Regulation of Aortic Smooth Muscle Relaxation in Spontaneously Hypertensive Rats

by

Andraéle Reed

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biological Sciences

Program

YOUNGSTOWN STATE UNIVERSITY

August, 2014

Regulation of Aortic Smooth Muscle Relaxation in Spontaneously Hypertensive Rats

Andraéle Reed

I hereby release this thesis to the public. I understand that this thesis will be made available from the OhioLINK ETD Center and the Maag Library Circulation Desk for public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research.

Signature:

Andraéle Reed, Student Date

Approvals:

Robert E. Leipheimer, Ph.D., Thesis Advisor Date

Jill M. Tall, Ph.D., Committee Member Date

Gary R. Walker, Ph.D., Committee Member Date

Dr. Salvatore A. Sanders, Associate Dean of Graduate Studies Date

Abstract

Hypertension may be caused by excessive vasoconstriction that can occur through a variety of dysfunctions in cellular mechanisms. Estrogen has been postulated to have protective properties against various cardiovascular pathologies and reported to play numerous direct and indirect roles within vascular smooth muscle. Therefore, the goals of this study were to examine two specific cellular mechanisms, the Rho-kinase pathway and the sarcoplasmic reticulum Ca^{2+}-ATPase pump, that regulate smooth muscle activity in Spontaneously Hypertensive Rats (SHR). We also investigated whether ovariectomy caused differences in the effectiveness of these cellular pathways in aortic smooth muscle. The aortas from ovariectomized SHR were isolated, attached to force transducers, and placed in water jacketed chambers. All chambers were contracted with phenylephrine (PE) and treated with cyclopizonic acid (CPA), a sarcoplasmic reticulum Ca^{2+}-ATPase pump inhibitor, or Y-27632, a Rho-kinase inhibitor. The tissues treated with CPA were relaxed with sodium nitroprusside (SNP), whereas Y-27632 was the relaxing agent in that group. As a result of our study, it was found that the cellular mechanisms that regulate contraction and relaxation in aortic smooth muscle are altered in SHR and were also significantly affected by ovariectomy. Our results also demonstrated that CPA and Y-27632 treatment significantly inhibited relaxation of aortic rings from ovariectomized spontaneous hypertensive rats. Together, these results suggest: (1) estrogen plays an important role in maintaining the function of the SR Ca^{2+}-ATPase pump; and (2) estrogen has a facilitatory role in maintaining the integrity of the Ca^{2+}-desensitization pathway (Rho-kinase – myosin phosphatase interaction) in spontaneous hypertensive rats.

Acknowledgements

I would first like to thank my advisor, Dr. Robert Leipheimer, for all your guidance and mentorship throughout the years, which lead to the completion of this project. You helped me to realize how much my contribution or "my pebble" means to the greater tapestry that is science.

To my committee members, Dr. Jill Tall and Dr. Gary Walker, I would like to thank each of you for your assistance and encouragement as I actively worked my way through the writing process of this thesis.

Dawn Amolsch, thank you so much for helping me learn the "ins and outs" of the laboratory and how a lab is properly ran. I also want to thank for all your help in showing me the proper ways to care for and handling of the animals used throughout this project. Without all your help I would not have made it this far.

To my extended family and friends, Grandma Williams and Grandpa Reed, my aunts and uncles, my cousins and little nephews, as well as my fellow graduate students, thank you all for your support and being a part of my life.

Lastly and most importantly, I would like to thank my immediate family. To my mom and dad, thank you for all your much needed support and raising me to know that in God all things are possible. You taught me to never give up and to keep pressing towards the finish line. I love you both very much. To my siblings, Andrae, Cristen, and Carissa, let us keep moving forward down our respective paths and continue to keep our parents proud of us in everything we decide to do. To my fiancé, Darian Scott, thank you so much for your much needed patience, support, and believing in me that I could accomplish my goals. I love you and with you, I finally made it.

Table of Contents

Title Page i
Signature Page ii
Abstract iii
Acknowledgments iv
Table of Contents v
Introduction 1
Materials and Methods 22
Results 27
Discussion 38
References 43

Introduction

Vascular smooth muscle plays a critical role in the body by regulating blood pressure and aiding in the transport of blood and other vital nutrients necessary for sustaining life. However, impairments to this tissue occurs in hypertension or atherosclerosis, which can often hinder the functioning of vascular smooth muscle and lead to devastating cardiovascular ailments, if left untreated. Therefore, in order to effectively understand how significant vascular smooth muscle is, it is necessary to first examine smooth muscle in general and its functions.

Smooth muscle lines most internal hollow organs in the body, for example the arteries, intestinal tract, and bronchioles to name a few. The purpose of smooth muscle in these locations is to involuntarily regulate movement of substances from one place to another, in addition to decreasing or stopping the movement of a substance in the body. An example of this would be blood in the arteries being transported from one part of the body to another, via the constriction and relaxation of the smooth muscle within the vessel. During times of metabolic need in the body, smooth muscle can decrease blood flowing to one organ and simultaneously increase blood flow to the organ requiring more nutrients.

At one time, smooth muscle's structure was categorized into two distinct fiber types, single-unit and multi-unit (Somlyo & Somlyo, 1968). These fibers support the various ways smooth muscle is assembled throughout the body depending on how autonomic motor neurons innervate them. Single-unit smooth muscle, which is commonly associated with the viscera, are tightly packed sheets of muscles. They are connected by gap junctions that spread action potentials from a single neuron to a large

group of cells, stimulating the cells to contract as a single unit. Multi-unit smooth muscle also occurs in sheets; however, the ratio one neuron per cell is preserved because there are few, if any gap junctions found between each muscle fiber. This implies the depolarization of multi-unit smooth muscle cannot occur by way of action potentials alone, but also from an external mechanical response (Casteels et al., 1977). These types are usually found in organs as in the bronchioles, iris, the walls of large arteries, etc. (Hilgers & Webb, 2005). At present, smooth muscle is not as rigidly classified as being in one group versus the other, because some smooth muscle can fall into both categories, depending on where it is located in the body. The autonomic nervous system (ANS) primarily controls its function, where neural innervation initiates various signal transduction pathways to elicit contraction or relaxation. In addition to autonomic regulation, many other local factors can also influence smooth muscle activity. The present study will focus more specifically on aortic smooth muscle.

Arterial Smooth Muscle

The arteries use smooth muscle for the primary function of regulating the diameter of the lumen within the vessels, effectively regulating blood flow. Smooth muscle contraction in the walls of these vessels leads to vasoconstriction, decreasing the diameter of the blood vessels as well as blood flow (Owens, 1995; Hilgers & Webb, 2005). In contrast, smooth muscle relaxation leads to vasodilation, resulting in the increase of vessel diameter and blood flow. The smooth muscle in these tissues, primarily in the larger vessels, could be classified as multi-unit smooth muscle because they express many of the characteristics for this muscle type as previously discussed.

These tissues lack gap junction and are mainly stimulated externally by either hormones, nerves, or other chemicals released by local tissue factors.

Arteries have a ridged exterior (tunica externa), a thick muscular middle layer (tunica media), and a smooth inner endothelial layer (tunica intima), which all allow for blood to flow through the vessels with ease. The endothelium also helps to regulate blood pressure and flow by releasing either vasodilating or vasoconstricting factors in response to signals in the blood (Harris and Matthews, 2004). Once the heart pumps out blood, the large arteries with their elastic structure expand and fill with blood. When the heart relaxes, these arteries use elastic recoil to decrease in diameter and exert a force strong enough, to maintain tissue blood flow during diastole.

Smooth Muscle Structure

Because smooth muscle is not striated, there are no organized myofibrils or sarcomeres as there are in skeletal and cardiac muscle (Foucrier et al., 2001). Each smooth muscle fiber consists of the thick (myosin) and thin (actin) filaments. Myosin is composed of 2 heavy and 4 light chains, the heavy chain being associated with the pivoting head in the cross bridge cycle, to be expanded on later, and are distributed throughout the sarcoplasm. Actin is attached to dense bodies, which are dispersed throughout the sarcoplasm intermixed within a network of proteins called desmin, or intermediate filaments (Tang, 2008). Many dense bodies are also found to be anchored to the sarcolemma as well. Once a contraction occurs, which will also be described later, the thin filament attaches to myosin and pulls the surrounding dense bodies closer together allowing the smooth muscle cell to twist on itself and shrink in length. In

general, contraction and relaxation of smooth muscle cells depend directly on changes in cytosol calcium ion concentrations. Calcium is a key ion needed in all muscle types, which in turn activates the cross bridge cycle. This cycle initiates the coupling of the thick and thin filaments to create the force of contraction. It allows muscle to be a chemomechanical transducer, taking the chemical energy stored in the terminal phosphate group of ATP and converting it into mechanical work (Webb, 2003).

The Autonomic Nervous System

The autonomic nervous system (ANS) is primarily responsible for most involuntary actions and is devoted to the regulation of normal internal functions within the body. The ANS is subdivided into the sympathetic and parasympathetic nervous systems. These systems are responsible for the fight or flight and the rest or digestion responses, respectively, in the body. In addition, these systems are used to regulate homeostasis as well as individual organ function (Gabella, 1995). Both of these systems consist of myelinated preganglionic fibers which make synaptic connections with un-myelinated postganglionic fibers. It is these post-ganglionic fibers which then innervate the effector organs (Gabella, 1995). The neurotransmitter that is released at cholinergic synapses for these preganglionic nerve fibers is acetylcholine (ACh) which binds to nicotinic receptors on the postganglionic excitatory neurons. Depending on the pathway of stimulation, postganglionic excitatory neurons will either release ACh in the parasympathetic or mainly norepinephrine (NE) in the sympathetic nervous system as its neurotransmitter in the particular effector tissue. The adrenal medulla is an endocrine

organ that releases epinephrine and NE in response to sympathetic stimulation and therefore augments the effects of the sympathetic nervous system. Because most vascular smooth muscle is primarily regulated by sympathetic stimulation, the postganglionic neurons will release the neurotransmitter NE.

The sympathetic nervous system utilizes the cell's excitatory signal transduction pathway to bring about physiological changes in response to an external stimulus, or stress, to the body (Harris and Matthews, 2004). It is through increased or decreased sympathetic regulation that determines vascular smooth muscle's contracted or relaxed states, respectively. Norepinephrine can be found as a hormone and a neurotransmitter in the body. When NE is released as the neurotransmitter from the postganglionic sympathetic neurons it is released from specialized sacs along the axon called varicosities. These varicosities will release NE directly on to the surrounding vascular smooth muscle cells. The body's response to stressors would be to deploy the binding of norepinephrine to its adrenergic receptor further propagating the contractile response. These receptors are activated in various organs containing smooth muscle with the ultimate action depending on the specific location and function of the organ or tissue. In vascular smooth muscle stimulation of adrenergic receptors generally leads to contraction and vasoconstriction. The pathway of adrenergic receptors will be further discussed in the following section.

The parasympathetic nervous system, which is not the actual focus of this paper, is more responsible for restoration and conservation of energy. This system causes a reduction in heart rate and blood pressure mainly by its action in the atria. This is accomplished through the binding of ACh to muscarinic acetylcholine receptors found

primarily in SA node and atrial myocardium. As a result, there is a decrease in heart rate and force of atrial contraction. Therefore, together these actions lead to a decrease in cardiac output and ultimately a decrease in arterial pressure.

