the blood, the amount of ATP could also increase. This possible mechanism for HBO was tested using the addition of exogenous ATP on approximately five millimeter wide rings of fresh porcine gut arteries. The vascular rings were attached to a force transducer which measured the change in force exerted by the vascular rings when exposed to ATP. The addition of ATP to the vascular rings that did not undergo any hyperbaric treatment did not result in a statistically significant change in force ($p>0.05$). The addition of ATP to the vascular rings that did undergo hyperbaric treatments also did not result in statistically significant change in force ($p>0.05$). These results suggest that the HBO mechanism is independent of changes in ATP.

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Abbreviations

Ach – Acetylcholine

ANOVA – Analysis of Variance

ANS – Autonomic Nervous System

Atm – Atmospheres

ATP- Adenosine Triphosphate

cGMP – Cyclic Guanosine Monophosphate

DI – Deionized

eNOS – Endothelial Nitric Oxide Synthase

GTP – Guanosine Triphosphate

K-ATP – ATP Sensitive Potassium Channel

KCl – Potassium Chloride

NO – Nitric Oxide

SNP – Sodium Nitroprusside

Introduction

Many types of arteries within the body function to move oxygenated blood from the heart to the living tissues, i.e. the systemic circulatory system, and to move deoxygenated blood from the heart to the lungs, i.e. the pulmonary circulatory system. All arteries are controlled by at least one of the two divisions of the autonomic nervous system which allows involuntary control over the narrowing of the vessel, vasoconstriction, or widening of the vessel, vasodilation. Vasodilation occurs during tissue damage to increase the volume of blood to the damaged area, thus providing increased oxygen, cells of the immune system to fight off pathogens, and cellular fragments of megakaryocytes, called platelets, to form clots. All of these are necessary to initiate and maintain the healing process.

At the histologic level, the artery is composed of three layers from most lumenal to outermost: the tunica intima, tunica media, and tunica adventitia. The tunica intima is composed of a single layer of squamous epithelial cells surrounded by a basal lamina (together comprising the endothelium), subendothelial connective tissue, and an internal elastic membrane. The middle layer, tunica media, is the thickest of the three layers and contains layers of smooth muscle arranged circumferentially or helically and can contain lamellae of elastin in certain types of vessels. The outermost layer, tunica adventitia, is a connective tissue layer that is the least vasoactive and relatively thin in arteries. In larger arteries, the tunica adventitia may contain vasa vasorum and nervi vascularis which supply blood and autonomic innervation to the artery, respectively.

At the gross level, the arterial anatomy of the gastrointestinal system begins as the abdominal aorta emerges through the aortic hiatus of the diaphragm at the level of the twelfth thoracic vertebra. Three midline branches extend anteriorly off the abdominal aorta:

the celiac trunk, the superior mesenteric artery, and the inferior mesenteric artery. The celiac trunk quickly branches into the common hepatic artery which supplies blood to the liver, duodenum, and pancreas; the left gastric artery which supplies blood to the lesser curvature of the stomach and lower portion of the esophagus; and the splenic artery which supplies blood to the spleen. The superior mesenteric artery supplies blood to the small intestine, ascending colon, and transverse colon while the inferior mesenteric artery supplies blood to the descending colon, the sigmoid colon, and the rectum. The possible homologous arteries used in this research are the branches of the three abdominal aorta midline branches which can include the splenic artery, left gastric artery, gastroduodenal artery, right gastroepiploic artery, right colic artery, middle colic artery, left colic artery, superior rectal artery, and the jejunal arteries.

Arteries branch into smaller vessels called arterioles. Arterioles branch into capillaries. Blood flows from the capillaries into the venous system by way of venules which flow into veins back to the heart. The arterioles, capillaries, and venules make up the microcirculation. Capillaries are delicate and cannot withstand the high pressures of the arterial system so the microcirculation must be adept at regulating the blood pressure and blood flow at the local level. This regulation is accomplished through a process called autoregulation. Autoregulation regulates vessel vasodilation or vasoconstriction without the influence of other organs or nervous system input at the normal physiologic blood pressure range of 50-110 mmHg. There are two main regulatory methods: metabolic and myogenic. Metabolic regulation is related to metabolism. As metabolism increases, blood flow increases to meet the increased demand on the tissues. The exact mechanism regulating metabolic regulation is unknown. Myogenic regulation uses the pressure exerted by the blood against the wall of the artery, called transmural pressure, to cause a response in the vascular smooth

muscle. With a high transmural pressure, the blood vessel vasocontricts to increase the resistance on blood flow which lowers the blood pressure as the blood enters the capillaries. With a low transmural pressure, the blood vessel vasodilates to decrease the resistance on blood flow which increases the blood pressure as the blood enters the capillaries. The two mechanisms of autoregulation allow the blood vessels to stabilize blood flow through the capillaries with only a slight altering of the blood pressure in the larger blood vessels.

Vasodilation and vasoconstriction are a part of vascular reactivity controlled by the autonomic nervous system (ANS). The cardiovascular control center, located in the medulla oblongata, receives information from afferent neurons that arise from baroreceptors, the primary acute blood pressure regulator, and chemoreceptors. Baroreceptors respond to increases in blood pressure by decreasing heart rate and initiating reflexes that cause blood vessels to dilate. Chemoreceptors monitor primarily oxygen, carbon dioxide, and proton levels in the blood. To cause vascular changes, the ANS is divided in to two divisions: the sympathetic nervous system and the parasympathetic nervous system.

