Identification of Myosin Light Chain, Myosin Light Chain Phosphatase, and Rho Kinase in the Corpus Cavernosum of the Rat

by

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ABSTRACT

Myosin light chain kinase, Myosin light chain phosphatase, and Rho kinase are three important regulatory proteins that are involved in regulating penile erection. This study focused on identifying these regulatory proteins in the resting (contracted) state of the corpus cavernosum. Rat corpus cavernous tissues were isolated and collected for protein analysis. Protein samples were then subjected to quantitative protein analysis, one dimensional gel electrophoresis, two dimensional gel electrophoresis and western blotting. The results clearly confirmed that all three regulatory proteins are expressed in this tissue. Future work will be carried out to identify these proteins during different phases of contraction and relaxation under a number of experimental conditions.

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I. Introduction

Erectile dysfunction (ED) or impotence is a sexual dysfunction characterized by the inability to develop or maintain erection of the penis. The cause of erectile dysfunction can be physiological or psychological and can be defined by diagnostic modalities such as color duplex sonography, administration of vasoactive agents into one of the two corpora cavernosa, nocturnal penile tumescence testing, and measurements of the pressure in the cavernosa (Lue et Tanagho, 1987). Erectile dysfunction may result from impairment from psychogenic, neurogenic, hormonal, vascular, cavernosal, drug induced, or a combination of these etiologic elements (Burnett, 1995). Erectile dysfunction is a widespread disorder that affects approximately 52% of men between the ages of 40 to 70 years of age (Vitezic & Pelcic, 2002). This percentage equates to an estimated 20 to 30 million men in the United States and 150 million men worldwide (Kim et al. 2002).

Many men in the United States suffer from some type of chronic systemic illness that can cause erectile dysfunction (Behrends, 2000). Damage to nerves, arteries, smooth muscle, and fibrous tissue can cause erectile dysfunction. Vascular diseases like atherosclerosis, smoking, diabetes mellitus, heart disease, hypertension, and venous leakage are all commonly associated with erectile dysfunction (Feldman et al., 1994). Results from the Massachusetts male aging study showed that the age adjusted prevalence of complete impotence was 28% in

treated diabetic patients, 39% in those with treated heart disease, and 15% in men taking antihypertensive treatment (Feldman et al., 1994).

Neurogenic disorders like neuropathies, spinal cord injury, prostatectomy, and multiple sclerosis can cause erectile dysfunction. Kidney disease can also associate with erectile dysfunction due to chronic renal failure and liver failure. Other diseases such as untreated ulcer, untreated allergies, and even untreated arthritis can cause erectile dysfunction (Feldman et al., 1994). It is estimated that 25% of erectile dysfunctions seen in patients were caused by common medicines for blood pressure, hypotensive agents, antidepressants, tranquilizers, and appetite suppressants (Korenman, 1995).

Several endocrine abnormalities, such as hyperthyroidism, hypogonadism, and hyperprolacinemia commonly cause erectile dysfunction. Interestingly enough, erectile dysfunction is also found in approximately 30% of men with chronic obstructive pulmonary disease (Jarow et al., 1998). The role of testosterone in erectile dysfunction is not clear (Carrier et al., 1994). Alcorn et al., 1999 showed that castration inhibits the relaxation of isolated corpus cavernosum following treatment of sodium nitroprusside (Nitric Oxide donor). It is clear that testosterone acts within the smooth muscle cells of the corpus cavernosum to modulate relaxation of this tissue. Testosterone replacements in castrated rats were able to restore relaxation (Alcorn et al., 1999). Greenstein et al., 1995, reported that some men continue to achieve erection even after

castration. Patients with hyperprolacinemia, frequently associated with low testosterone values, can develop low libido and erectile dysfunction by unknown mechanisms. Testosterone replacement treatment without correction of concurrent hyperprolactinaemia, does not resolve erectile dysfunction associated with hyperprolactinaemia (Carrier et al., 1994).

