

Author



Nadeem Goraya has always been interested in the intricacy and consistency of the vascular system, and considers himself blessed to be able to explore this interest under the guidance of Dr. Purdy. He has enjoyed the chance to pursue this animal-model project from the beginning through performing the analysis and probing for important proteins. He is thrilled to be a part of this project, with its potential significance in better understanding vascular function. Nadeem feels that his research experience has been a vital part of preparing for his ultimate goal of becoming a physician, and that the skills he has learned in the lab will help him remain well informed and productive throughout his career.

Key Terms

- ◆ Alpha (α)-actin
- ◆ Alpha (α)-tropomyosin
- ◆ Calponin
- ◆ Heavy (h)-caldesmon
- ◆ Hindlimb unweighting (HU)
- ◆ Myosin
- ◆ Simulated Microgravity

The Role of Contractile Proteins and Cellular Phenotype in Rat Abdominal Aorta Exposed to Simulated Microgravity

Nadeem A. Goraya

Biological Sciences

Abstract

This study evaluated crucial contractile proteins and the role of vascular smooth muscle cell phenotype in the abdominal aorta of male Wistar rats exposed to simulated microgravity. Myofilament proteins myosin heavy chain (MHC), myosin light chain-20 (MLC20), and α -actin were measured along with myofilament binding proteins calponin, h-caldesmon, and α -tropomyosin, which are markers for phenotype change. Microgravity was simulated through use of the hind limb unweighting (HU) model for 20 days. Male Wistar rats, designated either HU or control (CTL) at random, were euthanized and the abdominal aortas were removed and stored for Western blot analysis. The aforementioned proteins were probed, and relative protein levels were quantified and compared between HU and CTL rats. Results indicate an HU-induced downregulation in all three contractile proteins, MHC, MLC20, and α -actin. However, HU-induced upregulation of the myofilament binding, contraction inhibitory protein h-caldesmon was observed. No significant change was observed in either calponin or α -tropomyosin. These results suggest that downregulation of contractile proteins and upregulation of the contraction inhibitory protein h-caldesmon could be possible mechanisms for HU-induced vasoconstrictor hyporesponsiveness.

Faculty Mentor



The UROP-sponsored research project carried out by Nadeem Goraya was an extraordinary, positive experience for both of us. I was able to provide a supportive environment in which Nadeem could learn techniques, discuss problems and ideas, delve into the literature, and undertake an exciting research project. On Nadeem's side, his energy, initiative, imagination, and persistence were critical to his success. Nadeem's study was the first to identify cellular mechanisms for the impairment of vasoconstriction and the resulting orthostatic intolerance experienced by astronauts and long-term bed rest patients. He found that microgravity can downregulate the expression of the very proteins involved in vasoconstriction, myosin and actin. Based on his work, we are now investigating mechanism-based therapeutic interventions.

Ralph E. Purdy

School of Medicine

Introduction

When astronauts enter space, their cardiovascular systems adapt to zero gravity conditions. This adaptation, called cardiovascular deconditioning, may cause astronauts returning to the gravity of Earth to experience fainting symptoms or actual fainting (syncope) (Hargens and Watenpaugh, 1996). Cardiovascular deconditioning may be caused by the hemodynamic effects of exposure to microgravity. For instance, in a person standing upright under normal (sea level) Earth gravity, the mean blood pressure at the head is 70 mmHg, 100 mmHg at the heart, and 200 mmHg at the feet. Under microgravity conditions, this gradient is eliminated; the blood pressure becomes 100 mmHg throughout the body, and there is a cephalad fluid shift (Hargens and Watenpaugh, 1996). This causes the cardiovascular system to adapt so that the return to gravity causes resting tachycardia, decreased stroke volume, reduced exercise capacity, and, most important to our research, orthostatic intolerance. This disorder—characterized by headache, dizziness, and actual fainting on standing upright—commonly affects long-term bedridden patients. Although it is not well understood, orthostatic intolerance is thought to be caused by an inability of one's blood pressure to acclimate appropriately to orthostatic challenge, leading to insufficient blood perfusion to the brain. Although there are several specific underlying mechanisms involved, one very important factor in orthostatic intolerance is impaired vascular contractility.