Smooth Muscle Contraction

Smooth muscle is generally kept at a relatively constant tension, where the muscle is not allowed to reach complete relaxation. This is done so that smooth muscle stays at a basal state of contraction, which makes each subsequent contraction easier to attain. This is called smooth muscle tone and is regulated by sympathetic tone, which determines the activity of two main regulatory enzymes, myosin light chain kinase and myosin light chain phosphatase. A contraction is the shortening of a muscle while exerting a force in order to perform work (Hilgers &Webb, 2005). In order for smooth muscle to contract, an initial stimulus is needed to activate Ca^{2+} release into the sarcoplasm. One process of contraction, which is referred to as excitation-contraction coupling, begins when an electrical impulse causes a change in membrane potential large enough to generate an action potential in an axon. The action potential travels down the axon of the preganglionic neuron, until it reaches the axon terminal to release ACh on to the post-synaptic neuron. Excitatory agonists (like hormones in the case of the adrenal sympathetic pathway, and neurotransmitters) are then released from the postganglionic neuron onto the sarcolemma at the varicosities, where they bind to their respective receptors and trigger the Ca^{2+}-dependent pathway for Ca^{2+} release (Hilgers &Webb, 2005). Once the [Ca^{2+}] has been increased in the cytosol, Ca^{2+} can then form a complex with the calcium binding protein calmodulin. This complex has several functions when

formed; however, one of its primary roles is to activate myosin light chain kinase (MLCK), which phosphorylates the Ser 19 of the 20-kDa regulatory light chain of myosin initiating the cross bridge cycle (Shen et al., 2010). While contractions are occurring, myosin light chain phosphatase (MLCP) is actively dephosphorylating the myosin light chain. During a prolonged contraction or continued stimulation, additional signal transduction pathways are activated, to counteract the oscillating nature of myosin light chain kinase and myosin light chain phosphatase, and inhibit dephosphorylation of the myosin light chain (Hilgers & Webb, 2005). However, it is important to note here that other factors can stimulate the same contractile response because in vascular smooth muscle, neural input is not necessarily required. Contractions can occur without an action potential, and this can be referred to as excitation-free contractions and it is the initial stimulus that causes Ca^{2+} release and the process of contraction to occur. Those factors can be local chemical changes (such as in oxygen, carbon dioxide, or adenosine), circulating hormones (Angiotensin II, AVP, etc.), or even physical change (as in extreme stretching or irritation) (Hilgers & Webb, 2005). These factors enable vascular smooth muscle to assume immediate local control over the blood flow to independent tissues depending on current metabolic demands of that tissue.

Mechanism of Ca^{2+} dependent contraction

At the onset of an initial stimulus, sarcoplasmic [Ca^{2+}] begins to increase, as seen from example in figure 1. This is accomplished when an agonist, such as NE, binds and activates adrenergic receptors, which are also linked to the activation of G-protein-coupled receptor transduction pathways. This transmembrane G-protein, located on the

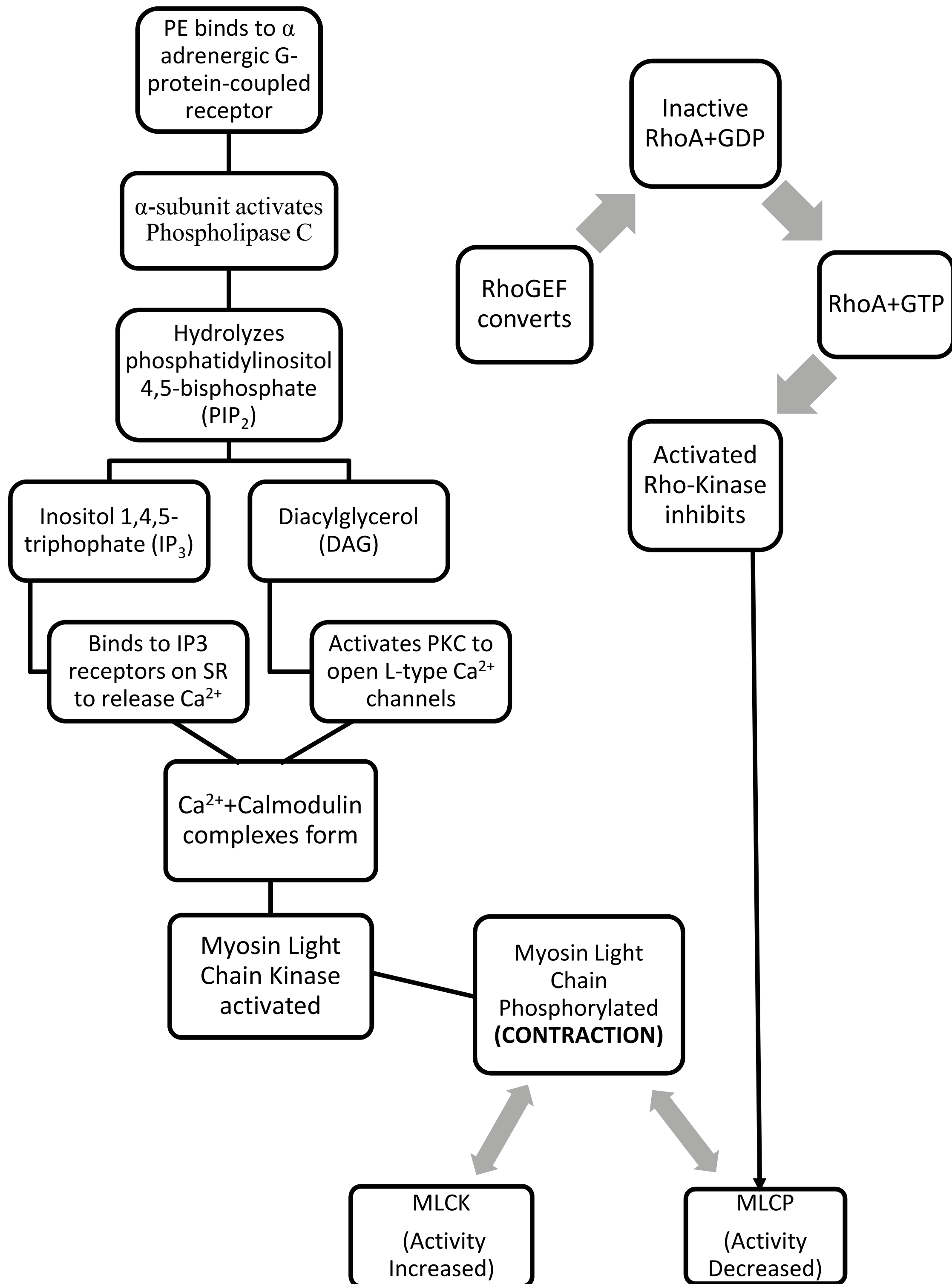
surface the sarcolemma, is made up of three subunits; alpha (α), beta (β), and gamma (γ), with an inactive GDP attached to the alpha subunit. Once the agonist is bound to the receptor, the G-protein goes through a conformational change, initiating the dissociation of the α-subunit and coupled GDP from the β and γ-subunits. This is achieved once the guanosine exchange factor, also located on the α-subunit, exchanges the GDP for GTP. Once the exchange occurs, the α-subunit, in turn, activates phospholipase C in the same cascade. Phospholipase C is an enzyme that catalyzes the hydrolysis of the target phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) into the two secondary messengers, diacylglycerol (DAG) and inositol 1,4,5-triphophate (IP_3) (Ochocka et al., 2008).

The second messenger, DAG, phosphorylates protein kinase C (PKC). Once PKC is activated, it can then phosphorylate the cascade of various other specific proteins, such as the activation of a CPI-17, as well as the transient receptor potential channels for cations, namely Ca^{2+} L-type channels (Large et al., 2009; Webb, 2003). CPI-17 is a MLCP inhibitor protein that is present within vascular smooth muscle (Dimopoulos et al., 2007). L-type Ca^{2+} channels are voltage gated and enables most of the Ca^{2+} to enter into the cell under normal contraction. It is these channels, however, that are associated with the Ca^{2+} release from the sarcoplasmic reticulum (SR) (Large et al., 2009). Asano and Nomura (2001) investigated L-type Ca^{2+} channels in spontaneously hypertensive rats. They reported that increased channel activity can lead to calcium mishandling in these animals, further demonstrating how vital these channels are to calcium regulation in smooth muscle.

The other second messenger, IP_3, will then bind to its receptors on the SR membrane and help to mediate the release of additional calcium from this cellular store into the sarcoplasm (Wray, 2010). With the now large concentration of Ca^{2+} now present in the cytosol in the smooth muscle, calcium/calmodulin complexes can form and phosphorylate MLCK. The greater the $[Ca^{2+}]$ in the sarcoplasm, the stronger the contractile response once the cross bridge cycle is initiated.

The cross bridge cycle is triggered when MLCK phosphorylates myosin, activating a series of contractile events. In stage one, when there is no stimulus, the actin thin myofilament and the globular head of the myosin thick filament are not attached. ADP and an inorganic phosphate are attached to myosin head at this time and myosin is in its high energy state ready for attachment. The second stage, when intracellular Ca^{2+} concentrations are high, the myosin head then attaches to actin by the calcium-regulated MLCK phosphorylation of the 20-kDa catalytic site of myosin. At this point, the myosin head is in a high energy configuration with ADP, while the inorganic phosphate is released. With the release of the inorganic phosphate, the bond between actin and myosin strengthens. The third step or power stroke, ADP is now released causing the myosin head to bend and pivot, which slides the actin. This movement causing the dense bodies to be pulled and ultimately shortens the cell. In the fourth step, another ATP binds to myosin, weakening the link and detaching the myosin from actin. In the last step, ATP is hydrolyzed, reactivating the myosin head and returning it to the high energy configuration, its ready position.

Figure 1: Ca^{2+}-dependent Contraction and Sensitization



Mechanism of Ca^{2+} sensitization

Once the contraction has been initiated, it can be maintained for a relatively long period of time, if needed. In vascular smooth muscle, a Ca^{2+} sensitization pathway is activated in parallel to the Ca^{2+} dependent contractions. This Ca^{2+} sensitization pathway has been reported to be mediated by Rho kinase, which in turn is regulated by the membrane bound G-protein, RhoA (Ogita et al., 2003). Activation of this RhoA/Rho-kinase pathway functions to sustain and strengthen the contraction, which was generated by the rise in sarcoplasmic $[Ca^{2+}]_i$. This is achieved by inhibiting the activity of MLCP, conversely increasing MLCK activity (Webb, 2003). As previously mentioned, two primary enzymes regulate the state of MLC phosphorylation and they are MLCK and MLCP. During the initial contraction, the dephosphorylating effects of MLCP are avidly countered by this the Rho-kinase pathway.

The activation of the Rho-kinase pathway (figure 1) usually begins with the stimulation of the monomeric G-protein RhoA bound to an inactive GDP (Somlyo & Somlyo, 2003). Rho-specific guanine nucleotide exchange factor (GEF), which is an intracellular molecular switch that catalyzes the exchange of the GDP bound to RhoA, for the active form of GTP. However, the complete activation and regulation of these intracellular GEFs is still relatively unknown (Wettschureck and Offermanns, 2002). Once RhoA has been activated, it is then able to stimulate the activation of its downstream target, Rho kinase, which will then phosphorylate MLCP (Denniss, 2010). Härte et al. (2007) reported, MLCP is a holoenzyme made up of three subunits; a 37-kDa catalytic subunit, a 20-kDa variable subunit, and a 110- to 130-kDa myosin-binding subunit, otherwise known as MYPT1. Rho-kinase inhibits the activity of MLCP by

phosphorylating the myosin binding subunit, which will enable the contraction to be prolonged.

The Rho-kinase pathway can act in two primary ways. When Ca^{2+} is present, this pathway intensifies the muscle's tone, by augmenting the smooth muscle's cellular responsiveness to Ca^{2+}. This augmentation of the contraction, regardless of the amount of Ca^{2+} in the sarcoplasm, this is known as Ca^{2+} dependent sensitization. The other way Rho kinase can work is through Ca^{2+} independent sensitization as discussed in Dimopoulos et al. (2007). In their study they reported a contraction can also be generated without an initial increase in Ca^{2+} concentration in the intracellular environment. Activation of the Rho-kinase pathway has been shown to cause phosphorylation of the myosin light chain stimulating contraction (Ogita et al., 2003), independent of Ca^{2+}concentration.