Erectile dysfunction can also be caused by psychological factors. These factors could be due to specific situations, such as guilt, low self esteem, loss of employment, strained relationships, and lack of sexual arousability (Cole, 1993). Overt psychiatric disorders such as depression, stress, and schizophrenia could also be contributors (Jarow et al., 1998). The Massachusetts male aging study revealed that complete impotence has been observed to increase with the severity of depression; almost 90% of severely depressed men reported complete erectile dysfunction (Feldman et al., 1994).

In the past, men have failed to report erectile dysfunction because of embarrassment and the belief that little could be done to alleviate it. The availability and marketing of new therapies for erectile dysfunction have greatly increased. The two current general methods used to treat erectile dysfunction are nonpharamacologic and pharmacologic treatments. Nonpharamcologic treatments consist of sex therapy, vacuum erection devices, penile prostheses implantation, and penile vascular surgery. Pharamacologic treatments consist of oral agents, penile injections, and intraurethral medications.

In nonpharamacologic treatments, sex therapy alone may resolve psychogenic erectile dysfunction and can be an important adjunct to other treatments for erectile dysfunction of organic and mixed organic /psychogenic origin (Lizza, et al., 1999). Vacuum erection devices may have significant adverse effects and questionable acceptability, but studies have shown that they result in erections satisfactory for intercourse in a high percentage of users (Sidi, et al., 1990). Penile prostheses are available in inflatable and noninflatable forms that approximate the action of a naturally occurring erection. Three piece inflatable penile prostheses have improved significantly in design over the last decade, and studies have reported high rates of satisfaction among users and their partners (Beutler, et al., 1984). Penile vascular surgery has shown reasonable success within the first year after surgery, but late failures occur. The procedure appears to be most efficacious in young men with erectile dysfunction resulting from pelvic or perineal trauma (Goldstein, 1986).

In pharmacologic treatments, Intracavernosal administration of alprostadil (Caverject) has reported success rates of 67% to 85% and has been used in the treatment of erectile dysfunction for several years (Engelhardt, et al., 1998). Alprostadil is injected directly into the corpus cavernosum, which will cause arteriolar smooth muscle cells to relax. This produces an erection within several minutes. Its mechanism of action is to stimulate an increase in the levels of intracellular cyclic nucleotides that cause relaxation. Several side affects were

reported such as penile pain, hematoma, and priapism (Morgentaler, 1999).

Transurethral Alporstadil (MUSE) has reported a success rate of 65% to treat erectile dysfunction. A pellet is inserted into the urethra an inch deep. The pellet dissolves and diffuses into the corpus spongiosum, which passes into corpus cavernosum. This causes arteriolar smooth muscle to relax, resulting in an erection. Erection will begin within 8 to 10 minutes and may last 30 to 60 minutes. Common side effects are aching in the penis and the testes. The urethra may also experience burning or redness due to increased blood flow to the penis (Morgentaler, 1999).

Sildenafil, Varendafil, and Tadalif are all oral phosphodiesterase type 5 inhibitors. These inhibitors are a group of new therapeutic agents that block cGMP degradation via phosphodiesterase inhibition. Prolonged cGMP enhances the effect of Nitric Oxide (NO) in inducing smooth muscle relaxation and erection (Boolell et al., 1996). Five different types of phosphodiesterase isoforms have been described and found to be present at various concentrations in human tissues. Penile erectile effects are influenced primarily by phosphodiesterase 5 in the corpus cavernosum, with phosphodiesterase 2 and 3 also being identified (Stief et al., 1996). The observation of specific phosphodiesterase isoform distribution inside human corpus cavernosum tissue, led to the study of a possible use of a potent and selective inhibitor of the cGMP-PDE5. In the treatment of male erectile dysfunction, Ballard et al. 1996 were the first to report that

sildenafil, a selective inhibitor of phosphodiesterase 5, stimulates smooth muscle cell relaxation in vitro.