Sympathetic innervation almost exclusively uses α-adrenergic receptors for vasoconstriction and β-adrenergic receptors for vasodilation of blood vessels. However, the parasympathetic system can cause vasodilation of blood vessels that is regulated by muscarinic cholinergic receptors located within the endothelial lining of the blood vessel. Acetylcholine (Ach), released from parasympathetic neurons, binds to the muscarinic receptor which activates the enzyme endothelial nitric oxide synthase (eNOS), thus producing nitric oxide (NO). The NO diffuses into the adjacent smooth muscle cells where it activates guanylyl cyclase to convert GTP into cyclic GMP (cGMP) causing a local smooth muscle relaxation, or vasodilation. The drug, sodium nitroprusside (SNP) which is used in

this study, dissociates immediately into NO, bypassing the need to activate the eNOS and the endothelial lining of the blood vessel (Figure 1).

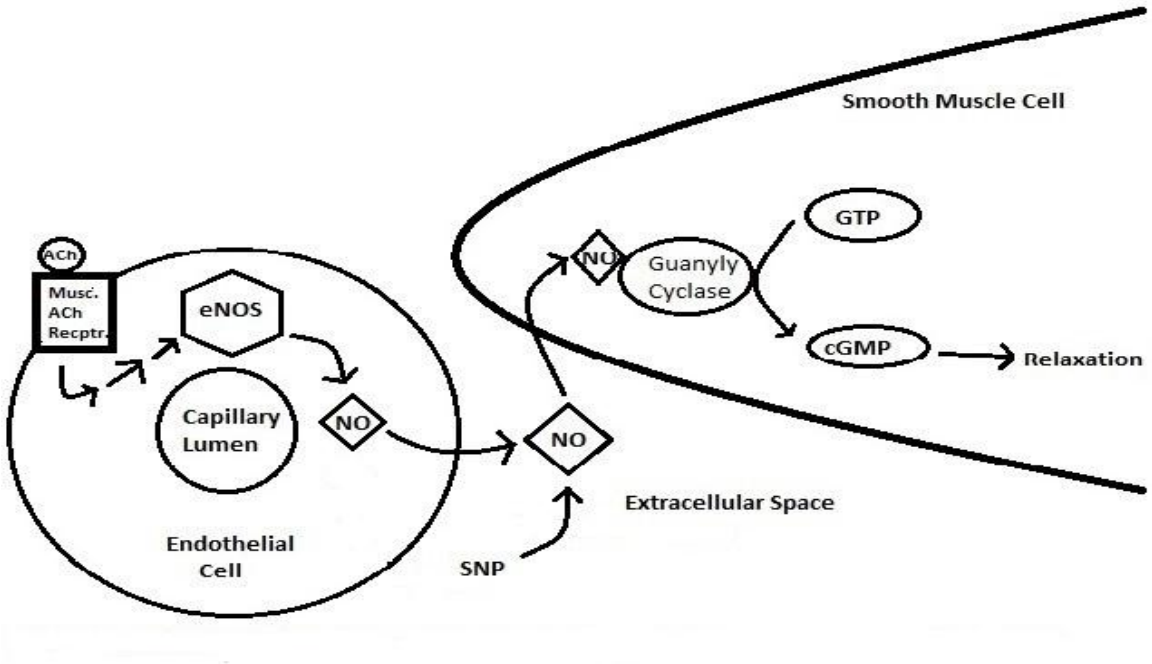

Figure 1. The acetylcholine signal transduction pathway in arteries mediated by the endothelium and vascular smooth muscle. Modified from Boron & Bulpaep, 2009.

Hyperbaric oxygen therapy is defined as breathing a high concentration of oxygen in a pressurized room, or smaller chamber. It has been used in the medical field to increase the partial pressure of oxygen in the blood to allow the tissues to absorb more oxygen and enhance healing ability (Aydin, et. al. 2013). However, the mechanism and efficacy of hyperbaric oxygen therapy are not fully understood. The air in the atmosphere has a pressure of approximately one atmosphere, or 14 psi, and contains approximately 78% N_2, 21% O_2, 0.9% Ar, and 0.1% other molecules. The pressure of air during hyperbaric oxygen therapy can range between greater than 1 atmosphere to 3 atmospheres and contains 100% O_2. At greater than 3 atmospheres of 100% O_2, hyperbaric oxygen therapy becomes toxic to the

body. However, between the effective range, hyperbaric oxygen therapy has been used to treat carbon monoxide poisoning (Weaver, et. al. 2002), traumatic brain injury, and compromised wound healing situations such as those associated with diabetics (Abidia, et. al. 2003; Kessler, et. al. 2003). Although the exact mechanism is unknown, these uses of hyperbaric oxygen therapy have been thought to be successful due to the increased concentration of oxygen in the blood. In normal circulation, oxygen binds to hemoglobin within the erythrocyte while the cell is within the lung's respiratory membrane. Hemoglobin's affinity for oxygen prevents dissociation until the erythrocyte reaches body tissues requiring oxygen. At one atmosphere, hemoglobin is approximately 98 percent saturated with oxygen with only a small amount of oxygen dissolved in the blood plasma. Increasing the pressure increases the amount of oxygen dissolved in the blood plasma. The increase in the oxygen concentration in the blood plasma allows more oxygen to be delivered to the tissues (Lambertsen, et. al. 1953). More oxygen reaching the tissues could allow an increase in the production of ATP by aiding the electron transport chain. This increased ATP could be causing an alteration in the vascular reactivity in the affected tissues leading to the perceived enhanced wound healing.