Smooth Muscle Relaxation

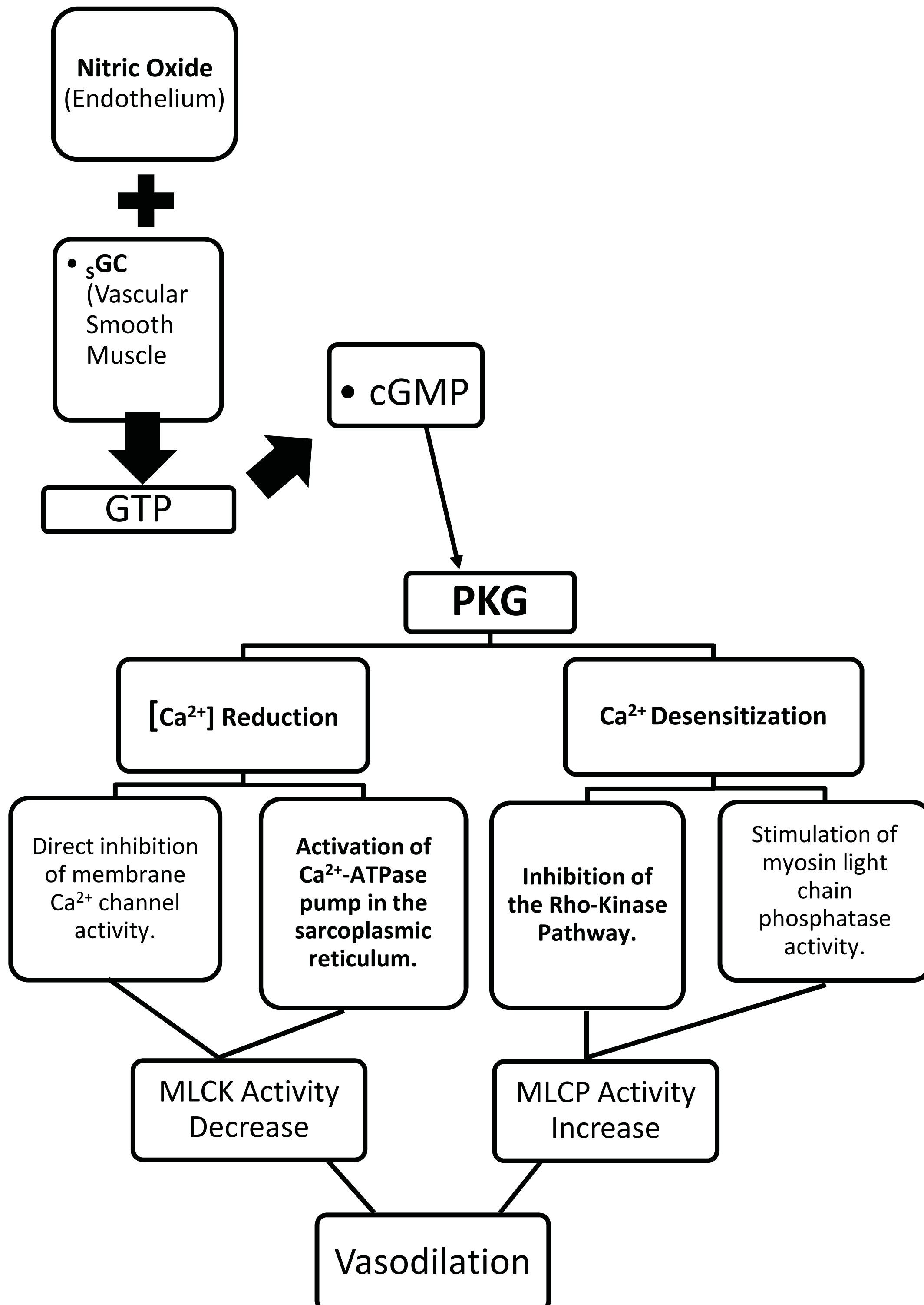
Relaxation can be achieved in two main ways: One, via the re-uptake of Ca^{2+} from the sarcoplasm through overall calcium efflux out of the cell and/or its reuptake back into the SR; and two, activation of MLCP which dephosphorylates myosin resulting in detachment of the myosin heads from actin. This latter mechanism is referred to as Ca^{2+} desensitization. Furthermore, smooth muscle cells may relax through passive or more active mechanisms.

Passive relaxation occurs once the stimulus has ceased or been removed following receptor activation. For example, once NE dissociates from its receptor, all the events described that stimulate contraction are reversed. This in turn, lowers cytosolic [Ca^{2+}],

which then leads to the decrease of MLCK activation and increase in MLCP activity. In some smooth muscle cells, the phosphorylation of the light chain of myosin is maintained at a low level in the absence of external stimuli (Floyd & Wray, 2007). This occurrence is due to vascular smooth muscle being a tonic smooth muscle. Tonic smooth muscle remains contracted most of the time while only relaxing for a brief time. However, dephosphorylation of the myosin light chain does allow relaxation to occur. Also, as a result of decreased calcium levels, myosin light chain phosphatase activity is increased further desensitizing the muscle to calcium.

The other method, active relaxation (figure 2), is devoted to triggering smooth muscle relaxation relatively more rapidly based on the metabolic needs or function of the tissue. This method is activated when a stimulus, such as a decrease in blood pressure, is sensed within the blood vessel. This will stimulate a sequence of events to enhance vasodilation, increasing blood flow. Nitric oxide (NO) is produced within the endothelial cells from the conversion of l-arginine and oxygen into NO and l-citrulline (Chitaley and Webb, 2002). Once NO is released, it diffuses from the endothelial cell into the smooth muscle cell where it associates with soluble guanylate cyclase and stimulates its activation. The activated guanylate cyclase will then catalyze the conversion of GTP into cyclic-GMP. This stimulates the rise in levels of the second messenger cGMP within smooth muscle cells (Chitaley and Webb, 2002). cGMP activates protein kinase G (PKG), which in turn activates several pathways; to cause relaxation. These pathways function to decrease cytosolic Ca^{2+} concentrations and/or lead to Ca^{2+} desensitization (Lee et al., 1996; Somlyo and Somlyo, 2003).

Figure 2: Active Relaxation



Once PKG has been activated, it is able to act on a variety of downstream targets committed to causing relaxation in the cell. Those targets are able to act on both the reduction of cytosolic Ca^{2+} concentrations as well as desensitizing the cell to calcium ultimately decreasing vascular tone (Lee et al., 1996). In order to reduce the Ca^{2+} levels, PKG will directly phosphorylate ion channels to inhibit Ca^{2+} diffusion into the sarcoplasm and activates pumps in the cell membrane to help extrude Ca^{2+} out. This is accomplished by the active pumping of Ca^{2+} out of the cell via pumps like the Na^{+}/Ca^{2+} exchanger and Ca^{2+}-ATPase pump in the sarcolemma, as well as the closing of ion channels, such as the L-type Ca^{2+} channels, decreasing calcium influx into the cell (Carvajal et al., 2000). PKG will also affect the cells membrane potential via the opening of Ca^{2+} -activated K^{+} channels. When these channels are opened, the cell becomes hyperpolarized, inhibiting the entrance of Ca^{2+} into the cell (Neylon, 1999). There are also second messengers, like IP_3, that PKG can act on in a couple of ways. One way, is when PKG phosphorylates PLC it will decrease the production of IP_3. The other way is for PKG to directly phosphorylate the receptors of IP_3, in the sarcoplasmic reticulum, which will then inhibit the binding of IP_3 (Tertyshnikova et al., 1998). This will then decrease Ca^{2+} diffusion from the SR and further limit the amount of Ca^{2+} found within the cytosol.

Another location in the sarcoplasmic reticulum, which leads to the reduction of calcium, is the Ca^{2+}-ATPase pump, located in the cellular membrane of the sarcoplasmic reticulum (Cornwell et al., 1991). This pump can be regulated by PKG when it is stimulated by the direct phosphorylation of PKG on the regulatory protein, phospholamban (Carvajal et al., 2000). This protein binds reversibly to the pump

depending on type of phosphorylation (Wray and Burdyga, 2010). When phosphorylation favors relaxation, this protein will switch on the Ca^{2+}-ATPase pump which allows the rebinding of calsequestrin to the Ca^{2+} ions inside the SR. This calcium complex will stay bound until the next contraction cycle. Pharmacological agents, such as cyclopizonic acid (CPA) have been found to inhibit this reuptake pump. CPA is found as a myotoxin produced from certain molds strains such as *Aspergillus flavus* and is a potent inhibitor of the Ca^{2+}-ATPase pump (Laporte et al., 2003). Kwan et al. (1994) found this drug decreased the affinity for ATP to bind to the pump, further decreasing the pump's ability to re pump Ca^{2+} back into the SR. It has also been found that in vasculature, the amount of SRs decrease in quantity smaller blood vessels (Levitsky et al., 1992). Therefore, the role of the SR in regulating [Ca^{2+}] becomes diminished in the smaller diameter blood vessels.

Regulation of Ca^{2+} can also be influenced by external manipulations. Pharmacological agents, such as, fasudil and Y-27632 are used to contribute to the desensitization of Ca^{2+}. This desensitization is accomplished by inhibiting Rho kinase from phosphorylating MYPT1. Fasudil is a potent Rho kinase inhibitor. The drug Y-27632 is a selective Rho kinase inhibitor and is vasodilator that can inhibit smooth-muscle contractions induced by a variety of agonists, for example phenylephrine (Dimopoulos et al., 2007). With MLCP not being inhibited, this enables its enzymatic activity to continue, dephosphorylating the MLC. It has also been postulated that PKG also phosphorylates RhoA directly, destabilizing the membrane binding activity of RhoA (Chitaley and Webb, 2002). Therefore, Ca^{2+} desensitization and ultimately relaxation can occur.

Hypertension

Mean arterial pressure (MAP) is regulated primarily by two components: cardiac output and total peripheral resistance. The heart utilizes various cellular mechanisms to establish how fast and forceful blood will be pumped out of the heart. These activities makeup heat rate and stroke volume and the product of these processes is cardiac output (CO). Blood vessel diameter, determined by the radius of the vessels ($1/(\text{radius})^4$), is the total peripheral resistance (TPR). The arteries make up most of the TPR. Resistance drops exponentially as the radius increases, this inversely affects the MAP. Thus, when CO remains constant, MAP is directly dependent on TPR. Increased TPR, associated with vasoconstriction, leads to increased MAP. In contrast, decreased TPR, associated with vasodilation, leads to decreased MAP. Therefore, these changes in the diameter of blood vessels alter the pressure that blood exerts on the vessel's walls. Other conditions such as atherosclerosis affect the walls of the arteries and the degree of TPR, further influencing the MAP.

Blood pressure is measured largely based on the systolic and diastolic activities of the heart. A normal blood pressure is a systolic pressure of about 100-120 mmHg and diastolic pressure is about 60-80 mmHg. When systolic and diastolic pressures are found to be at about 140 mmHg and 90 mmHg, respectively, or greater, hypertension is apparent. Hypertension or high blood pressure is a serious medical condition, where blood is persistently forced through and against the vascular walls of the body at elevated pressures. This chronic medical condition can arise from excessive or prolonged vasoconstriction. Hypertension can occur through variety dysfunctions in cellular mechanisms, such as pathways in cardiac output, the Rho kinase pathway and other

pathways devoted to vascular function (Denniss et al., 2010). Over time, this often leads to more serious cardiovascular pathologies like myocardial infarctions, arteriosclerosis, and even stroke (Johansson, 1999). It has been found that hypertension can come in different forms. In essential or primary hypertension, the exact cause of is still relatively unknown. However, some environmental implications can also influence this chronic elevation of blood pressure over time, such as stress, ethnicity, as well as diet. In secondary hypertension, the cause is known, because it usually develops after an already preexisting condition has arisen for instance in some kidney diseases. In these kidney pathologies, hypertension usually stems from salt and water imbalances, which in turn affects blood pressure. When salt and water increases in the body, blood pressure also tends to rise as well.

In terms of ethnicity, hypertension over the years has tended to disproportionately favor more the African American community than any other ethnic group in places like the United States (Nesbitt and Victor, 2004). Nesbitt and Victor, 2004 reported this may be due to a mix of genetic and environmental influences that links to hypertension in this particular community. It also is one of the leading factors related to cardiovascular deaths that have been seen in men and post-menopausal women in the U.S (Lindsey and Chappell, 2011; Philpott, 2014). The decrease in blood levels of estrogen has been postulated to be a contributing factor to this increase in cardiovascular problems in men and postmenopausal women.