Penile erection is a neurovascular event controlled by corporal smooth muscle tone. In the flaccid state, the corporal smooth muscle of cavernous arteries, helicine arteries, and trabeculae are contracted (Wagner, 1992). There are four physiological components necessary to achieve penile erection: Intact neuronal innervation, intact arterial supply, appropriately responsive smooth muscle, and intact veno-occlusive mechanism. To better understand theses physiological components of penile erection, it is important to review the anatomy of the penis.

The penis anatomy is made up of three cylindrical bodies, the corpora cavernosa and the corpus spongiosum. The corpora cavernosa is a paired structure lying lateral to the corpus spongiosum that makes up two thirds of the penis. The corpora cavernosa is composed of a series of contiguous, endothelial-lined, and sinusoidal spaces, which are referred to as corporal smooth muscle (Anderson, 2003). The corpus spongiosum contains the urethra and the head of the penis, which lies medially in the penis. The corporal bodies are covered by a dense fascia structure known as the tunica albuginea (Jarow et al., 1998).

The tunica albuginea plays an important role in penile erection. It tightly surrounds the corpus cavernosa and corpus spongiosum to trap increased blood flow during erection. The tunica albuginea that surrounds the corpora cavernosa

is a bilayered structure. It has an inner layer that is composed of circularly oriented bundles that acts as struts to support the erectile tissue. The tunica albuginea surrounding the corpus spongiosum is much thinner than the corpora cavernosa. During an erection, the corpus spongiosum is not as rigid as the corpora cavernosa. The urethra, within the corpus spongiosum must be slightly free in order for semen to pass through during ejaculation. A single deep dorsal vein and a pair of dorsal arteries are located between the tunica albuginea and Buck's fascia.

The internal pudendal artery is primarily responsible for the blood supply to the deep structure of the penis (Benoit et al., 1987). The internal pudendal runs in a curve along the dorsalateral pelvic sidewall and enters the lesser pelvis through the lesser sciatic notch. In humans, the internal pudendal gives rise to the scrotal and bulbar branches and then continues as the common penile artery. The common penile artery continues along the medial margin of the inferior ramus of the pubis. The common penile artery subdivides into three arteries; the bulbourethral artery, the dorsal artery, and the cavernosal artery.

The bulbourethral artery supplies blood to the bulbourethral gland and the urethral bulb. The dorsal artery gives off circumflex branches to supply blood to the corpus spongiosum (Breza et al., 1989). The cavernosal artery is responsible for the blood flow to the corporal bodies. At the time of an erection, these arteries will dilate and straighten, increasing blood flow to the lacunar spaces (Montorsi et

al., 1998). As the corpus cavernosum sinuses relax and fill with blood, intracavernosal pressure and volume increases. The influx of arterial blood flow increases, exceeding the capacity of the veins to drain the blood (Kim et al., 1993).

There are three sets of veins that drain the penis: superficial veins, intermediate veins, and deep veins (Moscovici et al., 1999). The venous system drains both the corpora cavernosa and the corpus spongiosum. The superficial dorsal vein drains the skin and the subcutaneous tissue superficial to Buck's fascia. The intermediate set, which is the emissary vein, the circumflex vein, and the deep dorsal vein, below Buck's fascia drains the glans and two third of the corpora cavernosa and corpus spongiosum. The deep penile vein drains the cavernosa and bulbar veins, which then empties into the internal pudendal veins.

At the time of erection, the dilation of the cavernosal arterioles and sinuses results in increased blood flow and a subsequent rise in intracavernosal pressure (ICP). This initial rise in ICP activates a veno-occlusion mechanism. This mechanism is developed by the stretching and compressing forces by expandable corpus cavernosum tissue on subtunical venules. This limits the outflow of blood and further increases the pressure inside corpus cavernosum, causing the penis to become a blood filled capacitor (Anderson and Wagner, 1995).

The bulbocavernous muscle surrounds the penile bulb at the end of corpus spongiosum, which is supplied by the deep branch of the perineal nerve. The

ischiocavernosus muscle is supplied by the perineal branch of the pudendal nerve. It covers the corpus cavernosa and proximal part of the penile shaft. At the onset of an erection, the bulbocavernosus reflex is triggered thus causing the ischiocavernosus muscles to forcefully compress the base of the blood filled corpora cavernosa and the penis. The penis becomes very rigid, with an intracavernous pressure reaching several hundred mm Hg. These muscles and erectile bodies play a significant role in penile erection.