In an experiment performed by Buckley *et al.* (1994), 14 astronauts who had returned to earth were subjected to 10-min stand tests. Only five astronauts were able to complete it successfully. After measuring numerous cardiovascular parameters, researchers determined that the only difference between those who completed the test and those who failed was their bodies' ability to raise total peripheral resistance (TPR), which is the cumulative resistance generated by the network of arterioles in the body. Since resistance (and TPR) is dependent on the ability of these peripheral blood vessels to constrict, the study suggests that post-flight orthostatic intolerance may be due to microgravity-induced vascular hyporesponsiveness.

A rodent hindlimb unweighted (HU) model was used to simulate a microgravity environment. In this model, the rat's hindlimbs were elevated above the cage floor by the use of a tail harness tethered to the ceiling of the cage. This orientation produced a chronic cephalad fluid shift similar to that experienced by astronauts in space.

It has been shown that HU treatment causes a reduction of contractility in all arteries outside the blood-brain barrier of male Wistar rats (Delp *et al.*, 1993; Purdy *et al.*, 1998; and Wilkerson *et al.*, 2002). HU-induced depression of contractile force, specifically in the abdominal aorta, does not seem to be caused by changes in mechanical properties of the vessel as occurs during vascular remodeling (Papadopoulos and Delp, 2003). Moreover, loss of contractile force in the HU abdominal aorta has not been attributed to endothelial dependent vasodilators. Both Delp *et al.* (1993) and Sangha *et al.* (2000) have demonstrated that abdominal aorta contractility induced by a known vasoconstrictor, norepinephrine, followed the same trend in both endothelium denuded and endothelium intact abdominal aorta, of both control (CTL) and HU rats. Thus, lower vasoconstriction response in the abdominal aorta of HU rats cannot be attributed to endothelium dependent vasodilator mechanisms. This is in contrast to the response of many other blood vessels, including the thoracic aorta (Summers *et al.*, 2005).

Although data suggest that mechanical change and vasodilation do not contribute to the vascular hyporesponsiveness in the HU abdominal aorta, alternate factors could account for this change. For instance, changes in α -adrenergic receptors, Ca^{2+} availability and sensitivity of myofilaments to Ca^{2+} , and, most important to this study, the expression of myofilaments, are all possible mechanisms that can be affected by simulated microgravity. The fundamental hypothesis of this study is that there will be a decrease in the contractile proteins—myosin heavy chain (MHC), myosin light chain twenty (MLC20) and α -actin—providing a mechanism for vasoconstrictor hyporesponsiveness under simulated microgravity. Moreover, it is known that these filaments decrease under conditions in which the vascular smooth muscle (VSM) cellular phenotype changes from mature contractile to immature noncontractile. This leads to the second hypothesis: that simulated microgravity causes VSM cellular phenotype change. To address these hypotheses, the aforementioned mature contractile proteins were measured. Then data were collected on other marker proteins for phenotypic change, such as the myofilament binding proteins h-caldesmon, calponin and α -tropomyosin, which are predicted to decrease during phenotypic change from mature contractile to immature noncontractile phenotype.

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of UCI, under protocol #1997-1588.

Animal Model

The HU model was used on the male Wistar rats to mimic cardiovascular-deconditioning effects. The rats, weighing 250–300 g (Simonsen Laboratories, Gilroy, CA), were caged individually at 23 °C in a 12:12-hr light-dark cycle, and were given water and easily accessible chow. Rats were chosen at random and were assigned either to HU or CTL. The HU rats were fixed with tail harnesses that raised the hindlimbs 1 cm off the cage floor, producing a 35° head-down angle in the body of the rat. To apply the tail harness, the tail was sprayed with benzoin tincture (Smith and Nephew, Memphis, TN) and air dried, then an adhesive strip of Moleskin Plus (Dr. Scholl's, St. Louis, MO) was placed around the tail and secured with three ~1-cm wide adhesive strips (Beiersdorf-Jobst, Rutherford College, NC). A plastic ring was placed at the center of the moleskin and was tethered to a swivel and loop, which moved freely along the length of a horizontal aluminum tube fixed to the top of the cage. They retained this position for 20 days.

Tissue Harvest

After the 20-day HU treatment, rats were exposed to 100% CO₂ (Glen, JB, and Scott WN., 1973) to induce deep anesthesia, and were then euthanized by opening the chest cavity and removing the heart. Abdominal aortas were removed and cleaned under a dissecting microscope to remove fat and connective tissues. The aortas were then weighed and frozen in 2-methylbutane in dry ice, and kept at -20 °C until they were used in further experimentation.

