2011

Gene Expression Changes in Response to Hyperbaric Oxygen Therapy in the Murine Model

Kousane Chheda
University of Connecticut - Storrs, kousanee.chheda@uconn.edu

Gene expression changes in response to hyperbaric oxygen therapy in the murine model

Kousanee Chheda

Department of Molecular and Cellular Biology

University of Connecticut

Abstract

A study was conducted to determine the effects of acute hyperbaric oxygen therapy (HBOT) at different pressures on gene expression in various organs in a murine model. It was hypothesized that the expression of stress-response genes would increase as the HBOT pressure increased. Twenty-four mice were either left untreated, or treated with HBO at 1.0 ata (100% O_2, normal pressure), 1.5 ata, 2.0 ata, 2.4 ata, or 3.0 in groups of four. Four hours after treatment, various tissues were harvested and utilized for gene expression analysis. The focus of these gene expression studies were genes involved in the cellular stress response, including HMOX-1, MT1, Nrf2, PECAM, and Hsp70. All genes responded differently in each organ with increasing, decreasing, or fluctuating trends as pressure increased. Of particular note was the robust activation of anti-oxidant genes in the large aorta. The results from this study provide a good foundation for further studies about how and why different HBOT pressures affect specific genes *in vivo*. This data may be the initial step towards expanding clinical applications of HBOT.

Introduction

Hyperbaric oxygen therapy (HBOT) is the administration of oxygen at a concentration and pressure higher than ambient air (Bent 2011). HBOT is currently used in the clinic to facilitate healing for chronic wounds such as diabetic ulcers (Londahl 2011). It has been shown to induce angiogenesis, and a state of cytoprotection in human microvascular endothelial cells through the transient formation of reactive oxidative species (ROS)(Godman 2010, Thom 2009). HBOT causes oxidative stress, and therefore the release of ROS, which activates a series of

signaling pathways that ultimately helps the cell become cytoprotected from more serious forms of stress (Thom 2009).

The four tissues studied in this experiment were the liver, spleen, kidney, and aorta. These organs were chosen because of their medical importance and distinct cellular compositions. The liver filters body wastes and hormones, as well as foreign toxins from the blood. It also synthesizes plasma proteins important for clotting, such as albumin (Sherwood 1997). Hepatocytes make up 80% of the organ, and serve multiple functions, including synthesizing proteins, bile salts, cholesterol, and detoxifying and excreting toxins that are presented in the blood (Zeisberg 2007). The spleen is a secondary lymphoid organ that produces antibodies, and destroys old and damaged erythrocytes. The bulk of the spleen consists of the "red pulp" that contains erythrocytes and macrophages. The "white pulp" contains B lymphocytes, which produce antibodies in response to antigen recognition, and T lymphocytes which attract other immune cells to the location of antigens (Sherwood 1997). Circulating dendritic cells present the antigen to lymphocytes. Since the function of the spleen revolves around the blood, there is a lot of trafficking in this organ. The kidney is another filtering organ that excretes wastes such as urea and ammonium. It also maintains water and electrolyte balance in the blood. Endothelial cells are primarily responsible for filtration and reabsorption (Rookmaaker 2004). Depending on the location of the cell in the kidney, it will contain proteins that aid in its function. For example, cells with sodium-potassium-2 chloride cotransporter (NKCC) recognize low water volumes in the blood (Hallows 2010). Another type of cell contains thiazide-sensitive sodium chloride cotransporter (TSC), which is responsible for making the cell reabsorb about 5% of the sodium in the blood (Eladari 2011). The final tissue selected for study was the aorta. It is the first vessel that takes oxygenated blood out of the heart, and is

made of endothelial and smooth muscle cells. It is the most pressurized artery in the body, and plays in important role in directing coronary blood flow.

The genes analyzed in this experiment include major antioxidant and cyto-protective genes. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor that plays an important role in activating the genes that protect the cell from oxidative stress (Chan 2001). Heme oxygenase-1 (HMOX-1) catabolizes heme into biliverdin in the reticuloendothelial system of the liver, spleen, and bone marrow (Durante 2011). It also has been shown to protect cells from oxidative stress, and is therefore a reliable stress response marker (Poss 1997). Metallothionein (MT1) is a major stress responder that binds heavy metals and protects the cell from metal-generated radicals, and has been shown to have high expression in the liver and kidneys (Ashino 2003). Finally, Heat shock protein 70 (Hsp70) aids in protein folding processes within the cell, including new protein folding and repair of misfolded or denatured protein (Mayer 2005). The oxidative stress signaling pathway involves three of these genes; HBOT activates Nrf2, which activates target genes such as HMOX-1 and MT1 (Fig.1). Oxidative stress caused by HBO triggers *Keap1* to oxidize and form disulfide bonds, which then leads to Nrf2 stabilization and activation. HBOT may also activate heat shock genes, including Hsp70. Treatments may trigger large scale protein oxidation causing proteins become denatured and misfolded, thereby contributing to Hsp70 signaling pathway activation (Fig. 2). This pathway allows Hsp70 to fix misfolded proteins and stimulates the production of more Hsp70. PECAM, found mainly in intercellular junctions of cells forming new blood vessels and is therefore considered to be an angiogenesis marker.