The innervations of the penis is both autonomic (sympathetic and parasympathetic) and somatic (sensory and motor). From the neurons in the spinal cord and peripheral ganglia, the sympathetic and parasympathetic nerves merge to form the cavernous nerves, which enter the corpus cavernosum and corpus spongiosum to affect the neurovascular events during detumescence and tumescence (Dean, 2005). The parasympathetic initiates penile erection and the sympathetic inhibits penile erection. The penis is supplied with afferent sensory fibers, which coalesce to form the dorsal penile nerve. These sensory signals reach the spinal cord via the pudendal nerves. The pudendal nerve is the prime somatic nerve (Christ, 1997). The somatic nerve is primarily responsible for sensation and the contraction of the bulbocavernous and ischiocavernosus muscles.

Penile erection is initiated by sexual stimuli, including auditory, visual, and olfactory stimuli and erotic activity. The medial preoptic area and the

paraventricular nucleus of the hypothalamus and hippocampus are important integration centers for sexual function and penile erection (McKenna, 1998). The sympathetic signals exit the spinal cord through nerve routes at T11 through L2 to travel via hypogastric nerve. Parasympathetic signals exit at S2 through S4 and travel through the pelvic plexus and cavernous nerve innervating the penis, which include cholinergic and nonadrenrgic-noncholinergic (NANC) terminals. NANC releases a neurotransmitter (explained later) that mediates the dilation of cavernosal and helicine arteries.

Detumescence can be triggered either by the cessation of sexual stimuli by the sympathetic burst or orgasm and ejaculation. Detumescence occurs when the corporal smooth muscle cells and helicine arteries are contracted, repressing a decrease in arterial blood flow and a normal venous outflow. Adrenergic nerve activation and release of norepinephrine from sympathetic nerve terminals is the primary mediator of this event (Christ et al., 1990). Norepinephrine has generally been accepted as the principal neurotransmitter in the control of penile flaccidity. Norepinephrine has been shown to contract cavernosum smooth muscle in vitro in the rat (Dail et al., 1987). Endothein-1 was found to be able to contract human corpora cavernosa smooth muscle tissue (Holmquist et al., 1990). Besides these two, other neurotransmitters such as vasopressin, substance P, and calcitonin gene-related peptide have been identified in contracting corporal smooth muscle tissue (Stief et al., 1991).

Penile erection is the result of a complex balance between intracellular events and extracellular signals controlling contraction and relaxation of smooth muscle cells tone (Anderson and Wagner, 1995). Neurotransmitters that participate in erection and detumescence largely modulate smooth muscle tone through their effects on ion channels, activation of downstream second messages, and gap junctions (Christ, 1995). The maintenance of adequate calcium homeostasis is important in the regulation of smooth muscle tone. This is achieved by one of three different mechanism; influx of extracellular Ca^{2+} via voltage –gated channels, activation of membrane bound receptors, allowing influx of Ca^{2+} through receptor–opened channels, and activation of specific signaling pathways stimulated by the release of Ca^{2+} from the sarcoplasmic reticulum. Contraction and modulation of corporal smooth muscle depends on the continuous transmembrane influx of calcium and calcium sensitizing pathways (Cellek et al.,2002).

Contraction follows the activation of α-adrenergic receptors by norepinephrine, which leads to calcium mobilization. Activation of these receptors by norepinephrine leads to activation of G protein, which then leads to activation of phospholipase C. Next, phospholipase C cleaves the membrane bound phosphotidylinositol 4,5 bisphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). DAG then activates protein kinase C (PKC). PKC phosphorylation of cellular proteins, such as L-type voltage-dependent

calcium channels, K^+ channels, and inhibition of myosin light chain phosphatase all lead to increased corporal smooth muscle cell tone.