ATP is the main energy-carrier molecule and also has many various roles as a second messenger in cells. The production of ATP occurs due to the aerobic breakdown of glucose through glycolysis, the Kreb's cycle, and the electron transport chain. Thirty-two of the 36 ATP molecules resulting from these processes are produced during the electron transport chain. The electron transport chain requires oxygen as the final electron acceptor; and as the final electron acceptor, oxygen is reduced to form water. Physiological levels of ATP (approximately one nanomolar) are known to alter vascular reactivity (Manfredi, et. al. 2002). In certain arteries, ATP sensitive potassium channels (K-ATP) have been shown to

cause vasodilation. However, the existence of K-ATP channels in porcine gut arteries has yet to be confirmed (Pan, et. al. 2010). The K-ATP channel is an octamer composed of four subunits of SUR1 and four subunits of Kir6.2 (Figure 2).

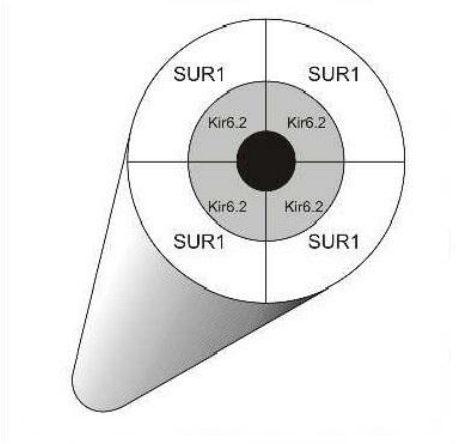

Figure 2. K-ATP Channel showing two distinct subunits. (Contreras, et. al. 2002)

The Kir6.2 subunits bind the ATP which prolongs the closure of the tonically active potassium channel (Inagaki, et. al. 1995). K-ATP channels are critical in smooth muscle contraction because these channels allow for the depolarization of the cell membrane. The depolarization activates (opens) voltage-gated calcium channels. Extracellular calcium molecules flow into the cytoplasm of the cell initiating contraction of muscle cells. The presence of ATP blocks the K-ATP channel causing the membrane to depolarize which activates the voltage-gated calcium channels, thus increasing free calcium within the cell and ultimately causes vasoconstriction. Conversely, in low ATP concentrations, the K-ATP channel is open allowing potassium to efflux and hyperpolarizes the membrane. With no depolarization of the membrane, the voltage-gated calcium channels close, thus blocking the movement of free calcium into to the cell that is necessary for muscular contraction and ultimately causing vasodilation (Maechler, et.al. 1998). The hypothesis for the mechanism of

HBO is that more oxygen is dissolved in the blood plasma due to both the increased content of oxygen and the increased pressure due to the HBO and could allow an increase in oxygen to reach the living tissues. If an increased amount of oxygen reaches the living tissues an increased production of ATP could occur and the increased levels of ATP could be responsible for the alteration in vascular biology and enhanced wound healing.

Methods

Porcine mesentery was obtained from the local abattoir and placed on ice. Mesenteric arteries were identified, dissected from surrounding tissues, and cut into five millimeter long segments, i.e. vascular rings. The mesenteric vessels chosen for use can include: the gastroepiploic, the superior mesenteric, the inferior mesenteric as well as branches of the celiac trunk. The vascular rings were placed in a Krebs-Henseleit solution composed of: 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 11 mM glucose. The vascular rings were then attached to the post and chain of a Grass force transducer, Model FT03C. The vascular rings and the post and chain of the force transducers were submerged in an organ bath filled with 25mL Krebs-Henseleit solution (heated to 37°C) while gas (composed of 95% oxygen and 5% carbon dioxide) was continuously bubbled through the solution. The opposite end of the force transducer was connected to the Iworx data acquisition system (Dover, NH) which uses LabScribe V2.336 software to record data. $CaCl_2$ was purchased from Fischer Scientific, Fair Lawn, NJ while all other reagents were purchased from Sigma-Aldrich, St. Louis, MO.

The vascular rings remained 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 $1x10^{-7}$ M, $1x10^{-6}$ M, $1x10^{-5}$ M, and $1x10^{-4}$ M sodium nitroprusside also waiting five minutes between doses. The vessels were equilibrated and tested for viability using KCl to verify vasoconstriction (an increase in tension on the force transducer) and SNP to verify vasodilation (a decrease in tension on the force transducer).

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 $1x10^{-8}$ M, $1x10^{-7}$ M, $1x10^{-6}$ M, and $1x10^{-5}$ M ATP (Enzo Life Science, Farmingdale, NY) or vehicle alone, 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 attain a preconstriction of the vessels. The pre-constricted vessels were then exposed to $1x10^{-8}$ M, $1x10^{-7}$ M, $1x10^{-6}$ M, and $1x10^{-5}$ 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.