This study aimed to reveal the effects of a range of HBOT doses on various organs in the murine model. Prior experiments indicated that maximum gene expression changes occur four

hours after HBOT *in vitro* (Godman 2010), therefore in this study, the genetic changes were monitored four hours after a single dose of HBOT. It was hypothesized that HBOT would upregulate all the genes in the organs as the dosage increased.

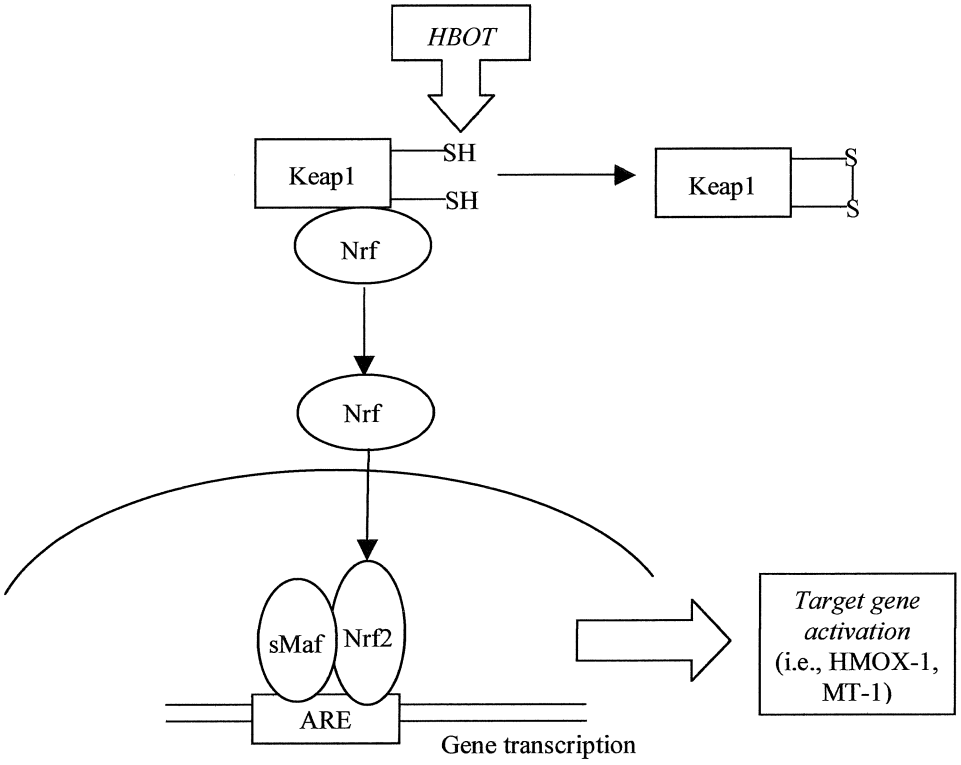

Fig 1. Nrf2 Signaling Pathway. HBOT oxidizes *Keap1*, resulting in a disulfide bond. Nrf2 travels into the nucleus, and attaches to antioxident response elements (ARE) and small Maf proteins (sMaf), which activates target gene transcription **(**Kensler 2007**).**