Estrogen is a hormone that has been found to produce protective properties against many known cardiovascular pathologies, for instance stroke and myocardial infarctions (Lindsey and Chappell, 2011). This hormone has three known receptors,

ERα, ERβ, and the g-protein coupled receptor, GPER30. These receptors have been found to be located in various cardiovascular tissues including the aorta (Meyer, et al., 2011). ERβ has been found in larger quantities than ERα, in rat aortas. All three receptors can be found distributed throughout the cellular membrane and nucleus of the endothelium and vascular smooth muscle, while the GPER30 can be found within the endoplasmic reticulum in the endothelium (Kahil, 2013). With the activation of these receptors, several intracellular mechanisms are stimulated for instance one leading to the production of nitric oxide. This was reported in the study by Mershon et al. (2002) that ERβ mediates estrogen-induced NO production through the stimulation of genomic pathways in the ovine coronary artery.

It has been found that estrogen plays numerous roles within vascular smooth muscle both directly and indirectly. As reviewed by Tostes et al. (2003) most of estrogen's effects are through acting directly on endothelial cells. It increases endothelium-derived relaxing factors such as NO. In contrast, estrogen decreases the endothelium-derived vasoconstricting factors, endothelin-1 and angiotensin II. Estrogen also indirectly regulates some intracellular components, for instance sarcoplasmic reticulum expression, to cause relaxation. In another study by Hill and Muldrew (2004) estrogen was found to increase the sarcoplasmic reticulum Ca^{2+}-ATPase pump expression allowing decreased Ca^{2+} in the sarcoplasm of vascular smooth muscle. Other relaxation effects of estrogen reportedly act directly on vascular smooth muscle. Ion channels such as activating K^+ channels and blocking Ca^{2+} channels within smooth muscle can be regulated by estrogen (Freay et al., 1997). This is done by estrogen binding to the ERβ found along the sarcolemma, which was reported by Kahil (2013). Estrogen has been

known to exhibit various effects on regulation in intracellular transduction mechanisms related to both Ca^{2+} desensitivity and $[Ca^{2+}]$ reduction. In a study by Hiroki et al. (2004) estrogen was found to suppress desensitization in Rho-kinase in the signal transduction pathway, for example, of vascular smooth muscle. These inhibitory effects that are exerted on Rho-kinase are mediated via the activation of the classic ERα (Kahil, 2013). With these effects displayed by estrogen, vascular smooth muscle relaxation can be attained, allowing estrogen to be used as a beneficial therapeutic agent for many cardiovascular disorders like hypertension. Further research into vascular estrogen receptors and improvements to present day selective ER agonists could possibly increase the benefits of estrogen therapy for cardiovascular diseases in post-menopausal women. Estrogens effects on these pathways could potentially protect the vasculature from the excessive accumulations of intracellular Ca^{2+} that may be the cause of some unexplained hypertensive incidences.

Purpose

The purpose of this research was to further investigate the cellular mechanisms that regulate relaxation in aortic smooth muscle. Specifically, we investigated two pathways that regulate smooth muscle activity by two different mechanisms. One of those mechanisms is the Rho-kinase pathway, which alters calcium sensitivity in vascular smooth muscle cells. The other mechanism is via the Ca^{2+}-ATPase pump in the sarcoplasmic reticulum, which acts to decrease cytosolic Ca^{2+} concentrations.

We examined the role of these pathways in Spontaneously Hypertensive Rats (SHR) to investigate whether these mechanisms are altered in rats known to be

predisposed to hypertension, when compared to its control, the Wistar Kiyoto rat (WKY). Furthermore, we investigated whether ovariectomy and the associated decrease in blood estrogen levels, caused differences in the effectiveness of these relaxation pathways in aortic smooth muscle. The Rho-kinase pathway was investigated by treating the tissues with Y-27632 (a potent Rho-kinase inhibitor) and the sarcoplasmic reticulum Ca^{2+}-ATPase pump was investigated by treating the tissue with CPA, an inhibitor of this pump. Therefore, we proposed two general hypotheses. First, are there differences in cellular relaxation mechanisms between SHR and WKY rats? And second, does ovariectomy (loss of gonadal steroid hormones) result in differences in the relaxation mechanisms in SHR and WKY rats?

Material & Methods

*Animals***.** Female Spontaneous Hypertensive rats (SHR) and Wistar Kiyoto rats (WKY) aged about 3-6 months were used in these studies. The rats were housed at the Youngstown State University animal housing facility, in plastic cages containing 4 rats per cage. They were kept on a reverse 12-hour light-dark cycle with lights on at 1800 hours. The rats also had free access to water and a diet of standardized laboratory pellets. The temperature in the animal facility was maintained at 70° (+/- 2 degrees) Fahrenheit and the humidity was about 30%. These experiments were approved by the Institutional Animal Care and Use Committee of YSU.

Surgeries. All surgeries were performed with sterile instruments under aseptic conditions. The sexually mature rats were randomly placed into sub-groups within their particular strain, for the SHR ovariectomized (n=5) and sham surgery (n=5); and the WKY ovariectomized (n=5) and sham (n=5). The rats were anesthetized with the drug cocktail of ketamine (50mg/kg) and xylazine (8mg/kg) injected intramuscularly and allowed to reach the fourth plane of anesthesia. After the rats were fully anesthetized, the fur on both dorsal sides was shaved in the area proximal to the ovaries and skin was decontaminated using iodine swabs. A small skin incision was made to expose the underlying abdominal muscle, removing any excess fat that might have been between the two tissues. A small incision was also made through the abdominal muscle wall. Next, the uterine horn, lying within the ovarian fat pad, was exteriorized and was tied off with 3.0 surgical silk thread to ensure, complete removal of the ovary. The ovary was excised from the designated animals, and the uterine horn was carefully placed back in the abdomen. Both the muscle and skin incisions were closed using 4.0 surgical silk thread.

The same procedure was followed for the ovary on the contralateral side. The animals receiving the sham surgeries, also received the same surgical procedure as described, except the ovaries were not removed. After the surgeries, each animal was monitored closely for any behavioral or physical changes. The animals were allowed to recover for at least 2 weeks, to ensure complete clearance of the ovarian hormones.

Drugs and solutions. A modified Kreb's solution was used which contained solutions of the following concentrations: NaCl (130 mM), KCl (4.7 mM), KH_2PO_4 (1.18 mM), $MgSO_4$*$7H_2O$ (1.17 mM), $NaHCO_3$ (14.9 mM), $CaNa_2EDTA$ (0.026 mM), $CaCl_2$ (1.6 mM), and dextrose (5.5 mM) (Alcorn, Toepfer, Leipheimer, 1999). The pH of the Kreb's solution was adjusted to 7.30-7.40 using HCl. Phenylephrine (PE), Cyclopizonic acid (CPA), and Sodium Nitroprusside (SNP) were all purchased from Sigma-Aldrich, Inc., St. Louis, Missouri. Y-27632 dihydrochloride was purchased from Tocris Bioscience, R&D Systems Inc., Minneapolis, MN. CPA was dissolved in Dimethyl Sulfoxide (DMSO), while all other drugs were dissolved in modified Kreb's solution.

Tissue Preparation. On the day of the experiment, the rats were quickly euthanized with an overdose of CO_2, a method in compliance with the AVMA Guidelines on Euthanasia 2007. The thoracic aortic tissue was immediately removed and placed in a petri dish containing modified Kreb's solution. Superficial connective tissue was removed and the tissue was cut into rings approximately 3mm wide. Each tissue was carefully denuded with forceps, to remove the endothelium layer. Eight rings were then individually anchored to a stationary hook on a fixed tissue mounting bracket, as well as to a thin connecting wire which was attached to a C.B. Science Force Tranducer-302 (iWorx Systems, Inc., Dover, NH). This apparatus containing the tissue was placed into a water-

jacketed tissue chamber containing 10 mL of the modified Kreb's solution, and bubbled with a 95% oxygen / 5% carbon dioxide gas mixture. This was done to allow the tissue to be oxygenated and also to maintain pH. The chambers were heated with a constant flow of water, from a circulation/heater pump, at a temperature of 37°C.

Data Collection. The output from the force transducer was received by a data acquisition recording unit IWX-304T from the iWorx Systems, Inc., and was connected to a computer using the iWorx Systems, Labscribe2 software calibrated to record tension from the aortic rings. A resting baseline tension of about 2 g was established and the tissue was left to equilibrate in the chambers for about one hour. After the hour of equilibration, the Kreb's solution in the chambers was replaced with 9ml of fresh Kreb's solution. The 2 g baseline tension was then reestablished for about 3-5 minutes then data recording began. Aortic rings were stimulated to contract by the addition of PE to the tissue chambers at a final concentration of 10^{-4} M. After about 20 minutes, a relaxing agent was used, determined by the appropriate experimental design below, and the recording continued for about another 20 minutes to assess the effects of the agent on tissue tension.

Experimental Design.

Experiment 1: CPA/ Vehicle. Each experimental day one aorta was removed from either the sham or an ovarectomized (OVX) rat in either strain. Figure 3 is a timeline schematic of the CPA experiment. PE was added to all four chambers, to stimulate contraction in the aortic smooth muscle. The tissue maintained contraction for about 2-3 minutes, as indicted by the first arrow. Then 30µl of CPA (final concentration of 10^{-4} M) was added

to two chambers and DMSO (control) was added to the other two chambers. The tissue in theses chambers remained contracted for about a 20 minute period. SNP, the nitric oxide donor, was then delivered to all 4 of the chambers at a final concentration of 10^{-3} M to relax the tissues.

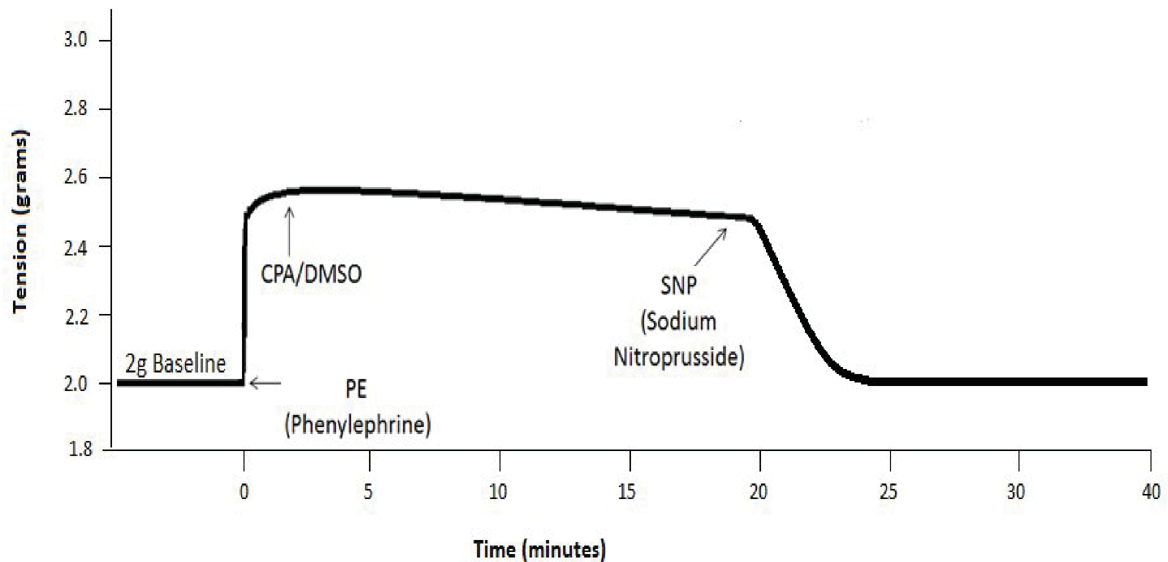


Figure 3: Schematic timeline of aortic tissues treated with CPA/DMSO. A two gram baseline tension was first established. PE induced contraction (final concentration of 10^{-4} M). 30µl of CPA or 30µl of the vehicle DMSO was added (final concentration of 10^{-4} M). Then tissue was relaxed with SNP (final concentration of 10^{-3} M).