The second type of data collected used a similar procedure to that described above, 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 and composition of: 140 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM HEPES (Sigma-Aldrich, St. Louis, MO), and 11.1 mM glucose. The vascular rings in the Krebs-Henseleit solution were placed in the hyperbaric oxygen chamber under one of the four gas/pressure treatments (100% O_2 at 26psi, 100% 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 pre-constriction 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.

Results

In order to assure each vascular ring is alive a potent vasoconstrictor followed by a potent vasodilator was administered. Potassium chloride (KCl) changes membrane potential of the cell to cause vascular smooth muscle contraction and was administered as the known, potent vasoconstrictor. The administration of KCl to the vascular rings, as seen in Figure 3, resulted in a pronounced increase in the mean change in force for all five treatments: 100% oxygen, room air/14psi, room air/26psi, 100% nitrogen, and no pretreatment (labelled ATP). This demonstrates strong vasoconstriction by the vascular rings. The two groups with the most robust vasoconstriction were the 100% oxygen (+17.31 g at 60 M KCl) and the room air at 26psi (+17.00 g at 60 M KCl, and the two least robust vasoconstricting groups were the 100% nitrogen (+12.96 g at 60 M KCl) and the room air at 14psi (+12.38 g at 60 M KCl). A one-way repeated analysis of variance (ANOVA) was performed and the results for each pretreatment were found to be statistically significant ($p<0.05$).

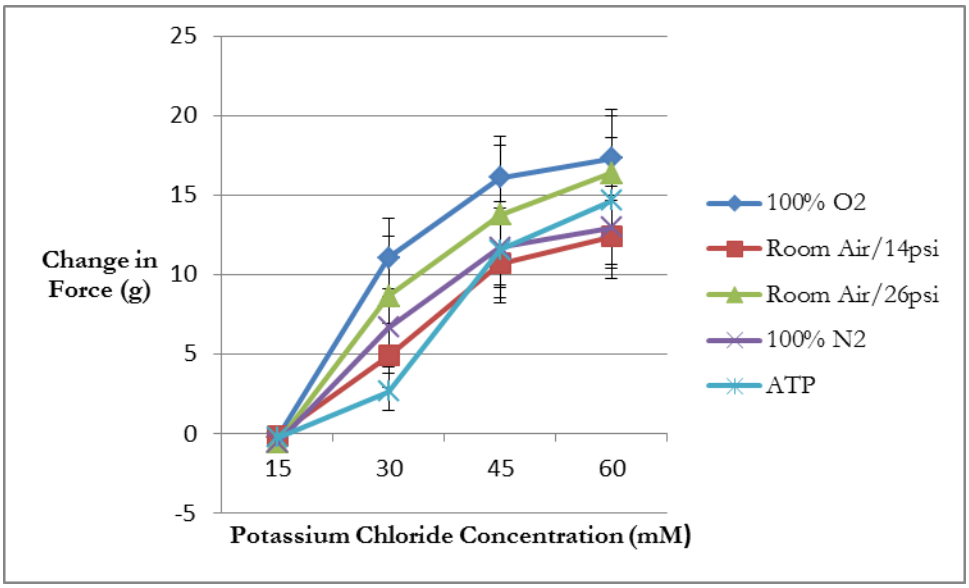

Figure 3. Effect of potassium chloride on vascular ring force after pretreatments of 100% oxygen, 100% nitrogen, room air at 14psi, and room air at 26psi, and no pretreatment (labelled ATP). Vascular rings of 100% oxygen, 100% nitrogen, and room air/26 psi were pretreated for two hours at 26psi (1.75atm). Vascular rings of room air/14psi were pretreated for two hours at 14psi (1.0atm). ATP vascular rings were not given any gas/pressure pretreatment. All treatment groups indicate KCl is a significant ($p<0.05$) vasoconstrictor. Values are means with vertical error bars representing the standard error of each mean.

Sodium nitroprusside (SNP) dissociates in solution into nitric oxide (NO) which bypasses the need for endothelial-derived NO produced by the activation of endothelial nitric oxide synthase within the endothelium. The NO diffuses to the vascular smooth muscle activating guanylyl cyclase to elicit a vasodilatory response in the artery. SNP was administered as the known vasodilator and, as seen in Figure 4, resulted in a pronounced decrease in the mean change in force of the vascular rings which indicates vasodilation is, indeed, occurring. The two most robust vasodilations occurred in the room air at 26psi (10.30g at 10^{-4} M SNP) and the 100% oxygen (9.23g at 10^{-4} M SNP) pretreatments, and the two least robust vasodilations occurred in the 100% nitrogen (6.31g at 10^{-4} M SNP) and room air at 14psi (6.11g at 10^{-4} M SNP) pretreatments. A one-way repeated analysis of

variance (ANOVA) was performed and the results for each pretreatment were found to show that SNP is a significant ($p<0.05$) vasodilator.