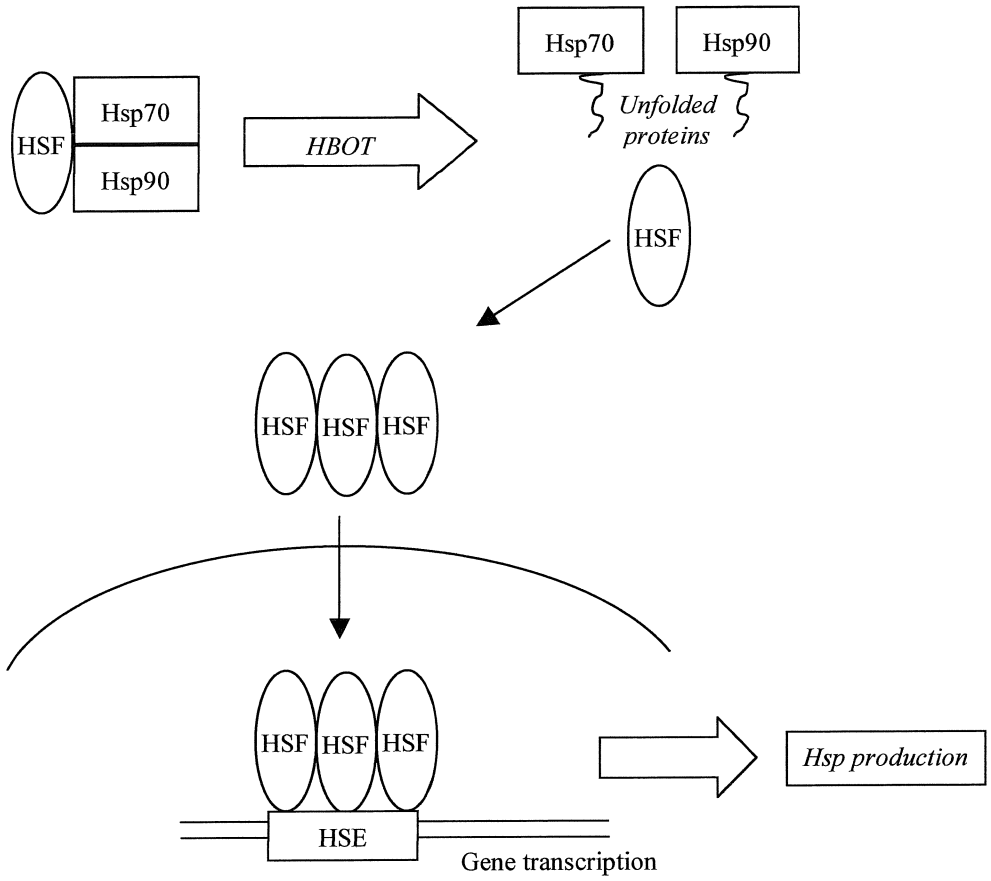

Fig 2. Hsp70 Signaling Pathway. Following HBO exposure, the heat shock factor (HSF) releases the heat shock proteins, which begin to refold unfolded or misfolded proteins. The HSFs collectively enter the nucleus and bind to the heat shock element (HSE) in the promotor region of the heat shock gene. This activates heat shock protein production (Erlichman 2009).

Materials and Methods

Animals

HBOT were performed on twenty-four female SKH1-Elite hairless mice (9-10 weeks, Charles River). Groups of four mice were caged in temperature, airflow, and light regulated rooms. They were provided with a standard rodent diet and water ad *libitum*. The animals were euthanized four hours after the completion of their HBO treatments by exposure to carbon dioxide at 2.5 L/min.

HBO Treatments

Treatments were conducted in OxyCure 3000 hyperbaric incubator (OxyHeal Health Group) with 100% O_2 at 1.0 atm, 1.5 atm, 2.0 atm, 2.4 atm, and 3.0 atm at 25°C. Groups of four mice were treated with HBO at each pressure dosage in their respective cages.

Tissue Harvest

After euthanization, samples of liver, spleen, kidney, and aorta were taken from each mouse by dissection. Organ samples were preserved in RNAlater solution (Ambien) to maintain RNA integrity.

RNA Extraction

All tissue samples were ground with a homogenizer in TRIzol reagent (Invitrogen), and RNA was extracted using the manufacturer's protocol.

Reverse transcription PCR and quantitative real-time PCR

Up to 4 μg of RNA was diluted in nuclease-free water, and combined with the necessary reagents as described by the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Foster City, CA). Quantitative real-time PCR was performed using an Applied Biosystems 7300 Real-Time PCR system and software. Reactions were made using TaqMan 2x Universal PCR Master Mix in 10 μL volumes with approximately 25 ng cDNA. The TaqMan gene expression assays (Applied Biosystems) used for these reactions include Mm02619580_g1 for beta-actin, Mm00516005_m1m1 for HMOX1, Mm01159846_s1 for HSPA1A, Hs00496660_g1 for MT1, and Mm01242584_m1 for PECAM.

Statistical Analysis

A one-way ANOVA was performed on all of the data, along with a Tukey comparison post-test between the control (1.0 ata, ambient O_2) and each of the other pressure doses. The confidence interval was 95%.

Results

Reverse transcription PCR was performed on all 24 individual samples. The cDNA from the samples were then pooled into six groups according to HBOT dosage. Quantitative real-time PCR was then performed for each tissue to look for changes in beta-actin, HMOX-1, Nrf2, MT1, PECAM, and Hsp70 levels. The results for all genes except for Hsp70 are based on averaging the data from two qPCR runs per tissue. The results for Hsp70 are based on one qPCR run per tissue.

In the liver, HMOX-1, Nrf2, and PECAM levels were highest when treated with HBOT at 1.0 ata with 100% O_2, and had 1.8, 2.0, and 2.1-fold increases relative to control. MT1 peaked at 2.4 ata with a 7.5-fold increase, while Hsp70 levels steadily declined as pressure increased (Fig. 3).

In the spleen, HMOX-1 and PECAM levels stayed approximately the same for all HBOT pressures. The trends for Nrf2 and MT1 were the same, and gene expression was maximized at 2.0 ata with 1.5 and 1.75-fold increases relative to control. Hsp70 showed a spike in expression at 3.0 ata with a 40-fold increase (Fig. 4).