IP_3 levels induce the release of calcium from intracellular stores such as sarcoplasmic reticulum. Calcium channels on the smooth muscle cell membrane opens, leading to an influx of calcium from extracellular space (Bivalacqua et al., 2004). The elevated levels of calcium will cause Ca^{2+} to bind to calmodulin and change the latter's conformation to expose sites of interaction with myosin light-chain kinase. The resultant activation catalyzes phosphorylation of myosin light-chain and triggers cycling of myosin crossbridges with actin filaments, which produces force. Phosphorylation of light chain also activates myosin ATPase, which hydrolyzes ATP to provide energy for muscle contraction (Dean and Lue, 2005).

Smooth muscle cell reveals thin, intermediate, and thick filaments. Thin filaments are composed of actin, intermediate filaments are composed of desmin, and thick filaments are formed of myosin. Smooth muscle contraction is regulated principally by receptor and mechanical activation of the contractile proteins myosin and actin. The actomyosin cycle begins with phosphorylation of myosin by adenosine triphosphate (ATP), leading to cross-bridges between the regulatory myosin light chain globular heads and actin (Kamm & Stull, 1985). For contraction to occur, myosin light chain kinase must phosphorylate the 20kDa and 24kDa light chain of myosin, enabling the molecular interaction of myosin with

actin (Gallagher et al., 1997). Sustained maintenance for contraction depends primarily on high concentration of cytoplasmic free Ca^{2+}.

Contractility of vascular smooth muscle is not only regulated by intracellular Ca^{2+}, but also by Ca^{2+} independent mechanisms. In addition to the Ca^{2+} dependent activation of MLC kinase, the state of MLC phosphorylation is further regulated by myosin light chain phosphatase. Myosin light chain phosphatase removes the high energy phosphate from the light chain of myosin to promote smooth muscle relaxation (Somylo, 2000). There are three subunits of myosin light chain phosphatase: a 37kDa catalytic subunit (PP1c), a 20kDa subunit of unknown function, and a 110-130kDa myosin binding subunit (MYPT1). The binding of MYPT1and PP1c inhibits the enzymatic activity of MLC phosphatase, allowing the MLC to remain phosphorylated and, thereby, promoting contraction. (Ichikawa et al., 1996). Experiments have shown that activated G-proteins are involved in the signaling pathway for this Ca^{2+} sensitization because GTPYS, a nonhydrolyzable GTP analog, increases MLC phosphorylation and promotes contraction. GTPYS also demonstrated to decrease the rate of MLC dephosphoryalation, which is consistent with an overall decrease in MLC phosphatase activity (Kitazawa et al., 1991).

Contraction of smooth muscle is primarily mediated by calcium-dependent activation of MLC kinase, resulting in phosphorylation of myosin light chain and actin. The calcium independent increase in vascular smooth muscle tone, known

as calcium-sensitization, is largely mediated by activation of the small GTPase, Rho A and its downstream effector, Rho kinase (Uehata et al., 1997). Rho Kinase is a relatively large protein (160kDa- 170kDa) consisting of an amino-terminal kinase domain and a central coiled-coil domain that includes a Rho-binding domain (Nakagaea et al, 1996). RhoA/Rho kinase activity has been found to sensitize smooth muscle contractile filaments to intracellular Ca^{2+}, promoting and maintaining vasoconstriction (Somlyo & Somlyo, 2000). Subsequent experiments have shown that the small G protein RhoA and its downstream target Rho kinase play an important role in the regulation of MLC phosphatase activity (Uehata et al., 1997).

RhoA is a monomeric G-protein that can be activated by several agonists including angiotensin II (Jackson et al., 2005). Its activity is regulated by the binding of GTP, a transition facilitated by Rho-guanine nucleotide exchange factors (Rho-GERs) that enable the exchange of nucleotide to activate RhoA-GDP to RhoA GTP (Schmidt et Hall, 2002). Activated RhoA binds to Rho kinase, which then phosphorylates and inhibits regulatory myosin phosphatase target subunit-1 (MYPT1) of myosin light chain phosphatase at Thr-696 and inhibits it's activity, promoting smooth muscle contraction (Kimura et al., 1996).