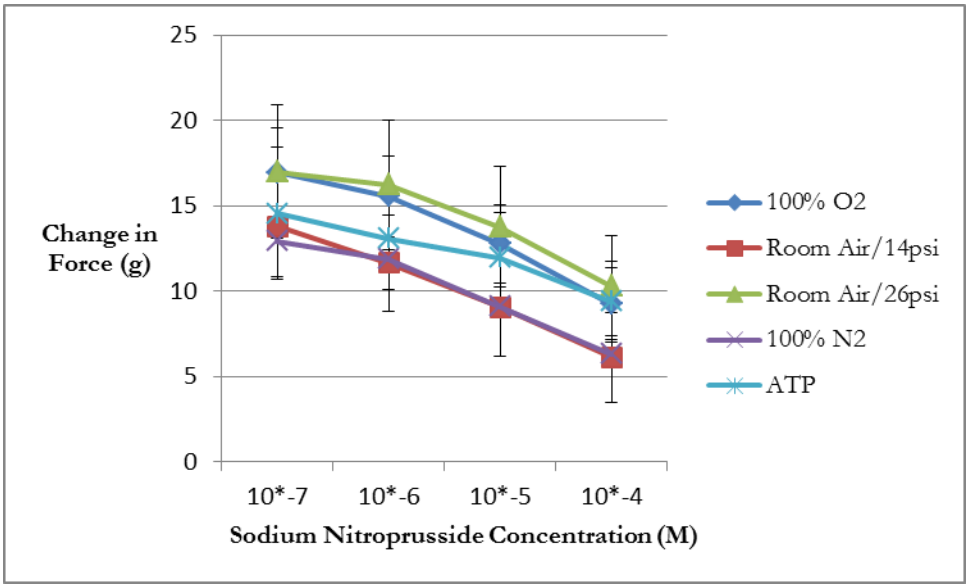

Figure 4. Effect of sodium nitroprusside on vascular ring force after pretreatments of 100% oxygen, 100% nitrogen, room air at 14psi, and room air at 26psi, and no pretreatment (labelled ATP). Vascular rings of 100% oxygen, 100% nitrogen, and room air/26 psi were pretreated for two hours at 26psi (1.75atm). Vascular rings of room air/14psi were pretreated for two hours at 14psi (1.0atm). ATP vascular rings were not given any gas/pressure pretreatment. All treatments indicate SNP is a significant ($p<0.05$) vasodilator. Values are means with vertical error bars representing the standard error of each mean.

Vascular rings receiving no pretreatments were exposed to vehicle or increasing concentrations of ATP. As shown in Figure 5, the mean change in force for the vascular rings exposed to either ATP or deionized (DI) water showed a slight decrease as the concentration of ATP or DI water increased, indicating vasodilation. However, a total mean decrease in force of approximately two grams after a 20 minute period is a very minor vasodilation. No significant difference ($p>0.05$) was found between the administration of increasing concentrations of ATP and DI water on vascular rings that had no pretreatment of any kind (hyperbaric oxygen, or preconstriction with potassium chloride).

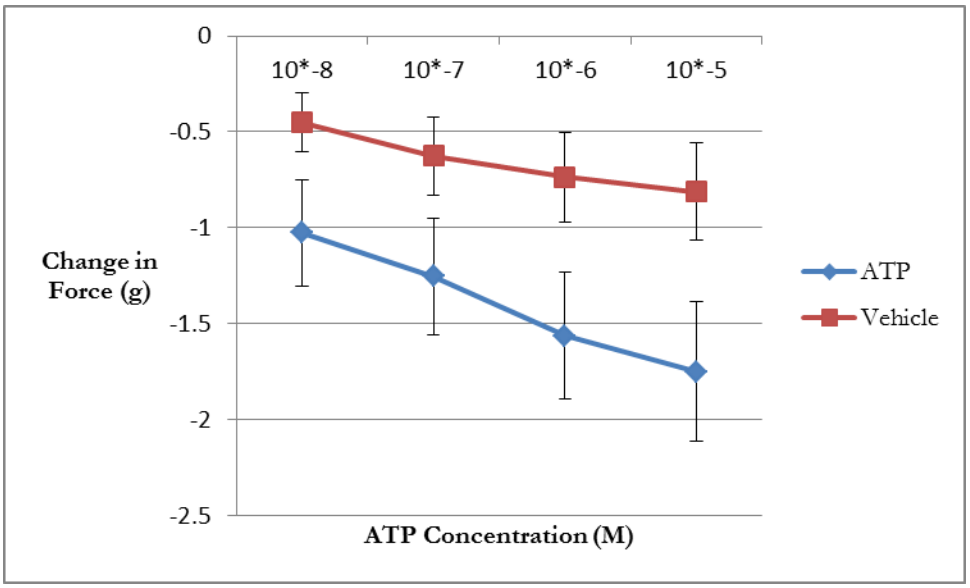

Figure 5. Vascular effect of ATP and vehicle on vascular rings with no hyperbaric oxygen therapy or preconstriction pretreatment with potassium chloride. Administration of ATP had no statistical significance ($p>0.05$). Values are means with vertical bars representing the standard error of each mean ($n=5-9$).

Vascular rings were pretreated with a single 30 mM dose of potassium chloride and allowed to vasoconstrict. After 15 minutes, the change in force on the vascular rings had plateaued and ATP or vehicle was administered in increasing concentrations. As shown in Figure 6, the mean change in force for both ATP and vehicle slightly increased as the concentration of each increased. An increasing change in force indicates vasoconstriction. Once again, the total mean change in force after the 20 minute period was only less than two grams which is a very minute change. The effect of ATP was not significantly different ($p>0.05$) than the effect of DI water in vascular rings that were preconstricted with KCl.