SDS-PAGE/Western Blot Analysis

Abdominal aortas were homogenized by hand grinding, using glass homogenizers with lysis buffer containing 50 mM β-glycerophosphate, 0.1 mM NaVO₃, 2 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, 1 mM DL-dithiothreitol, 0.02 mM pepstatin, 0.02 mM leupeptin, 0.1 U/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 500g for 20 min at 4 °C. The supernatant was centrifuged again at 4000g for 20 min at 4 °C and collected for Western blot analysis. Protein concentration was measured using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Tris-glycine SDS sample buffer (containing β-mercaptoethanol) was mixed with lysates and boiled for 4 min to denature the protein. Sample protein from both HU and CTL lysates (10–20 μg/lane) were subsequently loaded

equally onto four 20% Tris-glycine gels and separated by size through SDS-PAGE at 125 V for 2–3 hr in a mini-gel apparatus (Invitrogen, Grand Island, NY). Proteins were then transferred to nitrocellulose membrane (Amersham, Piscataway, NJ) at 500 mA for 90 min, blocked with 6.5% nonfat dry milk in Tween-20-Phosphate Buffered Saline (T-PBS; 0.1% Tween-20) and incubated overnight at 4 °C. The membranes were then drained and incubated with a primary antibody (specific for our protein of interest) for 30 min at 37 °C. T-PBS was then used to wash the membrane for 30 min, changing the T-PBS every 5 min. Membranes were incubated in secondary antibody for 1 hr in the dark at 20–25 °C and then washed in T-PBS for 30 min as above. The membranes were analyzed using the LI-COR Odyssey system (LI-COR, Lincoln, NB). Band density was quantified using a densitometry analysis program, UN-SCAN-IT (Silk Scientific, Orem, UT). Levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which had previously been shown by our lab to be unaffected by HU, were used to confirm equal loading of the lanes. The dilutions used for primary antibodies were: rabbit polyclonal anti-myosin light chain 20 (MLC20), 1:500; mouse anti-myosin heavy chain (MHC), 1:500; rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 1:250 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal anti-α-actin, 1:5,000; mouse monoclonal anti-α-tropomyosin, 1:500; mouse monoclonal anti-heavy caldesmon (hCAD), 1:500; and mouse monoclonal anti-calponin (CAP), 1:500 (Sigma Chemical Company, St. Louis, MO). The dilutions of secondary antibodies used were: IRDye 800 conjugated anti-mouse IgG 1:20,000 (Rockland, Gilbertsville, PA) and Alexa Fluor® 680 goat anti-rabbit IgG 1:20,000 (Molecular Probes, Eugene, OR).

Statistical Analysis

The values are presented as mean ± SE, and unpaired *t*-tests were made between groups. *p* < 0.05 was required for significance.

Results

The results of this study demonstrated an HU-induced decrease in expression of critical myofilament proteins MHC, MLC20 and α-actin. Figures 1 through 3 show the relative abundance of each protein on Western blots of the abdominal aorta with and without HU treatment. The Western blot of GAPDH is also shown to verify equal loading of total protein in each well.

To test this hypothesis three-myofilament binding proteins were measured by Western blot analysis. Contrary to our

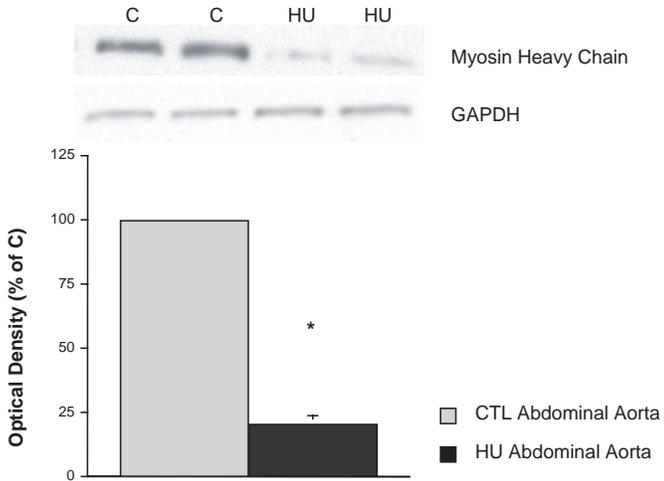


Figure 1
Sample Western blots of Myosin heavy chain and respective loading control GAPDH and mean data of Myosin heavy chain from CTL and HU rat abdominal aortas (n=4). HU=20.7 ± 3.42% (SEM) of control. * p<0.0001