Relaxation of corporal smooth muscle is essential for normal erectile function, and evidence exists to implicate neuronal and endothelial derived NO as the principal mediator of corporal smooth muscle relaxation (Hurt et al., 2002).

Other neurotransmitters such as acetylcholine and vasoactive intestinal polypeptide have the ability to relax corporal smooth muscle tissue. Numerous in vitro studies on the effect of muscarinic agonist and antagonist on strips of penile cavernosal smooth muscle from rabbits, rats, and dogs have shown that acetylcholine can influence the relaxation of penile smooth muscle tissue (Anderson et al., 1984). In vitro studies of human corpus cavernosum tissue have shown that vasoactive intestinal polypeptide has a relaxation producing effect (Adaikan et al., 1986). However, the most important physiologically active neurotransmitter during penile erection is NO (Burnett, 1995).

Various cellular processes are regulated through the release of NO from the endothelium, platelets, vascular smooth muscle cells, neurons, and other cell types (Ignarro et al, 1999). NO has many other important physiological roles, including neurotransmission, regulation of vascular tone, immunomodulatation, cell-mediated cytotoxicity against pathogens and tumor cells (Burnett, 1995; Ignarro et al. 1999; Bogdan, 2001).

The catalytic production of NO requires NO synthase (NOS), expressed in many biological tissues as three main isoforms: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Both nNOS and eNOS are coupled to Ca^{2+} and calmodulin and are the principal NOS isoforms involved in the induction of penile erection (Ignarro et al, 1990; Burnett et al, 2000; Rajfer et al, 1992). These enzymes use reduced Nicotinamide Adenine Dinucleotide

Phosphate (NADPH), flavin adenine dinucleotide, flavin mononucleotide and tetrahydrobiopterin (BH_4) as cofactors and heme as prosthetic group. These isoforms generate NO transiently and in low amounts, appropriate for cell-cell signaling, after a rise in intracellular Ca^{2+} and Ca^{2+}-calmodulin binding. The iNOS isoform is primarily expressed in immune tissue and involves alternative calcium independent mechanisms, is not perceived to be involved in normal penile erection. NO is an important mediator for erectile function through its ability to induce vasodilation and to inhibit vasoconstriction (Burnett, 1995).

Relaxation may be induced by two different pathways: (1) stimulation of the PGE1 receptor activates the adenylate cyclase enzyme, which catalyses the conversion of ATP to cAMP, leading to an increase in intracellular cAMP and stimulation of protein kinase A (PKA) and (2) Nitric Oxide. NO is released from endothelial cells, nonadrenergic- noncholinergic nerves (NANC) and cavernosal smooth muscle cells. NO is the major regulator of corporal smooth muscle relaxation. In vitro experiments have shown a relaxant effect of NO on strips of corpora cavernosa and helicine arteries from rats that have been precontracted by noradrenaline (Rajfer et al. 1992).

The most important physiological target of NO in the penis is the activation of cGMP. NO diffuses into adjacent corporal smooth muscle cells, binding to the iron atom within the heme moiety of soluble guanylate cyclase, which activates it to catalyze the conversion of guanosine triphosphate (GTP) to

guanosine 3'5'-cyclic monophosphate (cGMP) (Ignarro, 1990). The main function of cGMP is to target protein kinase G. PKG acts by phosphorylating several proteins to influence ion channel activity and contractile regulatory protein function (Bender and Beavo, 2006).

PKG phosphorylates numerous ion channels and pumps, each promoting a reduction in cytoslic calcium. PKG leads to cell membrane hyperpolarization and corporal smooth muscle relaxation via opening of potassium channels and the Na^+ / K^+ ATPase pump. PKG diminishes intracellular calcium levels by preventing influx and promoting calcium sequestration within the sarcoplasmic reticulum (Christ et al. 1999). Gap junctions, potassium channels, and calcium channels are major modulators of smooth muscle cell tone in the corpora.