In the kidney, HMOX-1 levels were slightly elevated at 1.5 ata with a 1.6-fold increase. Gene expression for MT1 and PECAM varied between 0.4 and 1.0 relative quantification. Nrf2 levels stayed the same except for a 0.6-fold decrease at 1.0 ata. Hsp70 peaked at 2.4 with a 5-fold increase (Fig.5).

In the aorta, all genes spiked at 2.0 ata, except for Hsp70, which spiked at 2.4 ata. HMOX-1, MT1, Nrf2, and PECAM showed 11, 6, 7, and 5-fold increases, respectively. Hsp70 showed a 2.75-fold increase.

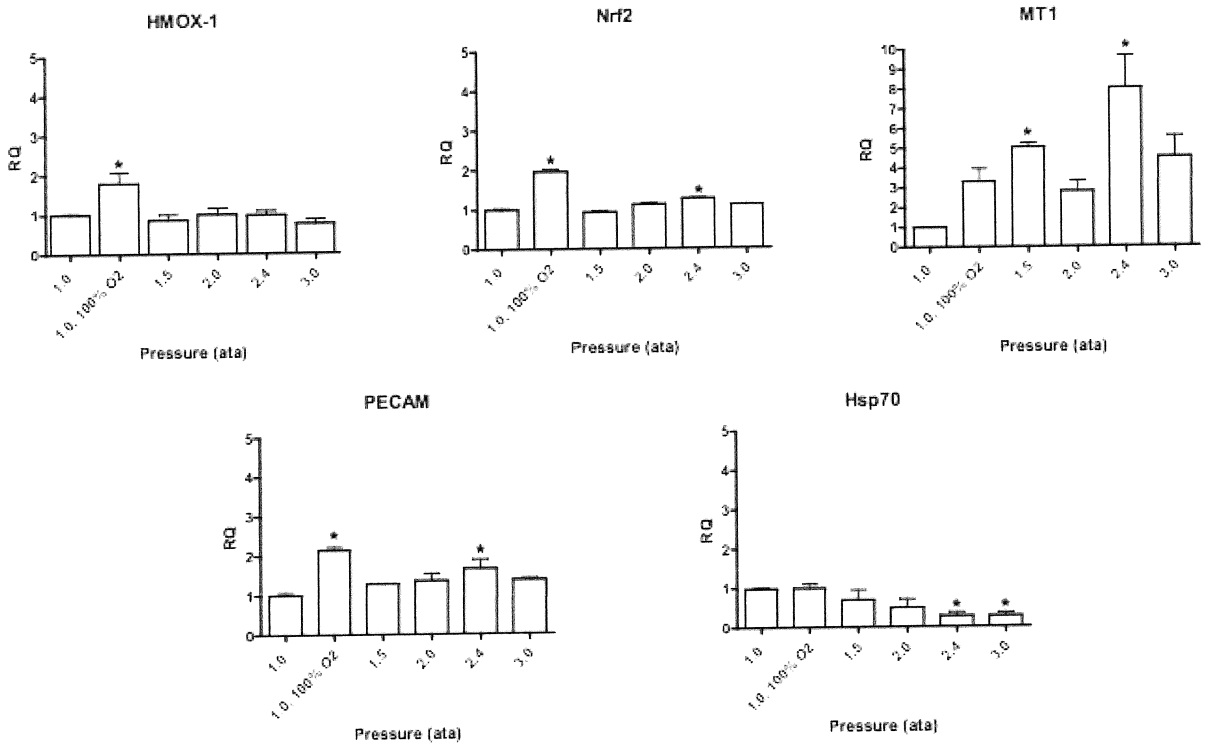

Fig 3. Gene Expression in the Liver

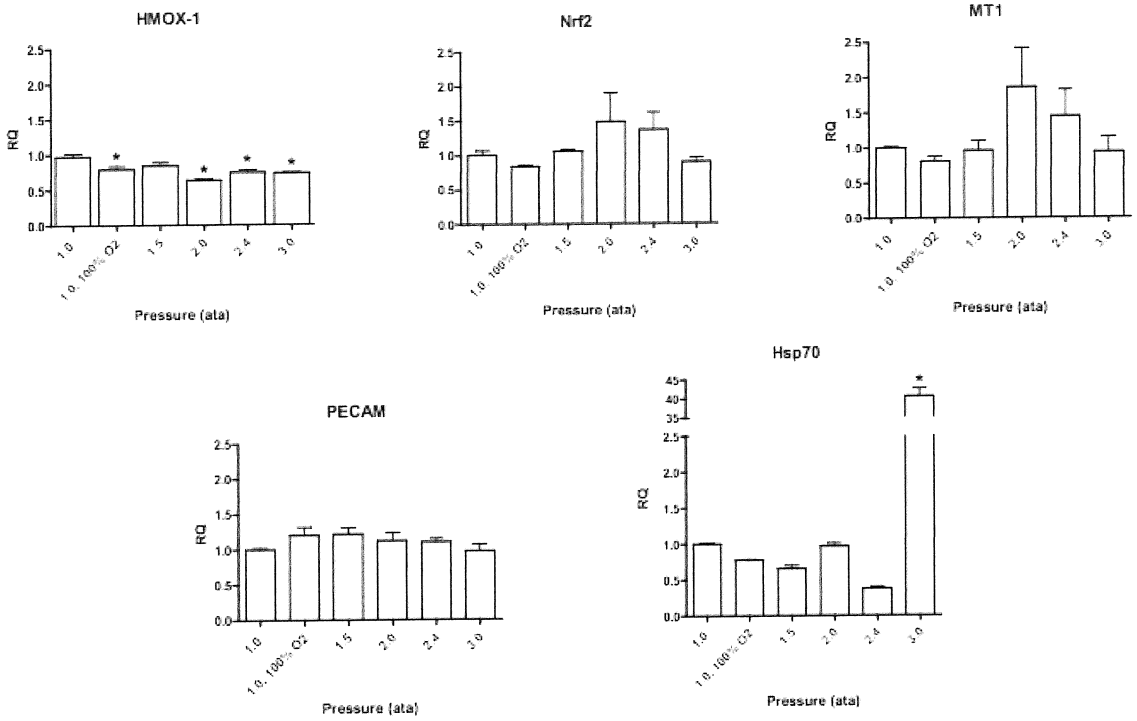

Fig 4. Gene Expression in the Spleen

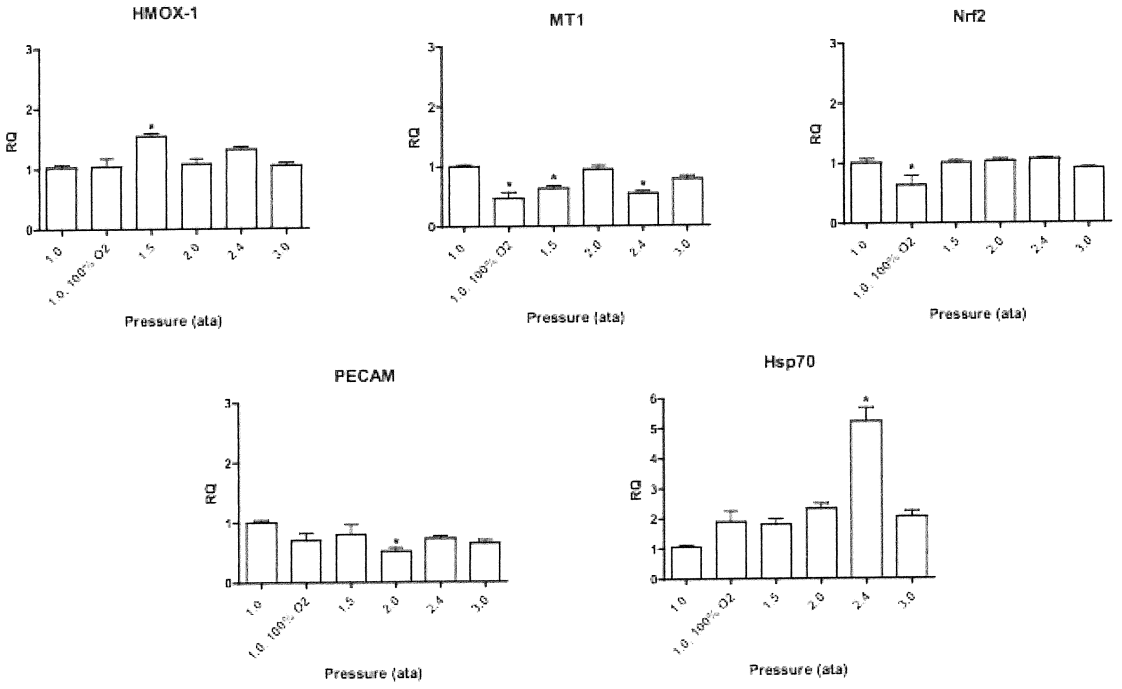

Fig 5. Gene Expression in the Kidney

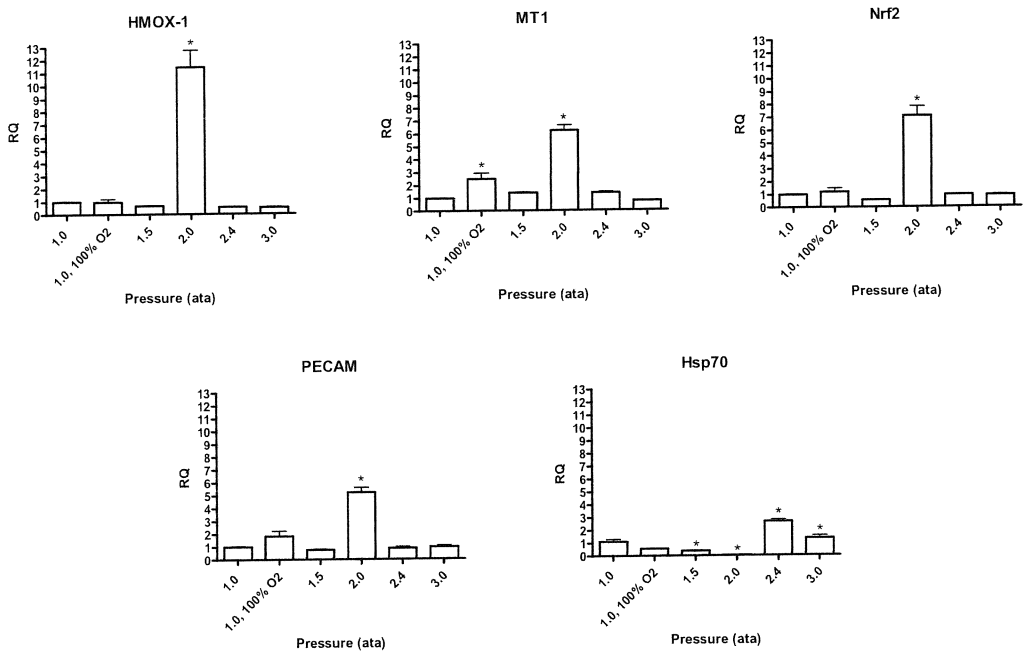

Fig 6. Gene Expression in the Aorta

Discussion

Organs are comprised of many types of cells, and gene expression depends on the function of the cells in the organ. Therefore, the results represent an average gene expression of all the cells in each organ. For example, the liver, which includes a wide variety of cells, may actually have a range of gene expression between cell types. Moreover, the cellular content of the spleen constantly changes based on the immune responses required by the body. Cells constantly enter and leave the organ through blood, which makes the variation between individual animals more prominent. At the same time, the aorta, which is primarily composed of endothelial cells, has less variation since most of the cells are the same. This data should be interpreted from a systemic perspective as well. For example, blood normally reaches the liver at the end of the systemic circulation cycle. Therefore, the blood is mostly deoxygenated. This could affect gene response in the liver as opposed to the other organs.