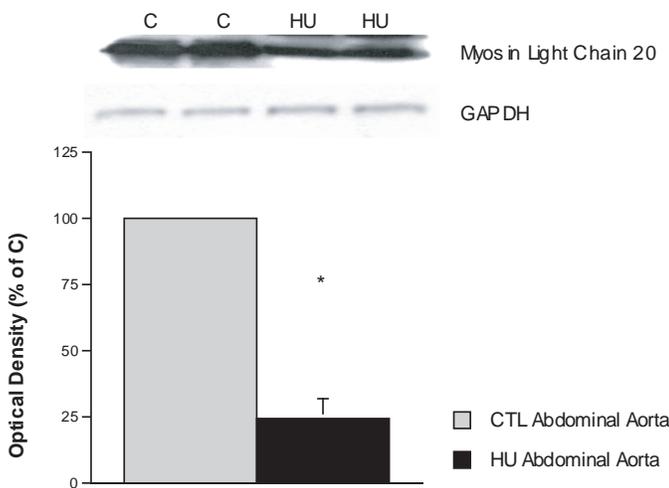


Figure 2
Sample Western blots of Myosin light chain 20 and respective loading control GAPDH and mean data of Myosin light chain 20 from CTL and HU rat abdominal aortas (n=4). HU=24 ± 7.71% (SEM) of control. * p<0.0001

hypothesis, there was an increase in expression of heavy caldesmon, as seen in Figure 4.

Moreover, HU had no effect on the expressions of Calponin (Figure 5) and α -tropomyosin (data not shown).

Discussion

The goal of this study was to determine the effect of simulated microgravity on the levels of the contractile proteins MHC, MLC20 and α -actin. The contractile element smooth

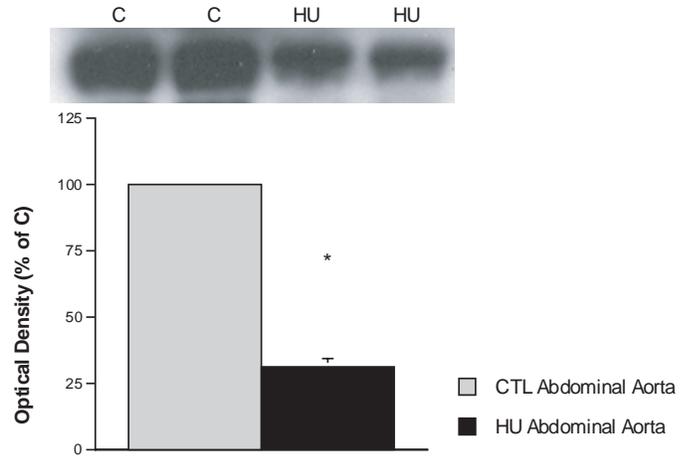


Figure 3
Sample Western blots and mean data of α -actin from CTL and HU rat abdominal aortas (n=3) HU=31 ± 3.29% (SEM) of control. * p<0.0001

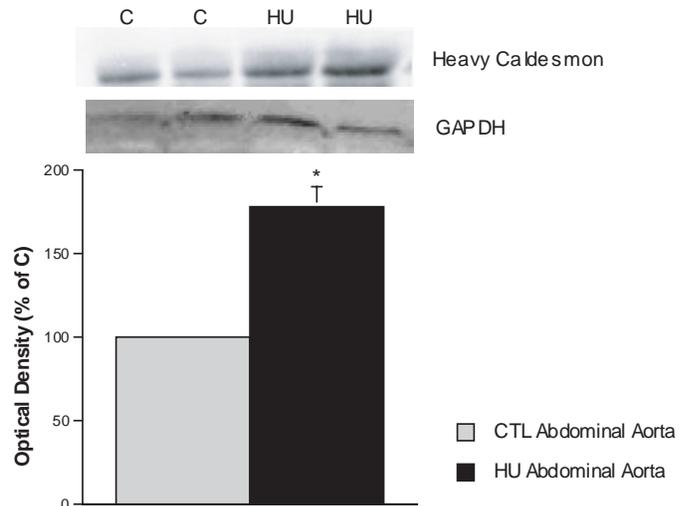


Figure 4
Sample Western blots of Heavy caldesmon and respective loading control GAPDH and mean data of Heavy caldesmon from CTL and HU rat abdominal aortas (n=5). HU=177.9 ± 11.5% (SEM) of control. * p<0.05

muscle myosin II contains two heavy chains (SM-2, 200 and SM-1, 204 kD) and a pair of light chains (MLC17 and MLC20) (Horowitz et al., 1996). We did not distinguish between the different isoforms of the heavy chain during Western blot analysis because their roles during contraction are not significantly different, unlike in skeletal muscle. Isoform change is not a major mechanism in regulating speed and strength of contraction in vascular smooth muscle (Horowitz et al., 1996). Rather, changes in total expression are more important, and were the focus of this study. The results demonstrate that MHC, MLC20 and α -actin are reduced by 70% or more in abdominal aorta from HU rats.