Experiment 2: Y-27632/ Vehicle. The same general experimental design outlined above was followed; however once the PE was added 20 minutes of sustained contractions were maintained, then the Rho-kinase inhibitor (Y-27632) was added. Figure 4 is a timeline schematic of the Y-27632 experiment. Two chambers received 30µl of Y-27632 (at a final concentration of 10^{-5} M) and the other two received the vehicle, buffer, as shown indicated by the arrow. This was done in order to relax the tissue for about 20 minutes.

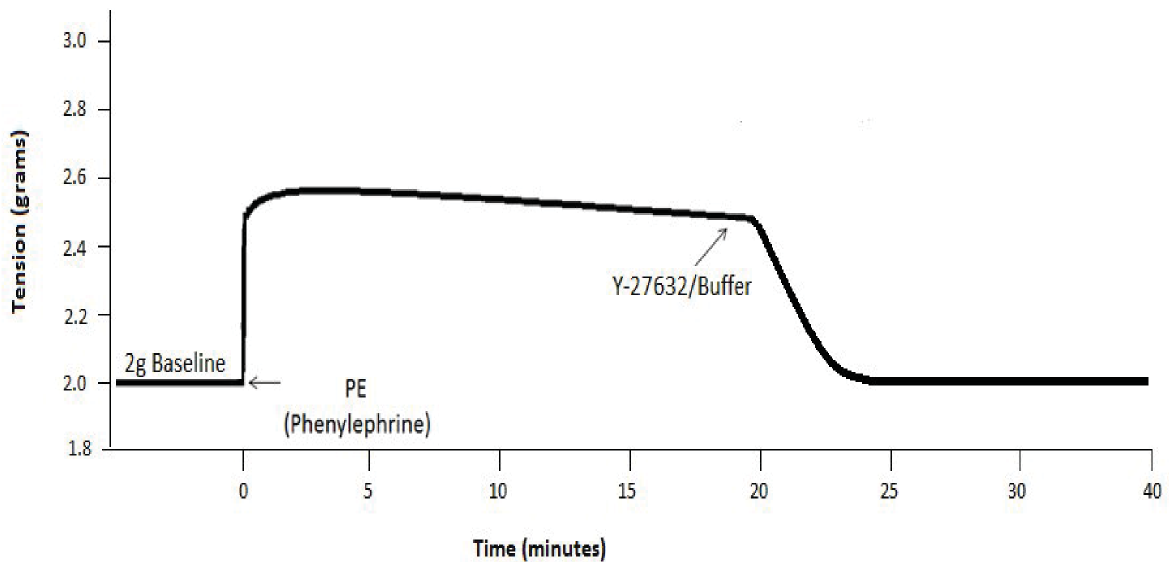


Figure 4: Schematic timeline of aortic tissues treated with Y-27632/buffer. A two gram baseline tension was first established. PE induced contraction (final concentration of 10^{-4} M). 30µl of Y-27632 or 30µl of the vehicle buffer was added to relax the tissue (final concentration of 10^{-5} M).

Data analysis. Raw data, in grams, for values measuring changes in tension during contraction and relaxation were kept in an Excel spreadsheet and was analyzed with the Sigma Plot statistical analysis software. Experimental chambers with technical difficulties and aortic rings that did not contract with PE were excluded from the study. The total peak tension after the addition of PE, the percent relaxation after the addition of SNP/ Y27632, and rate of relaxation were analyzed for all treatment groups. Means, standard deviations, and standard errors were determined for all groups. Differences between groups were analyzed by analysis of variance and Post-Hoc multivariate comparisons were determined by the Student-Newman Keuls test. The number of rings used for analysis is given for each treatment group and are found within the bars.

Results

Data collected from aortic rings, treated with CPA, isolated from SHR and WKY rats, are shown in figures 5-10. Figure 5 shows the initial peak contraction per tissue mass between the WKY and SHR strains, measured in grams. The data obtained from the SHAM and OVX aortic rings of both strains were combined and analyzed before either CPA or Y-27632 treatments were added. Figure 5 illustrates there was a significant decrease in contractile tension for tissue isolated from SHR when compared to the control WKY strain ($p<0.001$), showing SHR strain contracted less when lacking the hormone. Figure 5, also shows there was also a significant decrease found within SHR as compared to the WKY strain ($p<0.001$), resulting in the SHR contracting less as a result of the ovariectomy. A significant increase in tension was found within the WKY strain ($p=0.002$), when the hormone was removed, as seen in figure 5. This shows that WKY strain contracted more even when the hormone was removed. And finally, a significant decrease in tension found in figure 5, within the SHR strain when the hormone was absent ($p=0.034$) compared to the SHAM group.

Once the CPA was added, the aortic rings remained contracted until the relaxing agent SNP was administered (final concentration of 10^{-3} M). This was done to determine whether the effects of CPA alone had any effect on tension. Therefore, the percent differences in tension were calculated from the time right after the CPA was added, to right before the addition of SNP. In figure 6, there was a significant decrease in tension due to the drug's effect within WKY strain, in the OVX group as compared to the SHAM ($p< 0.001$). Figure 6 also shows that after CPA was added, within the SHR OVX, the tension remained relatively constant, as compared to the significant decrease in tension

found in the WKY OVX. Not shown is the percent tension difference in the aortic rings after the delivery of the vehicle DMSO (final concentration of 10^{-3} M), because no significance was found in either strain when the hormone was absent, indicating the presence of vehicle did not influence contraction. Also not shown are the sham groups for both strains, neither group showed significance in the percent tension difference upon same protocol administered with the treatment of CPA and vehicle.

Upon the addition of SNP, the data was recorded and calculated the decrease in tension of the rings during relaxation. The percent relaxation for the CPA experiment is summarized in figures 7-9. Figure 7 shows hormonal differences within the SHR strain. Although this data was not found to be significant, a trend in the data suggested, that the addition of CPA, inhibited the relaxation in both the sham and OVX groups (p= 0.079, and p= 0.066, respectively). Figure 8 displays percent relaxation differences between the strains. There was no significant difference found in the two strains among the sham group (p= 0.106). However, there was a significant decrease in tension for the SHR in the OVX group, when treated with the drug CPA before relaxation (p= 0.011). This shows that CPA inhibited the SHR strain from relaxing completely and CPA had no effect on relaxation in any the SHAM aortas. In figure 9, the control for the previous figure, showed percent relaxation differences between the strains when the vehicle DMSO was administered. For this data, there was no significant difference between either the sham or OVX groups (p= 0.333 and p= 0.077, respectively).

The slopes were also calculated to measure the rate of relaxation. However, none of the data from any of the experiments was found to be significant. Figure 10 shows the

slope, which corresponds with the data from figure 7, showing no significance in the rate relaxation between SHAM and OVX groups within the SHR strain.

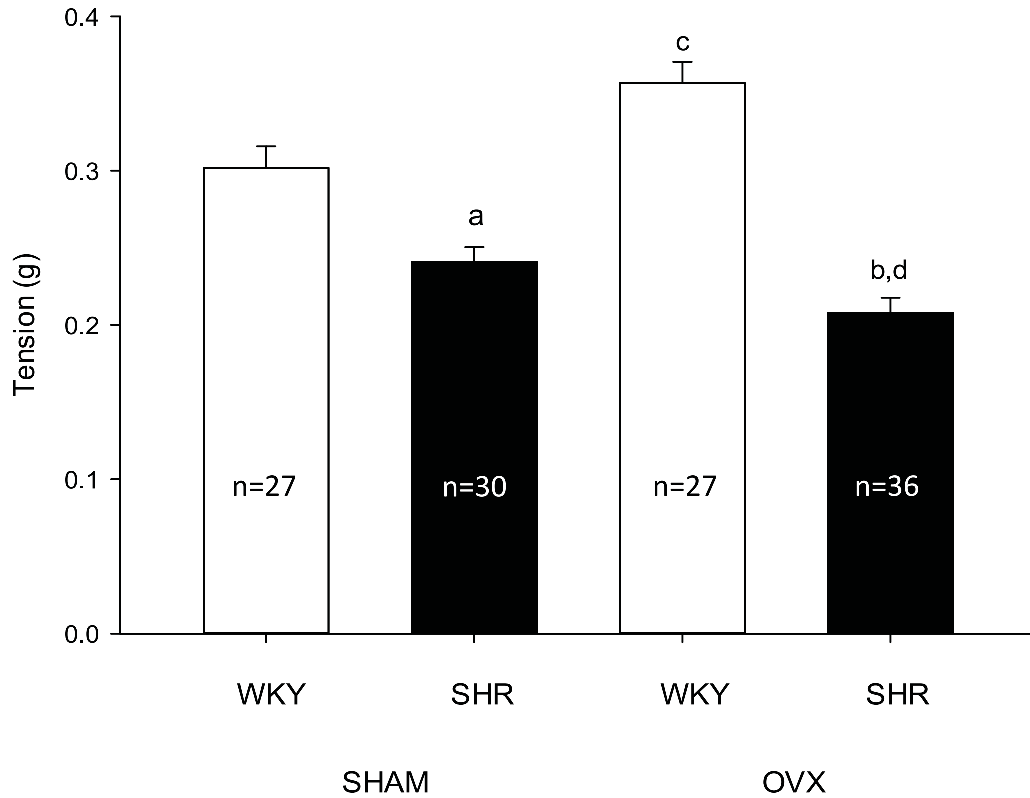


Figure 5: Display of calculated initial contraction per tissue weight in grams for control and experimental rat strains for both experiments, before either drug was added. (a.) Significant decrease in tension found within the control SHAM group for the SHR strain as compared to the WKY ($p<0.001$). (b.) Significant decrease found within SHR strain for the OVX group as compared to the control WKY ($p<0.001$). (c.) Significant increase in tension found within the OVX group as compared to the control SHAM WKY group ($p=0.002$). (d.) Significant decrease in tension found within the SHR strain for the OVX group as compared to the control SHAM SHR group ($p=0.034$). Values given are mean ± SEM for each treatment group. The number of aortic rings used for analysis is given for each treatment is found within the bars.

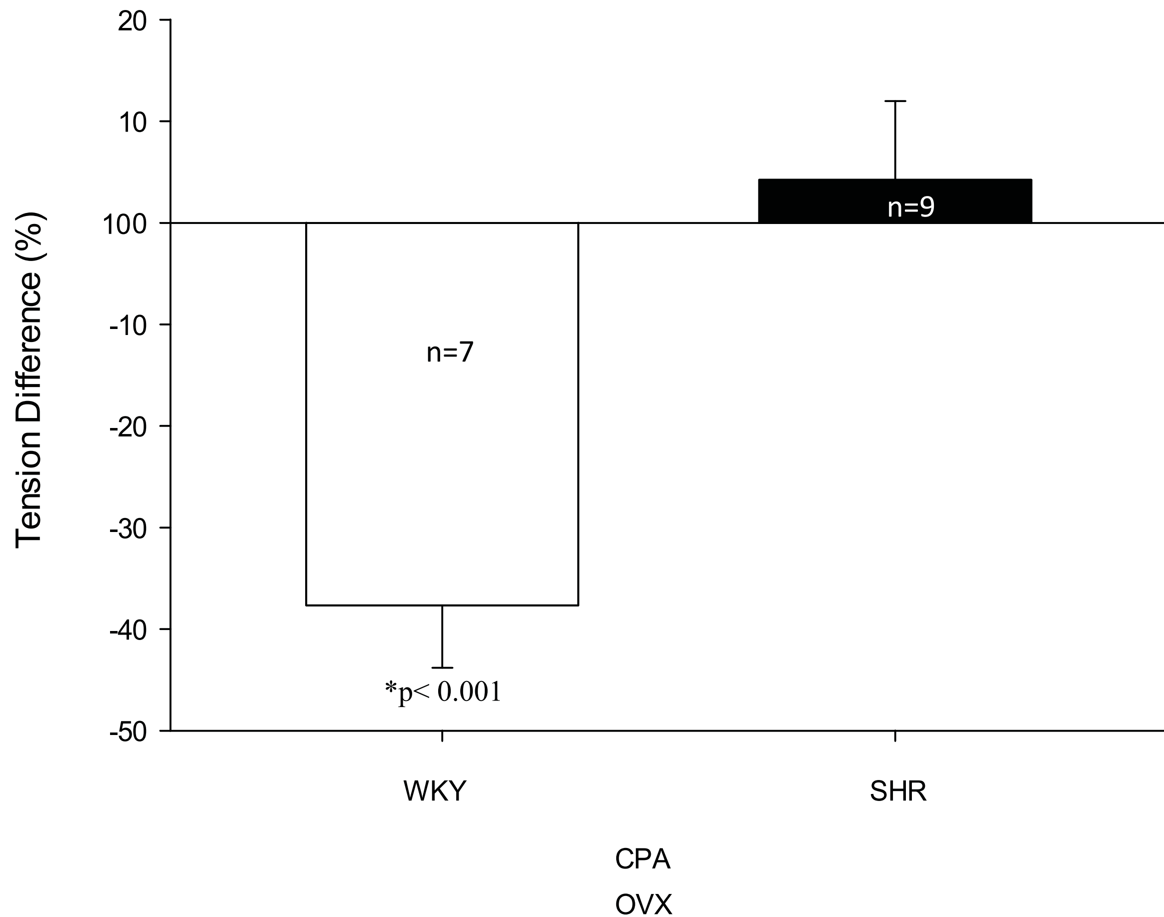


Figure 6: Display of calculated percent tension change based on the effects of CPA in grams for control and experimental rat strains for the OVX group. A significant decrease in tension was found due to the drug's effect within WKY OVX during the CPA treatment ($p < 0.001$). Values given are mean ± SEM for each treatment group. The number of aortic rings used for analysis is given for each treatment is found within the bars.