In the liver data (Fig. 3), HMOX-1, Nrf2, and PECAM levels are highest at 1.0 ata, 100% O_2. This is contradictory to previous *in vitro* experiments where elevated pressure was required to see genetic changes. This might suggest that other stressors or physiological interactions affect these genes as well. MT1 is activated at 2.4 ata, which could be attributed to the function of the liver. Hepatocytes constantly detoxify metals, and therefore normally contain high levels of MT1, which causes MT1 to be activated at a higher pressure. The decreasing trend of Hsp70 levels suggests that HBOT is less effective in activating Hsp70 signaling pathway at higher pressures. This data alone might suggest that HBOT is harmful to the liver, however histological slides from a study on chronic HBOT exposure to the liver (88 treatments) performed simultaneously with this study have shown no organ damage (data not shown).

The spleen data (Fig 4.) shows that HMOX-1 levels seem unaffected by HBOT. This might be because the heme released during phagocytosis of old erythrocytes in the spleen also affects HMOX-1 levels. Both MT1 and Nrf2 have the same activation trends, which reinforce the presence of the Nrf2 signaling pathway. Although the spike of Hsp70 levels at 3.0 ata is somewhat unclear, one possible explanation is cooperative protein folding, where misfolding of a few key proteins can trigger misfolding of many more proteins. This protein mis-folding would lead to more Hsp70 production. However, it is unclear whether this protein misfolding occurs in cells of the red or white pulp of the spleen.

The data from the kidney (Fig. 5) shows relatively flat gene expression changes in every gene except for Hsp70. HBOT does not seem to have activated Nrf2. Since Nrf2 activates other genes like HMOX-1 and MT1, the steadiness of Nrf2 levels explains the lack of expression change in those genes. The dramatic increase in Hsp70 levels at 2.4 ata might be explained yet

again by cooperative protein folding, but the reason for the decreased expression at 3.0 remains unclear.

The aorta data (Fig. 6) shows a general increase in gene expression at one of the pressures tested (2.0 ata). Since the aorta is exposed to the most oxygenated blood immediately coming out of the heart, it may be particularly sensitive to the increase in oxygen tension. In addition, the endothelial cells of the aorta may be more prone to respond to oxygen than other cell types.

These results imply that HBOT might be used to aid or facilitate medical treatments. For example, in the case of invasive surgeries, the organs may undergo varying levels of oxidative stress. The data from this study can be used to perhaps prepare patients undergoing surgery for stress to minimize organ damage. In another scenario, a patient with heavy metal exposure might be treated with HBO at 2.4 ata to achieve the maximum MT1 expression after 4 hours, which may help bind metals and prevent toxicity to the liver.