Activation of potassium channels (K^+) leads to the efflux of K^+, leading to corporal smooth muscle cell hyperpolarization and relaxation. Four distinct types of K^+ channels have been identified in corporal smooth muscle; the Ca^{2+} sensitive potassium (Kca) channel, the metabolically regulated Katp channel, the voltage regulated, delayed rectifier K channel, and the fast transient A-type K current (Fan et al., 1995). Recent studies have indicated that the Kca channel and Katp channels appear to be the most physiologically relevant in the modulation of smooth muscle cell tone (Christ et al., 1997). Recent studies in man, have demonstrated that the activation of Na^+ /K^+ ATPase by NO is involved in corporal smooth muscle relaxation (Anderson & Holmquist, 1990).

Smooth muscle cell responses are coordinated through gap junctions, which are intercellular communications that allow for the transmission of electrical or chemical signals between cells (Christ et al., 1993). Gap junctions mediate vasodilatation and vasoconstriction responses along the arteriolar wall in resistance vessels through smooth muscle and endothelial cells (Segal, 1992). Gap junction proteins, primarily connexin 43 provide the anatomic substrate for coordination of cell-to-cell responses among corporal myocytes. Connexin-43 derived gap junctions appear to modulate α1- adrenergic and ET- 1-induced contractility as well as NO induced relaxation responses of corporal smooth muscle (Campos de Carvalho, 1993).

Relaxation of corporal smooth muscle follows a decrease of free Ca^{2+} in the cytosolic cell. Calmodulin is then dissociated from myosin light chain kianse, which inactivates it. Myosin is dephosphorylated by myosin light chain phosphatase and detaches from the actin filament, promoting smooth muscle relaxation (Walsh, 1991). Chitaley and Webb, 2002, suggested that the mechanism by which NO causes vascular smooth muscle relaxation involves inhibition of Rho kinase signaling. An increased production of NO is known to increase the levels of cGMP and activate PKG. PKG has been shown to phosphorylate RhoA, thus inhibiting its Rho kinase activating ability, promoting smooth muscle relaxation (Sawada et al, 2001).

The overall purpose of this research was to identify proteins that are expressed in the resting (contracted) state of the corpus cavernosum. We hypothesized that these proteins, Rho kinase, Myosin light chain, and Myosin light chain phosphatase are expressed in the resting (contracted) state of the corpus cavernosum.

II. Materials and Methods

The animals that were utilized in this experiment were housed in groups of four in plastic cages. The animals were kept on a reverse light-dark schedule cycle. The lights were off from 1000 hours to 2200 hours at room temperature of 22°C. The animals were given a continuous supply of Lab Diet Rat Chow and water. The animals in the experiment were mature Long/Evans Rats between the ages of 6 to 8 months old. All experimental procedures were conducted in accordance with standard guidelines for animal experiments and were approved by the Institutional Animal Care and Use Committee (IACUC) at Youngtown State University.

Experimental Design

Six Long-Evans Rats were euthanized to obtain corpus cavernosum tissue. Each corpus cavernosum tissue was homogenized separately to make six individual protein samples. The following techniques were utilized: A Bradford assay to determine quantity of protein, One Dimensional Gel Electrophoresis to separate proteins based on molecular weight, Two Dimensional Gel Electrophoresis to separate proteins based on molecular weight and isoelectric point. Western blot analysis was performed for specific protein identification.

A. Preparation of Tissue

On the day of tissue harvest, the animals were placed in a plastic chamber and were quickly euthanized with CO_2. The penis was removed from the animal and placed into two dimensional electrophoresis (2DGE) buffer (8.4 M Urea, 2.4 M thiourea, 5% [3-(cholamidopropyl) dimehylammoniol-1-propanesulfonate] (CHAPS), 25 mM spermine base, 50 mM dithiothreitol (DTT)). The urethra, dorsal penile vein, and remaining connective tissue were removed. Once the corpus cavernosum tissue was isolated, it was cut vertically in half down the midsection into two pieces. The tissue was cut randomly into small fragments. The small, tiny tissue fragments were then placed into a brass homogenizer along with 1200 µl of 2DE buffer. After manually homogenizing the tissue sample for 30 minutes, the tissue sample was aspirated from the brass homogenizer and placed into an eppendorf tube. The tissue samples were microcentrifuged at 14,000 rpm for ten minutes then the supernatant was transferred to another clean eppendorf tube.