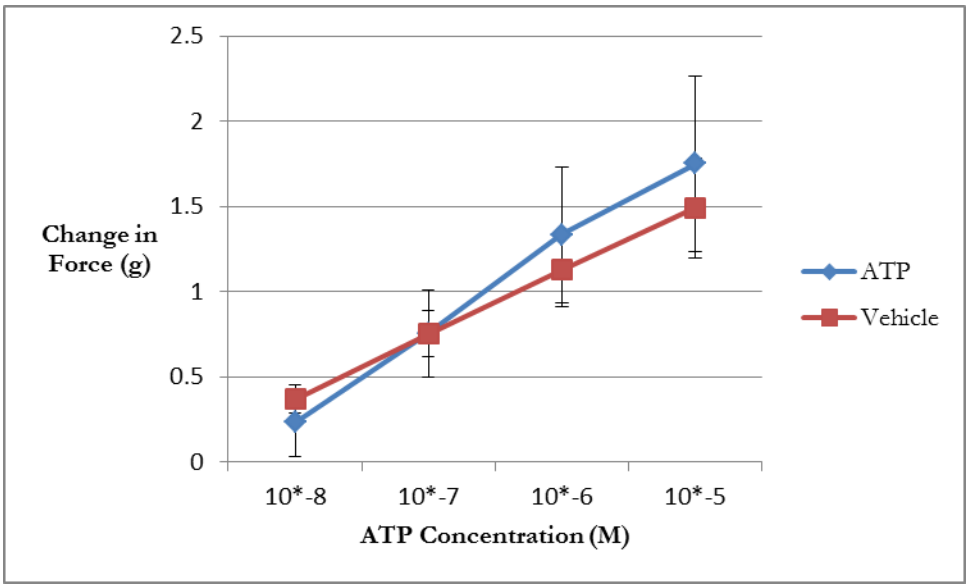

Figure 6. Vascular effect of ATP concentration and vehicle on vascular rings after a preconstriction with 30 mM potassium chloride. Vascular rings were equilibrated, administered a single dose of 30 mM potassium chloride and allowed to vasoconstrict for 15 minutes before administration of ATP or vehicle. Administration of ATP after vascular ring preconstruction was found to be not statistically significant ($p>0.05$). Values are means with the vertical bars representing the standard error of each mean ($n=5$-9).

Vascular rings were exposed to a two-hour gas/pressure pretreatment, with the 100% oxygen pretreatment representing the hyperbaric oxygen therapy, prior to the administration of the ATP or vehicle. As shown in Figure 7, all four pretreatments resulted in a slight 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 pretreatments is less than 1.5 grams. ANOVA analysis indicates that all four pretreatments are not significantly different ($p>0.05$) from one another when ATP is administered. A change in force of 1.5 grams is very small when compared to the mean force of potassium chloride, a known vasoconstrictor, obtained while determining vascular ring viability (Table 1) and would indicate that ATP is most likely not the cause of the vasodilation.

Gas/Pressure Pretreatment	Mean Change in Force after 10^{-5} M ATP (g)	Mean Change in Force after 60 mM Potassium Chloride (g)	n	Mean Change ATP/Mean Change Potassium Chloride (%)
100% Oxygen	-0.97	17.31	7	5.6
100% Nitrogen	-0.68	12.96	7	5.2
Room Air at 14psi	-0.84	12.38	8	6.7
Room Air at 26psi	-1.30	16.36	8	7.9

Table 1. Mean change in force of vascular rings after four doses of ATP being administered compared to the mean change in force after four doses of potassium chloride being administered. The mean change in force of the vascular rings after ATP administration was divided by the mean change in force of the vascular rings after potassium chloride administration and multiplied by 100 to find the percentage of potassium chloride vasoconstriction caused by ATP.

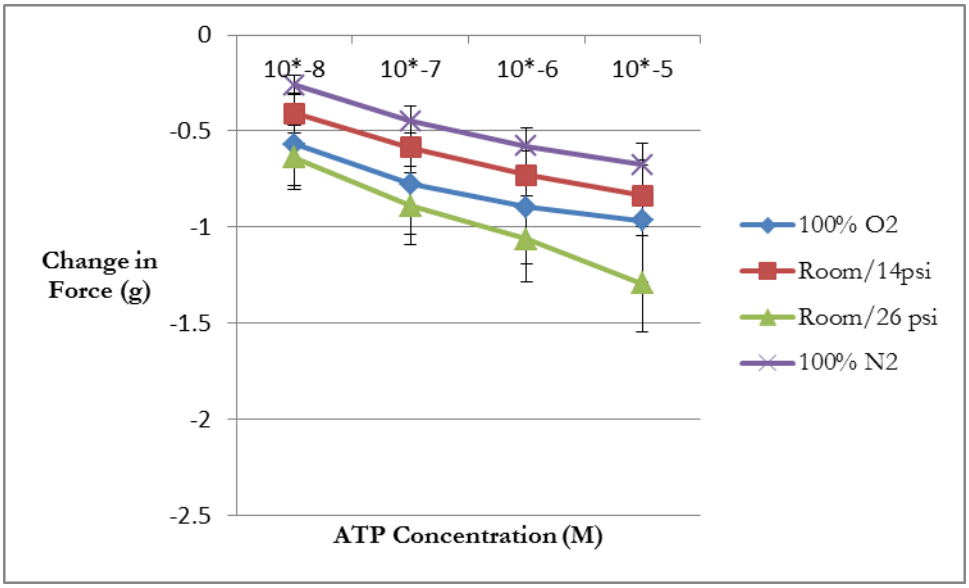

Figure 7. Vascular effect of ATP concentration and vehicle on vascular rings after one of four two-hour gas/pressure pretreatments. No statistical significance ($p>0.05$) was found among the treatments. Values are means with the vertical bars representing the standard error of each mean (n=7-8).