Although my hypothesis stating that gene expression will increase with higher HBOT pressures was not supported, this data provides a good foundation for continuing research on gene expression changes in response to varying pressures of HBOT. Hopefully in the future, this data will contribute to more efficient and widely applicable HBOT protocols in the medical field.

Acknowledgements

I would like to thank Mr. Robert and Carlotta Holster for funding this project, and UConn for providing the facility to conduct this research. Furthermore, I would like to thank Dr. Charles Giardina, Dr. Cassandra Godman, Dr. Lawrence Hightower, Dr. George Perdrizet, and Dr. Jill Deans for their support and guidance throughout this study.

References

Ashino, T., S. Ozawa, S. Numazawa, and T. Yoshida. 2003. "Tissue-Dependent Induction of Heme Oxygenase-1 and Metallothionein-1/2 by Methyl Methanesulfonate." *The Journal of Toxicological Sciences* 28 (3): 181-189.

Bent, S., K. Bertoglio, P. Ashwood, E. Nemeth, and R. L. Hendren. 2011. "Brief Report: Hyperbaric Oxygen Therapy (HBOT) in Children with Autism Spectrum Disorder: A Clinical Trial." *Journal of Autism and Developmental Disorders.*

Chan, K., X. D. Han, and Y. W. Kan. 2001. "An Important Function of Nrf2 in Combating Oxidative Stress: Detoxification of Acetaminophen." *Proceedings of the National Academy of Sciences of the United States of America* 98 (8): 4611-4616.

Durante, W. 2011. "Protective Role of Heme Oxygenase-1 Against Inflammation in Atherosclerosis." *Frontiers in Bioscience : A Journal and Virtual Library* 17: 2372-2388.

Eladari, D. and C. A. Hubner. 2011. "Novel Mechanisms for NaCl Reabsorption in the Collecting Duct." *Current Opinion in Nephrology and Hypertension* 20 (5): 506-511.

Erlichman, C. 2009. "Tanespimycin: The Opportunities and Challenges of Targeting Heat Shock Protein 90." *Expert Opinion on Investigational Drugs* 18 (6): 861-868.

Godman, C. A., K. P. Chheda, L. E. Hightower, G. Perdrizet, D. G. Shin, and C. Giardina. 2010. "Hyperbaric Oxygen Induces a Cytoprotective and Angiogenic Response in Human Microvascular Endothelial Cells." *Cell Stress & Chaperones* 15 (4): 431-442.

Hallows, K. R., P. F. Mount, N. M. Pastor-Soler, and D. A. Power. 2010. "Role of the Energy Sensor AMP-Activated Protein Kinase in Renal Physiology and Disease." *American Journal of Physiology.Renal Physiology.*

Kensler, T. W., N. Wakabayashi, and S. Biswal. 2007. "Cell Survival Responses to Environmental Stresses Via the Keap1-Nrf2-ARE Pathway." *Annual Review of Pharmacology and Toxicology* 47: 89-116.

Londahl, M., K. Fagher, and P. Katzman. 2011. "What is the Role of Hyperbaric Oxygen in the Management of Diabetic Foot Disease?" *Current Diabetes Reports* 11 (4): 285-293.

Mayer, M. P. and B. Bukau. 2005. "Hsp70 Chaperones: Cellular Functions and Molecular Mechanism." *Cellular and Molecular Life Sciences : CMLS* 62 (6): 670-684.

Poss, K. D. and S. Tonegawa. 1997. "Reduced Stress Defense in Heme Oxygenase 1-Deficient Cells." *Proceedings of the National Academy of Sciences of the United States of America* 94 (20): 10925-10930.

Rookmaaker, M. B., M. C. Verhaar, A. J. van Zonneveld, and T. J. Rabelink. 2004. "Progenitor Cells in the Kidney: Biology and Therapeutic Perspectives." *Kidney International* 66 (2): 518-522.

Sherwood, Lauralee. 1997. *Human Physiology: From Cells to Systems*. 3rd ed. Boston: Wadsworth.

Thom, S. R. 2009. "Oxidative Stress is Fundamental to Hyperbaric Oxygen Therapy." *Journal of Applied Physiology (Bethesda, Md.: 1985)* 106 (3): 988-995.

Zeisberg, M., C. Yang, M. Martino, M. B. Duncan, F. Rieder, H. Tanjore, and R. Kalluri. 2007. "Fibroblasts Derive from Hepatocytes in Liver Fibrosis Via Epithelial to Mesenchymal Transition." *The Journal of Biological Chemistry* 282 (32): 23337-23347.

Holster Project Weekly Reflections

Kousanee Chheda

6/27 – 7/1

This week I treated and harvested the tissues of three groups of mice. On Tuesday, Wednesday, and Friday mornings, I put the mice in the hyperbaric chamber for an hour at whatever pressure they were assigned. Four hours after the completion of the treatment, I euthanized the mice with CO_2 and took two samples of their skin, liver, spleen, and kidneys, and one sample of their aorta. I put these samples in RNAlater to maintain RNA integrity. I then put the samples in the refrigerator.