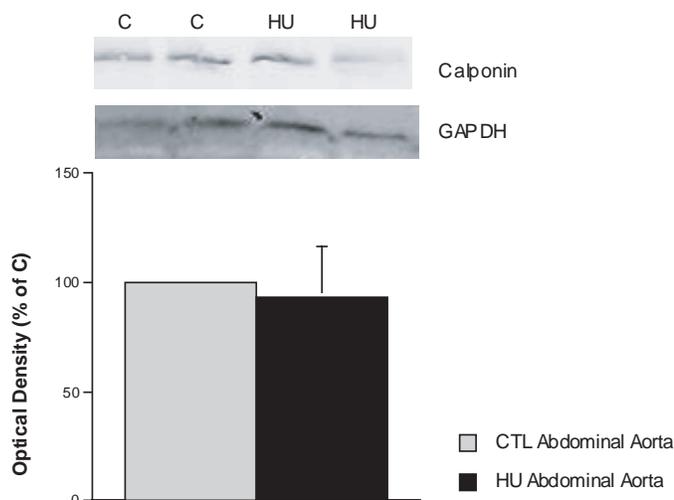


Figure 5
Sample Western blots of Calponin and respective loading control GAPDH and mean data of Calponin from CTL and HU rat abdominal aortas (n=5). HU = 93 ± 23.7% (SEM) of control.

Since phosphorylation of MLC20 is the main regulatory step initiating vascular contraction, the decrease in MLC20 alone could contribute to the HU-induced decrease in vasoconstriction. Phosphorylation of MLC20 leads to contraction, namely, crossbridge cycling of MHC on α -actin, the single event underlying vascular force development. Thus, these findings that MHC and α -actin expression are also reduced represents a further mechanism for the HU-induced decrease in force development. These results support our hypothesis that the decrease in the contractile proteins could contribute to HU-induced vasoconstrictor hyporesponsiveness.

Vascular smooth muscle can undergo a change from a mature contractile state or phenotype to an immature non-contractile phenotype. Moreover, a decrease in both contractile and myofilament-binding proteins characteristically accompanies such a phenotype change (Owens et al., 2004). This is because both contractile proteins share the same response elements, the cArg box, in their promoter region (Sobue et al., 1999). We took advantage of this known linkage between these two classes of proteins to test for HU-induced phenotype change. The criterion for such a change was a parallel decrease in both contractile and myofilament-binding proteins. However, our results indicated that there was no change in the proteins Calponin and α -tropomyosin, and an actual increase in h-caldesmon. Thus, these findings show that classical phenotype change does not occur as a result of simulated microgravity. Future experiments are required to determine the mechanisms of HU-induced decreases in contractile proteins.

It has been determined that h-caldesmon functions as a myofilament regulatory protein and has an inhibitory role on vascular smooth muscle contraction (Xiao et al., 2004). Specifically, h-caldesmon binds to α -actin and inhibits actin-activated myosin ATPase. The findings of this study show that h-caldesmon is increased in the HU rats. In turn, this could provide greater inhibition of contraction compared to control.

Conclusion

This study suggests two possible mechanisms for HU-induced vasoconstrictor hyporesponsiveness: 1) reduced expression of contractile proteins and 2) increased expression of the contraction inhibitory protein h-caldesmon.

These findings have implications for the treatment of orthostatic intolerance and other cardiovascular diseases. A putative treatment revealed by our study would be to increase the expression of vascular contractile proteins. Research conducted by Atkins *et al.* (2005) suggests a possible approach. They found that proteins h-caldesmon and MHC were significantly decreased in aortas of deoxycorticosterone acetate (DOCA) salt-hypertensive rats. Furthermore, when they treated these animals with peroxisome proliferator-activated receptor- γ (PPAR- γ) agonists, drugs normally used to treat type 2 diabetes, expression of the vascular contractile proteins returned to normal. PPAR- γ agonists have not been tested in HU rats. However, this appears to be a promising approach to the prevention and reversal of orthostatic intolerance. Further study is needed to assess the potential of this putative treatment.