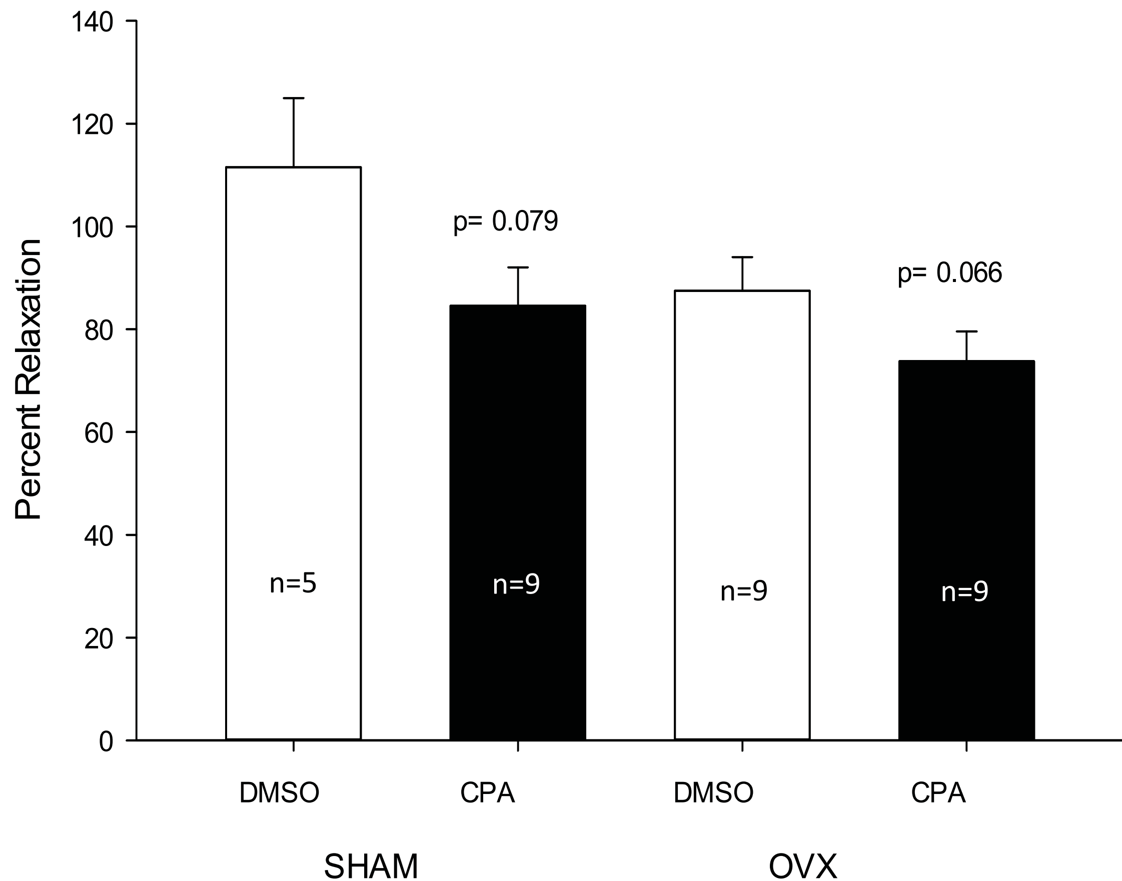


Figure 7: Display of calculated percent relaxation of CPA control experiment for experimental rat strain SHR. There was trend in the SHAM for the CPA treated group showing a decrease in percent relaxation compared to the control DMSO treated group ($p=0.079$). There was also an apparent trend in the OVX group showing a decrease in percent relaxation in the group treated with CPA as compared to the DMSO treated group ($p=0.066$). Values given are mean $\pm$ SEM for each treatment group. The number of aortic rings used for analysis is given for each treatment is found within the bars.

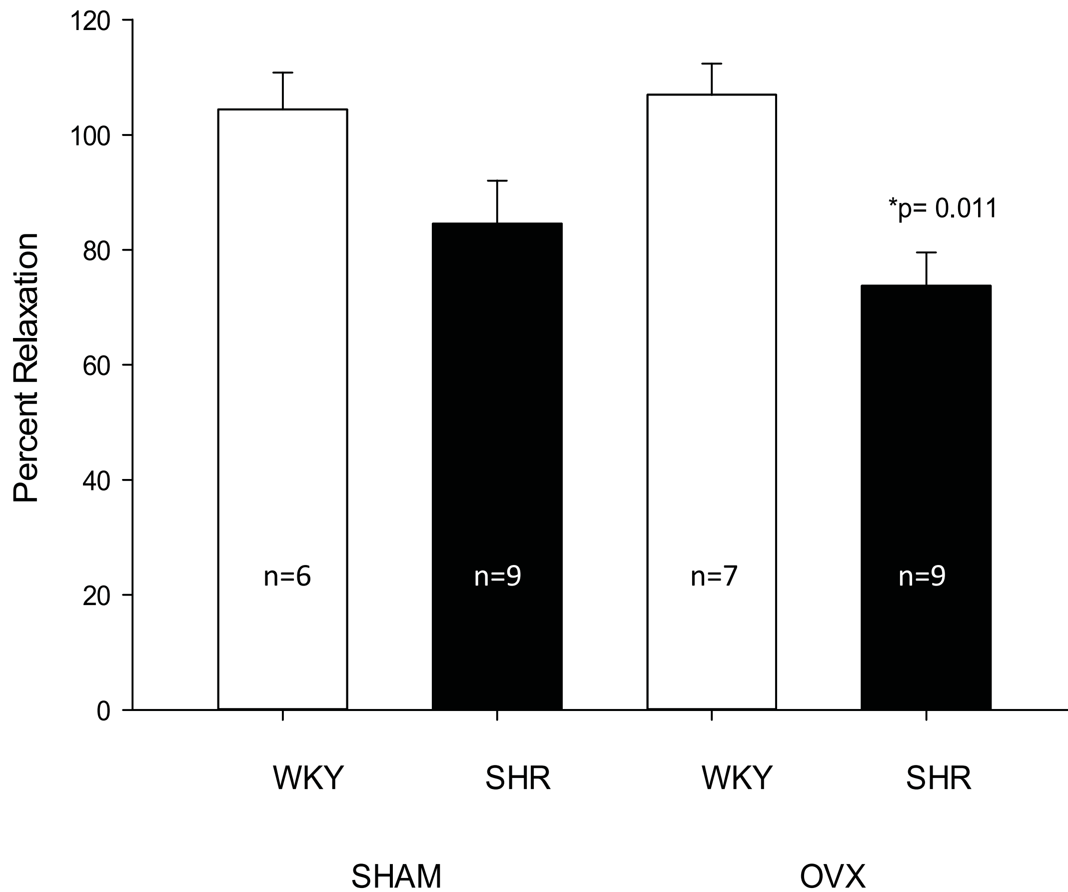


Figure 8: Display of calculated percent relaxation of CPA experiment for control and experimental rat strains. Significant decrease in the amount of relaxation was found within the experimental OVX group for the experimental SHR strain as compared to the control strain WKY ($p=0.011$). Values given are mean $\pm$ SEM for each treatment group. The number of aortic rings used for analysis is given for each treatment is found within the bars.

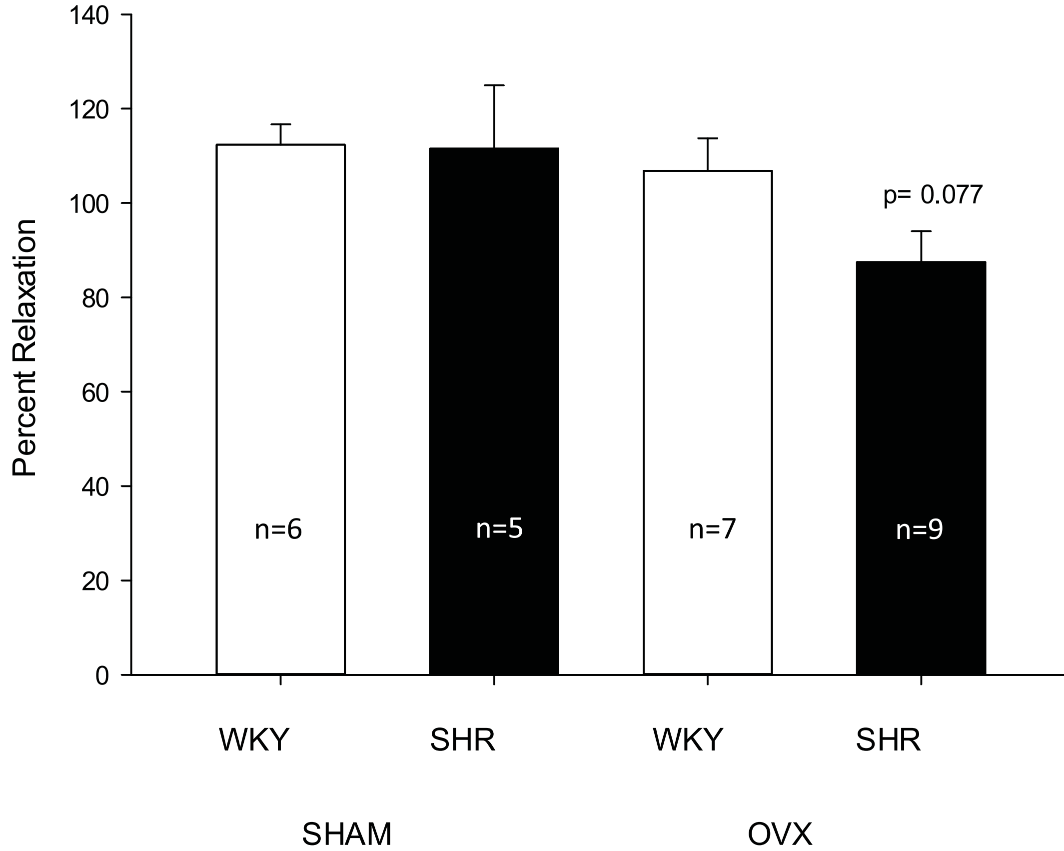


Figure 9: Display of calculated percent relaxation of DMSO control experiment for control and experimental rat strains. There was no significance found within the experimental OVX group for the experimental SHR strain as compared to the control strain WKY. However, there was an apparent trend of SHR OVX group showing a decrease in percent relaxation compared to WKY strain (p=0.077). Values given are mean $\pm$ SEM for each treatment group. The number of aortic rings used for analysis is given for each treatment is found within the bars.