Since I am afraid of mice, it was a challenge trying to muster the courage to touch the animals while transferring them between cages. It was also really hard to the watch the mice be euthanized. I did enjoy the dissections however, and it is amazing how small all the organs are in a mouse.

7/4 – 7/8

I continued to treat and harvest the mice this week on Tuesday, Wednesday, and Thursday. I obtained tissue samples from the 2.0 atm, 2.4 atm, and 3.0 atm groups. Handling the mice was easier for me this week, but the euthanizing step obviously was still the most difficult. I spent some time on Friday organizing the data collection process and creating a more concrete timeline.

7/11 – 7/15

This week I started collecting data. I began with the skin and extracted RNA from Monday through Wednesday. The extractions went smoothly and I am beginning to be able to repeat the protocol from memory. I then made cDNA and performed qPCR. My probes had not arrived by the time I wanted to conduct qPCR, so I used I just used ActB and HMOX-1. The results from the qPCR did not make sense. There was no data for the 0.0 atm and 1.0 atm samples, which made it difficult to conduct any analysis, since there was no baseline for comparison. Furthermore, there was a lot of variation in ActB between the samples, which is unusual.

After talking to Cassandra and Dr. Giardina, I decided to double the amount of cDNA I used for the qPCR, which I will repeat on Monday.

7/18 – 7/22

On Monday, I repeated the qPCR with double the amount of cDNA. The results however were totally whacky, and the graphs were parabolic instead of logarithmic. I decided to move on to other organs and come back to the skin. I also decided to run an RNA gel to judge the quality of the extracted RNA. The results from this showed that the problem is most likely occurring in the qPCR step of the process. On Tuesday, I extracted RNA from all of the liver tissue. While making cDNA on Wednsday, I ran into a little trouble dissolving the pellets because they were so large. However, I was able to make cDNA, and I performed a qPCR on Friday. I finally got data, and found that Nrf2 levels peak at 1.5 atm, while MT1 and PECAM levels peak at 2.0 atm. This is interesting because in the long run, it seems that HBOT dosages in a medical setting should be determined by which genes need to be activated to see improvements in a specific

condition. I plan on repeating the qRT-PCR next Friday to double-check the liver HMOX-1 levels.

7/25 – 7/29

This week, I did the same qRT-PCR procedure for the spleen. While grinding the tissues, I noticed that the spleen did not seem to dissolve away like the liver. Instead it broke into tiny, spongy, pieces, which made them more difficult to grind. The rest of the procedure went smoothly, as did the qRT-PCR on Wednesday. I also repeated the Liver qRT-PCR with HMOX-1, MT1, and Hsp70. The HMOX-1 data made more sense this time, and since the MT1 data was the same, I think this round was more accurate. I am beginning to research the different organs and the cells types that they are made of, so that I can better understand these results.

8/1-8/5

I began the week by extracting RNA from the aorta samples. I then made cDNA and performed a qRT-PCR. The results for HMOX-1, Nrf2, MT1, and PECAM were pretty exciting because there was a clear spike in gene expression at certain pressures. I've gotten really good at the entire qRT-PCR process since I've been doing it every week. I plan on repeating the qRT-PCRs on all of the organs one more time for the same genes. In the end, I will have two sets of data for all genes except for Hsp70, which I don't think I'll have time to repeat a second time this summer. I also won't have time to go back to the skin, so I'm going to eliminate any skin references from my paper and go back to it after this summer.

I started doing some background reading for the introduction to my paper, and hopefully I can begin writing this weekend.

8/8-8/12

I re-ran all of the qRT-PCRs this week, and graphed the data. I was a bit concerned because although a lot of the trends were the same, the relative quantifications were not. I talked to Cassandra about this, and she suggested I might average all of the results. I did this with my data and it worked out nicely. Some of the data is really interesting and surprising. It disproves my hypothesis, but based on the final graphs, I thought of many different follow-up questions to research. It was nice to see all of my work in these past two months produce actual data that I can do something with.

I've done a little bit of writing so far, but nothing concrete yet. I've finished reading for the most part, so now my focus will be to finish the paper

8/15-8/19

I spent all week writing. I didn't realize how much time and effort is required to write a scientific paper. The format of my paper mimics that of a journal paper so that I can practice how to write them. I found the discussion the hardest to put together because I had to find possible explanations for my data, which took extra reading and thinking. Overall though, I think the paper is coming out nicely. I met with Dr. Hightower and Cassandra and they gave me good suggestions and tips. One suggestion they gave was to make all the graphs the same y-axis scale, so that it would be easy to compare the data. I played around with this and equalized as much as I could, with the exception of the few graphs that had much higher fold increases. I will wrap my paper up this weekend, and hand it in on Monday!