Discussion

This study has shown that hyperbaric oxygen (100% O_2 at 26psi) therapy causes the greatest increase in the response of vascular rings to the known vasoconstrictor, potassium chloride (Figure 3). The second most reactive treatment, room air at 26psi (1.75atm), only vasoconstricted 0.31g on average less than the hyperbaric oxygen after the addition of 60 mM potassium chloride. This indicates a possible link between at least some oxygen content (21 percent in atmosphere or 100 percent in HBO) in the air and it being under a pressure greater than atmospheric pressure (14psi), and an increase in the vascular reactivity in porcine mesenteric arteries. In contrast, there is a large gap in the mean change in force between the two most robust treatments and the two least robust treatments. The 100% N_2 treatment surprisingly reacted more robustly than the room air at 14psi after 60 mM potassium chloride. This decreased vascular reactivity could be due to the lack of oxygen present in the 100% nitrogen treatment, and the lack of pressure in the room air at 26psi. The response to the potassium chloride by the vascular rings with no pretreatment of any kind which were used before the addition of ATP (Figures 5 & 6), and labelled ATP (Figure 3) falls right between the hyperbaric oxygen and room air, and the hyperbaric nitrogen and room air at room pressure. This may be because the vascular rings involved are not benefitting from the perceived enhanced vascular reactivity seen when oxygen content is under pressure. This keeps them from performing as well as vascular rings exposed to hyperbaric oxygen. In addition they also skip the two hour gas/pressure pretreatment which, with no oxygen/pressure benefits, would allow the more rapid onset of ring death.

Hyperbaric oxygen therapy also yielded the most robust vascular reactivity in response to the known vasodilator, sodium nitroprusside (SNP). Unexpected results were

obtained after the addition of SNP to the 10^{-4} M concentration because the most robust vasodilation occurred after the room air at 26psi treatment (Figure 4). This could be due to the smaller population size coupled with a few outlying numbers, and would most likely correct itself with the addition of more trials.

The results presented here are supported by a similar study performed by Ben Hake (2013) using hyperbaric oxygen, hyperbaric nitrogen, room air at 26psi, and room air at 14psi. The addition of KCl during the Hake experiment resulted in the same order of vascular reactivity seen in the results from this study: the most vascular reactivity observed from hyperbaric oxygen followed by hyperbaric nitrogen and room air at 26psi, and the least from room air at 14psi. However, the results of KCl addition during the Hake experiment caused a much more robust response for every gas/pressure treatment (+31.35g of mean force compared to my +17.31g of mean force after the addition of 60 M KCl for the hyperbaric oxygen treatment). The addition of SNP during the Hake experiment mimicked the results from the current study only in that hyperbaric oxygen caused the most vascular reactivity. Following hyperbaric oxygen, the Hake experiment resulted in the next best response to be room air at 14psi, then room air at 26psi, and lastly hyperbaric nitrogen. Whereas in this experiment the hyperbaric nitrogen resulted in the second best response to SNP followed by room air at 14 psi and lastly room air at 26psi. Again, the SNP results from the Hake experiment resulted in more robust response for every gas/pressure pretreatment (-23.66g mean force compared to my -9.23g mean force after the addition of 10^{-4} M SNP for the hyperbaric oxygen). However there are two differences between the SNP studies that could contribute to the differences in these results. First, this study had an n that ranged from six to nine whereas the Hake experiment had an n=12. These few extra trials may have attenuated the outlier effect and aligned my results more closely to the results of the Hake

study. Secondly, the Hake study preconstricted with phenylephrine, not KCl, before vasodilating with the SNP. This could alter the results since phenylephrine is an alpha-1 adrenergic agonist, while KCl changes the membrane potential to elicit the vaconstrictive response.

Although there was an increased response to KCl and SNP by the vascular rings after hyperbaric oxygen treatment, there was no significant vascular reactivity due to ATP addition with, or without hyperbaric oxygen treatment. The slight vasodilation seen during the addition of ATP with no pretreatment, and the addition of ATP after the gas/pressure was most likely due to the slow dying of some of the vascular ring which kept the ring from holding its muscular tone for the whole 20 minute experiment. Whereas the vascular rings pretreated with 30 mM KCl had a slight vasoconstriction most likely due to the KCl still causing an extended effect.