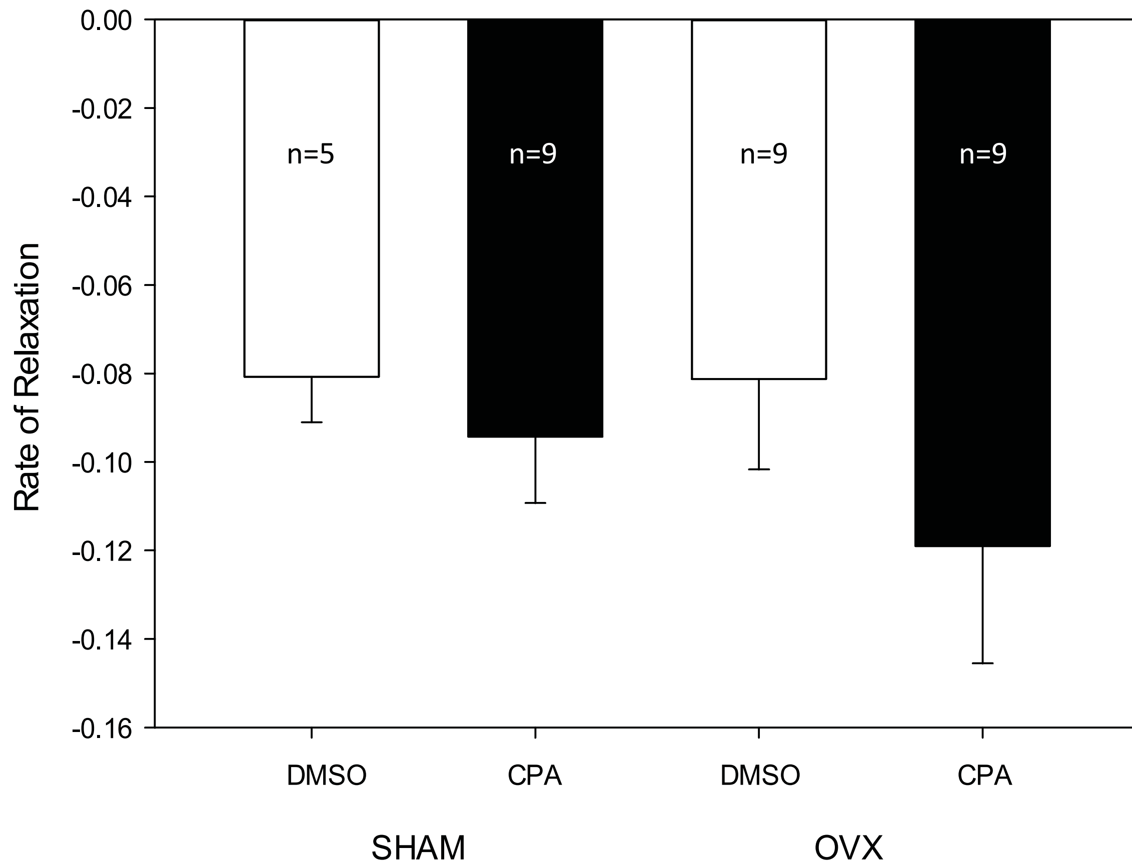


Figure 10: Display of calculated slope of the effects of CPA for experimental SHR strain. No significant difference was found between the SHAM and OVX groups. Values given are mean $\pm$ SEM for each treatment group. The number of animals for each treatment is found within the bars. The number of aortic rings used for analysis is given for each treatment is found within the bars.

Data collected from aortic rings, treated with Y-27632 isolated from SHR and WKY rats are shown in figure 11. The protocol was different from the CPA drug treatment groups because Y-27632 inhibits Rho-kinase and leads to smooth muscle relaxation. The data presented in figure 9 represents the percent relaxation calculated from the tension in grams remaining in the rings after Y-27632 was added. Figure 11 illustrates that within the SHR strain, ovariectomy significantly inhibited aortic smooth muscle relaxation ($p= 0.001$). In contrast, ovariectomy did not significantly inhibit relaxation in aortic tissue isolated from WKY rats.

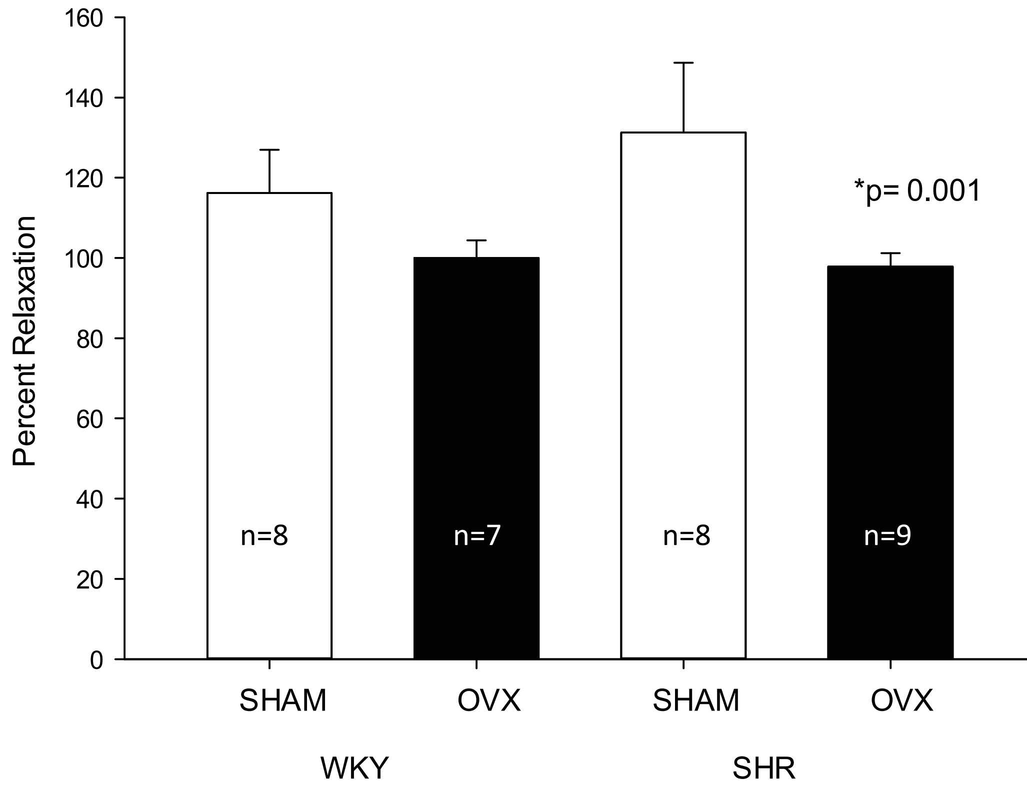


Figure 11: Display of calculated percent relaxation after 10 minutes. Percent relaxation was significantly inhibited in ovariectomized SHR treated with Y-27632 (p=0.001) compared to SHAM SHR. Values given are mean ± SEM for each treatment group. The number of aortic rings used for analysis is given for each treatment is found within the bars.

Discussion

The present study examined the differences in cellular mechanisms that regulate aortic smooth muscle relaxation within the SHR strain. This animal model was used because this stain of rat becomes predisposed to developing hypertension at about 5-6 weeks after birth. Spontaneously hypertensive rats are ideal for observing the effects of hypertension in various molecular mechanisms (Amenta et al., 2010), for instance, during the activation of relaxation mechanisms, such as the SR Ca^{2+}-ATPase pump and the Rho-kinase pathway as in this study. Also, ovarian hormones in females have been reported to have protective effects against various cardiovascular pathologies (Johansson, 1999). Results of the present study demonstrate that the SR Ca^{2+}-ATPase pump and Rho-kinase mechanisms are altered in aortic smooth muscle isolated from SHR. Furthermore, our results suggest that estrogen exerts direct actions on these cellular mechanisms during contraction/relaxation of this tissue.

Our results also demonstrated that relaxation was significantly inhibited in aortic tissue isolated from SHR that were ovariectiomized and treated with CPA when compared to ovariectiomized WKY control rats (Figure 6). These results suggest that the SR Ca^{2+}-ATPase pump mechanism responds differently in SHR in the absence of estrogen than the WKY controls. This is apparent, from the study by Kwan et al. (1994) that the SHR strain is more sensitive to the effects of CPA than the WKY strain. Also, Levitsky et al. (1993) reported in their study that Ca^{2+}-ATPase pump activity in SHR was found to exceed that of the WKY and that thoracic aortas of both strains do differ structurally. These studies further support the previous data that the SHR strain does indeed differ structurally in Ca^{2+} handling than the WKY. However, because the

endothelium in the aortic rings was removed prior to the experiments; it is unclear to what extent estrogen effects vascular smooth muscle when it relates to Ca^{2+} reuptake into SR. The endothelium is where estrogen has been found to exert many of its primary relaxing effects, causing an indirect effect on mechanisms within vascular smooth muscle. The study by Hill and Muldrew (2014) supports this by reporting estrogen's primary effects on the endothelium are found by upregulating SR Ca^{2+}-ATPase pump expression and aide in the reduction of cytosolic Ca^{2+} levels. Furthermore, as reported in Tostes et al. (2003) estrogen mainly exhibits its direct effects on vascular smooth muscle thru activating K^{+} channels and the blocking of Ca^{2+} channels in vascular smooth muscle, ultimately leading to relaxation via decrease in cytosolic Ca^{2+} concentrations. And lastly in a study by Townsend et al. (2010) reported that there was no evidence that estrogen's exposure on the SR affects Ca^{2+} reuptake into the SR. However, all these finding taken together allows us to further conclude that SHR relies heavily on the actions of the SR Ca^{2+}-ATPase pump for the removal of Ca^{2+} out of the cytosol. Estrogen is linked to this mechanism, however at present it is unclear how estrogen is working. Further testing would be needed to clarify estrogens actual role in the mechanism.

Our results also demonstrated that the Rho-kinase pathway is more sensitive to estrogen in SHR. We reported (Figure 9) that aortic tissue isolated from ovariectomized SHR relaxed less than control rats when treated with the Rho-kinase inhibitor, Y-27632. This suggests estrogen may be maintaining the Rho-kinase pathway during relaxation further indicating that estrogen may play an important role in regulating the activity of Ca^{2+}sensitization in the Rho-kinase pathway of intact animals. This is supported in the

study by Chrissobolis et al. (2004) which reported that after ovariectomy, vasodilation responses were less than the rats left intact when treated with Y-27632. Although, the study by Chrissobolis et al. (2004) focused on sex differences within vessel diameter of Sprague Dawley rats, estrogen's effects were clearly altered, in the presence of the Y-27632. However, it is unclear whether the differences between the Sprague Dawley rats and SHR, could account for the discrepancies in estrogen activity. In another study by Hiroki et al. (2004) it was found that estrogens inhibit Rho-kinase mRNA expression through estrogen receptor-dependent transcriptional mechanisms. While, their study induced contraction through angiotensin II in human coronary tissue and our study used PE on rat aortas; it still supports the notion that estrogens effects are mediated through ERα, the classic receptor-dependent mechanisms. Taken together, this allows us to conclude that estrogen is involved in Rho-kinase function within SHR. However, the exact role(s) this hormone plays is inconclusive.

Finally, our study also demonstrated, estrogen's effect during contractions and that the SHR handles Ca^{2+}differently than the WKY. We found estrogen plays a key role in contraction pathways in the SHR. Our results illustrate that SHR aortic rings contracted less than WKY control, with or without ovaries (Figure 3). In addition, SHR, lacking the hormone, contracted significantly less than SHR with intact ovaries. This indicates estrogen does play a substantial role during contraction in SHR, as well as Ca^{2+} regulation is greatly reduced when estrogen is not present. However, in contrast to our results, Asano and Nomura (2001) reported that SHR rats have been reported to have defects in Ca^{2+} maintenance mechanisms like those caused by increased L-type Ca^{2+} channel activity. This leads to large Ca^{2+} influxes and higher cytosolic Ca^{2+} concentrations as compared to the WKY strain. However, the previous study did not focus on the role estrogen plays on smooth muscle during contraction. Also, as reported in Kahil (2013) contraction stimulated by estrogen was found to be mediated through endothelium releasing factors that initiate contractions. Therefore, it is unclear how estrogen directly affects vascular smooth muscle during contraction. Taken together these results suggest that SHR are structurally different and deficient in Ca^{2+} handling and estrogen's role during contractions may be facilitatory.