Changes in vascular smooth muscle reported in this study are similar to those found in other cardiovascular ailments such as atherosclerosis and hypertension. Namely, the vascular changes in all these ailments include a decrease in the expression of the contractile proteins, α -actin and myosin. Thus, if it is found that PPAR- γ agonists can successfully treat orthostatic intolerance, these agonists also have the potential to be effective therapeutic agents in both atherosclerosis and hypertension.

Acknowledgements

I would like to express my sincere gratitude to Dr. Ralph Purdy for his kind support and editorial assistance during the course of this project. Thanks also to graduate student Sunup Hwang, for guidance and support throughout my research experience. Finally, I would like to recognize the Purdy Lab research team, Scott Summers, Dr. Adolph

Ulloa, Dr. Stanislav Shelkovnikov, and fellow undergraduate Valorie Willis for their help with experiments, support and friendship which made research a truly wholesome experience. This project was funded by NASA and the UCI Undergraduate Research Opportunities Program.

Works Cited

- Atkins, K.B., C.A. Northcott, S.W. Watts, and F.C. Brosius. "Effects of PPAR- γ Ligands on Vascular Smooth Muscle Marker Expression in Hypertensive and Normal Arteries." American Journal of Physiology—Heart and Circulatory Physiology 288 (2005): 235–243.
- Buckey, J.C. Jr., L.D. Lane, B.D. Levine, D.E. Watenpugh, S.J. Wright, W.E. Moore, F.A. Gaffney, and C.G. Blomqvist. "Orthostatic Intolerance after Spaceflight." Journal of Applied Physiology 81 (1996): 7–18.
- Delp, M.D., T. Holder-Binkley, M.H. Laughlin, and E.M. Hasser. "Vasoconstrictor Properties of Rat Aorta are Diminished by Hindlimb Unweighting." Journal of Applied Physiology 75 (1993): 2620–2628.
- Horowitz, A., C.B. Menice, R. Laporte, and K.G. Morgan. "Mechanisms of smooth muscle contraction." Physiological Review 76 (1996): 967–1003.
- Owens, G.K., M.S. Kumar, and B.R. Wamhoff. "Molecular Regulation of Vascular Smooth Muscle Cell Differentiation in Development and Disease." Physiological Review 84 (2004): 767–801.
- Papadopoulos, A. and M.D. Delp. "Effects of Hindlimb Unweighting on the Mechanical and Structural Properties of the Rat Abdominal Aorta." Journal of Applied Physiology 94 (2003): 439–445.
- Purdy, R.E., S.P. Duckles, D.N. Krause, K.M. Rubera, and D. Sara. "Effect of Simulated Microgravity on Vascular Contractility." Journal of Applied Physiology 85 (1998): 1307–1315.
- Sangha, D.S., N.D. Vaziri, Y. Ding, and R.E. Purdy. "Vascular Hyporesponsiveness in Simulated Microgravity: Role of Nitric Oxide-Dependent Mechanisms." Journal of Applied Physiology 88 (2000): 507–517.
- Sobue, K., K. Hayashi, and W. Nishida. "Expressional regulation of smooth muscle cell-specific genes in association with phenotypic modulation." Molecular Cell Biochemistry 190 (1999): 105–118.
- Summers, S.M., A.S. Ulloa, and R.E. Purdy. "Simulated Microgravity in Wistar Rats Markedly Increases Vasoconstrictor Capacity in Endothelium Denuded Thoracic Aorta." J. Gravitational Physiol. Abstract, In Press, 2005.
- Watenpugh, D.E. and A.R. Hargens. "The Cardiovascular System in Microgravity, in *Handbook of Physiology*." Environmental Physiology Bethesda, MD: American Physiological Society, (1996): 631–674.
- Wilkerson, M.K., P.N. Collieran, and M.D. Delp. "Acute and chronic head-down tail suspension diminishes cerebral perfusion in rats." American Journal of Physiology—Heart and Circulatory Physiology 282 (2002): 328–334.
- Xiao, D., W.J. Pearce, L.D. Longo, and L. Zhang. "ERK-Mediated Uterine Artery Contraction: Role of Thick and Thin Filament Regulatory Pathways." American Journal of Physiology—Heart and Circulatory Physiology 286 (2004): 1615–1622.