In contrast to the findings of this study, the Droogmans, et. al. study found ATP to be a vasoconstrictor using tissue cultures of porcine thoracic aorta smooth muscle cells. After the addition of 50 micromolar ATP, they saw a significant release of internal calcium which activated a chloride channel that was able to depolarize the cell and activate (open) voltage-gated calcium channels. The voltage-gated calcium channels allow free calcium to enter the cell causing vasoconstriction. This study, however, has key differences that may allow for the difference in results. In spite of the animal studied being the same in both of the studies, the receptors, channels, etc. of the cells can be vastly different by changing the location from which the arteries are harvested. Also, the aorta is considered an elastic artery whereas the mesenteric arteries are classified as muscular arteries, and there are histological differences between these vessels. Therefore, the porcine aorta may contain cellular

mechanisms not present in porcine mesenteric arteries that allow for regulation by ATP. The use of cell culture instead of intact, excised vascular rings also accounts for a discrepancy in the results. Cell culture is known to indicate results that are not always the same as can be indicated through *in vitro*, or *in vivo* studies. In the same Droogman, et. al. study, they admit that the calcium release due to ATP has only been documented in cultured smooth muscle cells.

A potential problem with the design of this study is the question of ATP permeability through the cell membrane. There are many conflicting studies with some studies claiming ATP is non-membrane permeable (Dieterle, et. al. 1978). However, a study using *in vitro* rat soleus muscle was conducted using ^{14}C labelled ATP that indicated ATP had successfully crossed from outside the cell membrane to within the cell, and that the ATP had not simply been assembled within the cell from its constituent parts (Chaudry & Gould 1970). This study, and others, that indicate ATP is membrane permeable have largely been overlooked and many more current studies have employed the use of agents that render the cell membrane more permeable, called permeabilizers, such as digitonin (Hara, et. al., 2009). This step, while unnecessary, would have ensured a more definitive result concerning ATP permeability.

One reason for choosing ATP in this study was because of its importance as an energy carrier molecule. The energy from ATP is necessary to initiate and power muscular contraction. This necessity led us to hypothesize ATP would be critical and therefore include a possible regulating function. However, smooth muscle contraction has unique differences from skeletal muscle contraction that may account for the lack of changes in vascular reactivity resulting from ATP administration. Both skeletal and smooth muscle utilize the

interaction between a thick (myosin) and thin (actin) filament. In skeletal muscle the myosin is attached to actin and requires the hydrolysis of one ATP molecule to give off the energy necessary to move the myosin along the actin filament forming a cross-bridge. The myosin then changes configuration and causes a muscular contraction by sliding along the actin filament. Since each cross-bridge requires one ATP, this process uses vast amounts of ATP. In contrast, smooth muscle uses an enzyme, myosin light chain kinase, that is activated by a complex of calcium and calmodulin. Once activated, myosin light chain kinase hydrolyzes one ATP molecule to phosphorylate the myosin and initiate the muscular contraction. Smooth muscle contracts less than one tenth that of skeletal muscle and, therefore, has a much lower demand for ATP. Smooth muscle can further lower its ATP consumption by using the latch-bridge state which is unique to smooth muscle. The latch-bridge occurs when myosin light chain kinase phosphorylates myosin initiating the muscular contraction, but detachment of the myosin occurs at a much slower frequency. While in the latch-bridge state, no extra ATP is consumed. This allows the smooth muscle to stay contracted for long periods of time with much lower demand for ATP (Wingard, et. al. 1994). These differences between skeletal and smooth muscle may account for why ATP could be less of a factor in my study, and why there was no change in vascular reactivity from ATP administration.

Since ATP does not alter vascular reactivity in porcine gut arteries, the next step would be to research if the enhanced vascular reactivity is due to endothelial-dependent factors, or smooth muscle-dependent factors. The endothelium produces many molecules that alter vascular reactivity. Nitric oxide, endothelium derived hyperpolarizing factor, and proctacyclin are a few examples of vasodilators. Endothelin and thromboxane A_2 are a couple of examples of vasoconstrictors. In addition to the release of certain molecules to induce a change in vascular reactivity, the endothelium can also elicit an effect through

mechanical stimuli. The endothelial cells can change the stiffness, or softness of their cytoskeleton in the cortex which is just beneath the cell membrane. With a soft cortex, the release of NO by eNOS has been shown to increase (Fels, et. al. 2012). Also, there have been mechanosensitive calcium channels found that are activated by shear forces put on the cell when the cortex softens. These mechanosensitive calcium channels then increase the intracellular free calcium concentration (Galan, et. al. 2011). Smooth muscle also has many methods to regulate vascular reactivity within the artery. A main molecule that is used by smooth muscle is calcium. Increasing intracellular calcium levels, as norepinephrine does by binding to an α_1 or α_2-adrenergic receptor that activates (opens) a calcium channel in the sarcoplasmic reticulum, will cause a muscular contraction, i.e. vasoconstriction. In contrast, pumps in the cell membrane can actively transport calcium out of the cell reducing the free intracellular calcium concentration and causing the relaxation of the muscle, i.e. vasodilation. Also, norepinephrine binding to a β_2-adrenergic receptor will inhibit the phosphorylation of myosin light chain kinase, inhibiting muscular contraction, and causing vasodilation. As can be seen, there are many, complex interactions and assuredly some that are not yet fully elucidated that occur in both endothelium-derived and smooth muscle-derived factors to bring about the changes seen in vascular reactivity. Looking next to narrow the search to only endothelial-derived factors, or only smooth muscle-derived factors will allow for a much more concerted effort in finding the mechanism responsible for hyperbaric oxygen therapy's enhanced vascular reactivity.

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