In summary, our results demonstrate that the cellular mechanisms that regulate contraction and relaxation in aortic smooth muscle are altered in SHR and were also significantly affected by ovariectomy. In terms of contraction in SHR, contractile mechanisms stimulated via α adrenergic receptors are impaired and ovariectomy further suppressed these contractile mechanisms. These results suggest that estrogen plays an important role in maintaining these contractile mechanisms in spontaneous hypertensive

animals. In terms of relaxation, our results demonstrated that CPA and Y-27632 treatment, significantly inhibited relaxation of aortic rings from ovariectomized spontaneous hypertensive rats. Together, these results suggest: (1) estrogen plays an important role in maintaining the function of the SR Ca^{2+}-ATPase pump; and (2) estrogen has a facilitatory role in maintaining the integrity of the Ca^{2+}desensitization pathway (Rho-kinase – myosin phosphatase interaction) in spontaneous hypertensive rats.

Further experiments using pharmacological modification of different transduction pathways can aide in better investigating the precise roles played by estrogen in modulating the complex mechanisms that regulate vascular smooth muscle activity. This study elucidates the need for the continual research into areas such as sexual differences in the cellular mechanisms that regulate smooth muscle function. Estrogen's clear effects on these cellular mechanisms in this study, further demonstrates its biological significance within females. With the increased prevalence of cardiovascular disorders seen in recent years (Philpott, 2014), studies similar to the present experiments could potentially result in better treatments for cardiovascular conditions such as hypertension within females. Furthermore, investigations of the various intracellular mechanisms for Ca^{2+} reuptake and Ca^{2+} desensitization and understanding dysfunctions in these pathways are key to better understanding the development of hypertension and other cardiovascular diseases in the future.

References

Amenta, F., Tayebati, S., Tomassoni, D. Spontaneously Hypertensive Rat Neuroanatomy: Applications to Pharmacological Research. *Italian Journal of Anatomy and Embryology*. 2010;115.1-2:13-17.

Asano, M., Nomura, Y. Calcium Buffering of Resting Voltage-Dependent Ca^{2+} Influx by Sarcoplasmic Reticulum in Femoral Arteries from Spontaneously Hypertensive Rats at Prehypertensive Stage. *Hypertens Res.* 2001;24.3:271-282.

Carvajal, J., Germain, A., Huidobro-Toro, J., Weiner, C. Molecular Mechanism of cGMP-Mediated Smooth Muscle Relaxation. *Journal of Cellular Physiology*. 2000;184.3:409-420.

Casteels, R., Kitamura, K., Kuriyama, H., Suzuki, H. Membrane Properties of Smooth-Muscle Cells of Rabbit Main Pulmonary-Artery. *Journal of Physiology-London*. 1977;271.1:41-61.

Chrissobolis, S., Klaudia, B., Marley, P., Sobey, C. Evidence That Estrogen Suppresses Rho-Kinase Function in the Cerebral Circulation InVivo. *Stroke*. 2004;35:2200-2205.

Chitaley K., Webb, R. Nitric Oxide Induces Dilation of Rat Aorta via Inhibition of Rho-Kinase Signaling. *Hypertension*. 2002;39.2:438-442.

Cornwell, T., Pryzwansky, K., Wyatt, T., Lincoln, T. Regulation of Sarcoplasmic Reticulum Protein Phosphorylation by Localized Cyclic GMP-dependent Protein Kinase in Vascular Smooth Muscle Cells. *Molecular Pharmacology*. 1991;40.6:923-931.

Denniss, S., Jeffery, A., Rush, J. RhoA-Rho Kinase Signaling Mediates Endothelium- and Endoperoxide-Dependent Contractile Activities Characteristic of Hypertensive Vascular Dysfunction. *American Journal of Physiology-Heart and Circulatory Physiology*. 2010;298.5:H1391-H1405.

Dimopoulos, G., Semba, S., Kitazawa, K., Eto, M., Kitazawa, T. Ca^{2+} Dependent Rapid Ca^{2+} Sensitization of Contraction in Arterial Smooth Muscle. *Circulation Research*. 2007;100.1:121-129.

Fernandez, T., Porras-Gonzalez, C., Castellano, A., del Valle-Rodriguez, A., Lopez-Barneo, J. Metabotropic Regulation of RhoA/Rho-Associated Kinase by L-type Ca2+ Channels New Mechanism for Depolarization-Evoked Mammalian Arterial Contraction. *Circulation Research*. 2011;8.11;1348-U130.

Floyd, R., Wray, S. Calcium Transporters and Signaling in Smooth Muscles. *Cell Calcium*. 2007;42.4-5;467-476.

Freay, A., Curtis, S., Korach, K., Rubanyi, G. Mechanisms of Vascular Smooth Muscle Relaxation by Estrogen in Depolarized Rat and Mouse Aorta. *Circulation Research.* 1997:81;242-248.

Foucrier, J., Bassaglia, Y., Grand, M., Rothen, B., Perriard, J. Prosomes Form Sarcomere-Like Banding Patterns in Skeletal, Cardiac, and Smooth Muscle Cells. *Experimental Cell Research*. 2001:266.1;193-200.

Harris, K., Matthews, K. Interactions between Autonomic Nervous System Activity and Endothelial Function: A Model for the Development of Cardiovascular Disease. *Psychosomatic Medicine*. 2004:66.2;153-164.

Härtel, F., Rodewald, C., Aslam, M., Gündüz, D., Hafer, L., Neumann, J., Piper, H., Noll, T. Extracellular ATP Induces Assembly and Activation of the Myosin Light Chain Phosphatase Complex in Endothelial Cells. *Cardiovascular Research*. 2007:74.3;487-496.

Hilgers, R., Webb, R. Molecular Aspects of Arterial Smooth Muscle Contraction: Focus on Rho. *Experimental Biology and Medicine. 2005:*230.11;829-835.

Hill, B., Muldrew, E. Oestrogen Upregulates the Sarcoplasmic Reticulum Ca^{2+} ATPase Pump in Coronary Arteries. *Clinical and Experimental Pharmacology and Physiology.* 2014:41;430-436.

Hiroki, J., Shimokawa, H., Mukai, Y., Ichiki, T., Takeshita, A. Divergent Effects of Estrogen and Nicotine on Rho-kinase Expression in Human Coronary Vascular Smooth Muscle Cells. *Biochemical and Biophysical Research Communications.* 2004:326.1;154-159.

Johansson, B. Hypertension mechanisms causing stroke. *Clinical and Experimental Pharmacology and Physiology*. 1999:26.7;563–565.

Khalil, RA. Estrogen, vascular estrogen receptor and hormone therapy in postmenopausal vascular disease. *Biochemical Pharmacology.* 2013*:*86;1627–1642.

Kwan, C., Chaudhary, R., Zheng, X., Ni, J., Lee, R. Effects of Sarcoplasmic Reticulum Calcium Pump Inhibitors on Vascular Smooth Muscle. *Hypertension.* 1994:23;I156-I160.

Laport, R., Hui, A., Laher, I. Pharmacological Modulation of Sarcoplasmic Reticulum Function in Smooth Muscle. *Pharmacological Reviews*. 2004:56.4; 439-513.

Large, W., Saleh, Sohag N., Albert, A. Role of Phosphoinositol 4,5-Bisphosphate and Diacylglycerol in Regulating Native TRPC Channel Proteins in Vascular Smooth Muscle." *Cell Calcium*. 2009:45.6;574-582.

Lee, M., Li, L., Kitazawa, T. Cyclic GMP causes Ca^{2+} Desensitization in Vascular Smooth Muscle by Activating the Myosin Light Chain Phosphatase. *Journal of Biological Chemistry*. 1997:272.8;5063-5068.

Levitsky, D., Clergue, M., Lambert, F., Souponitskaya, M., LeJemtel, T., Lecarpetier, Y., Lompre, A. Sarcoplasmic Reticulum Calcium Transport and Ca^{2+}- ATPase Gene Expression in Thoracic and Abdominal Aortas of Normotensive and Spontaneously Hypertensive Rats. *The Journal of Biological Chemistry.* 1993;268.11;8325-8331.

Lindsey, S., Chappell, M. Evidence that the G Protein-Coupled Membrane Receptor GPR30 Contributes to the Cardiovascular Actions of Estrogen. *Gender Medicine*. 2011:8.6;343-354.

Mershon, J., Baker, R., Clark, K. Estrogen increases iNOS expression in the ovine coronary artery. *American Journal of Physiology: Heart and Circulatory Physiology.* 2002:283.3;H1169-H1180.

Meyer, M., Prossnitz, E., Barton, M. The G protein-coupled estrogen receptor GPER/GPR30 as a regulator of cardiovascular function. *Vascular Pharmacology.* 2011:55.1-3;17-25.

Murphy, R. Myosin Phosphorylation and Crossbridge Regulation in Arterial Smooth-Muscle- State-Of-The-Art Review. *Hypertension*. 1982:4.3;3-7.

Nesbitt, S.,Victor, R. Pathogenesis of Hypertension in African Americans. *Congestive heart failure (Greenwich, Conn.)*. 2004:10.1; 24-29.

Neylon, C. Vascular Biology of Endothelin Signal Transduction. *Clinical and Experimental Pharmacology and Physiology*. 1999:26.2;149-153.

Ochocka, A., Grden, M., Sakowicz-Burkiewicz, M., Szutowicz, A., Pawelczyk, T. Regulation of phospholipase C-delta 1 by ARGHAP6, a GTPase-activating Protein for Rhoa: Possible Role for Enhanced Activity of Phospholipase C in Hypertension. *International Journal of Biochemistry & Cell Biology*. 2008:40.10;2264-2273.

Ogita, H., Kunimoto, S., Kamioka, Y., Sawa, H., Masuda, M. EphA4-mediated Rho Activation Via Vsm-Rhogef Expressed Specifically in Vascular Smooth Muscle Cells. *Circulation Research*. 2003:93.1;23-31.

Shen, Q., Rigor, R., Pivetti, C., Wu, M., Yuan, S. Myosin Light Chain Kinase in Microvascular Endothelial Barrier Function. *Cardiovascular Research*. 2010:87. 2;272-280.

Somlyo, A., Somlyo, AV. Electromechanical and Pharmacomechanical Coupling in Vascular Smooth Muscle. *Journal of Pharmacology and Experimental Therapeutics*. 1968:159.1;129.

Somlyo, A., Somlyo, AV. Ca^{2+} Sensitivity of Smooth Muscle and Nonmuscle Myosin II: Modulated by G Proteins, Kinases, and Myosin Phosphatase. *Physiological Reviews*. 2003:83.4;1325-1358.

Tang, D. Intermediate Filaments in Smooth Muscle. *American Journal of Physiology-Cell Physiology*. 2008:294.4;C869-C878.

Tertyshnikova, S., Yan, X., Fein, A. cGMP Inhibits IP_3-induced Ca^2 Release in Intact Rat Megakaryocytes via cGMP- and cAMP-dependent Protein Kinases. *Journal of Physiology. 1998:512.1;89-96.*

Tostes, R., Nigro, D., Fortes, Z., Carvalho, M. Effects of Estrogen on the Vascular System*. Braz J Med Biol Res. 2003:36.9;1143-1158.*

Townsend, E., Thompson, M., Pabelick, C., Prakash, Y. Rapid Effects of Estrogen on Intracellular Ca^{2+} Regulation in Human Airway Smooth Muscle*. Am J Physiol Lung Cell Mol Physiol. 2010:298;L521-L530.*

Webb, R. Smooth Muscle Contraction and Relaxation. *Advances in Physiology Education*. 2003:27.4;201-206.

Wettschureck N., Offermanns, S. Rho/Rho-kinase Mediated Signaling in Physiology and Pathophysiology. *Journal of Molecular Medicine-JMM*. 2002:80.10;629-638.

Wray, S., Burdyga, T. Sarcoplasmic Reticulum Function in Smooth Muscle. *Physiological Reviews*. 2010:90.1;113-178.