1. Bradford Assay

The Bradford protein assay is a simple method commonly used to determine the total protein concentration of a sample. This method is based on the proportional binding of the dye Coomassie blue to proteins. The Bradford assay utilizes Coomassie brilliant blue dye to specifically bind proteins primarily at arginine residues. The shift in absorbance of the proteins dye binding complex

was measured by a spectrophotometer (Hewlett-Packard 8453 UV-Visible System) (Twyman R, 2004). As the protein concentration increases, the color of the test sample becomes darker. The protein concentrations were determined by the absorbance of the protein dye binding complex at 595 nm in comparison to a series of protein standards of Bovine Serum Albumin (BSA). A standard concentration curve was constructed. The standard concentration quantifies the linear relationship between the protein dye binding and protein concentration (Bradford M, 1976).

The Bradford Assay procedures included preparing standards containing 10, 15, 20, 25, 30, 35, and 40 micrograms (µg) quantities of BSA. To each test tube, 80 µl of double distilled water (ddH_20), 20 µl of 0.1 M hydrochloric acid HCl, 10 µl of 2DGE buffer, and 4 ml of Bradford dye were added. To two other test tubes, the unknown sample concentrations of protein were 15 µl and 20 µl of sample previously suspended in sample buffer, 80 µl ddH_20, 20 µl of 0.1 M HCl, 10 µl of 2DGE buffer and 4 ml of Bradford dye. A blank was prepared by the addition of 80 µl of double distilled water (ddH_20), 20 µl of 0.1 M hydrochloric acid HCl, 10 µl of 2DGE buffer, and 4 ml of Bradford dye to a test tube. After 5 minutes for room temperature incubation and a 15 minute warming period for the spectrophotometer, the solutions were transferred to plastic cuvettes. Following the blanking procedure, the absorbances of the standard and unknown samples were measured using UV-visible light at 595 nm. The R^2 values that were

obtained from the standard curve had to be greater than or equal to 0.95. The R^2 values were used to determine protein concentrations.

2. Preparation of mini SDS-PAGE gels

The mini resolving gel plates were prepared in a gel pouring chamber at a concentration of 12% acrylamide. These gels were prepared in batches of 11. The 12% gels were made by the addition of 30 ml of resolving gel buffer (90.85gr Tris, 2gr SDS, 350mls H_2O and 8.8 Titrate w/HCI), 36 ml of 40%acrylamide, 480 µl APS (Ammonium Persulfate) and 120 µl TEMED (Sigma) to 54 ml of ddH_20. 20 ml of saturated butanol (poured first) and acrylamide solution was poured between 7 cm short glass plates and 1 mm long glass plates separated by 0.5 mm plastic spacer plates. The resolving gel was poured to a level 0.5 cm lower than the top of the shorter (7cm) glass plate. These gels were allowed to polymerize overnight at room temperature. The mini gel plates were removed from the pouring chamber and stored in an air tight container at 4°C.

Before use, the resolving gel was overlaid with stacking gel. The stacking gel was made by the addition of stacking gel buffer (12.1gr. Tris, 0.8gr. SDS, Titrate to 6.8 w/HCI, and 180 ml ddH_20), 1250 µl of 40% acrylamide, 12.8 µl Temed, 50uls APS, 6.250 µl ddH_20. The stacking gel was poured between the mini gel plates (7cm & 1mm) and a 1mm ten well comb was inserted in between the gel plates to allow space for the samples.

B. SDS-PAGE

The SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) is a technique used to separate proteins according to their molecular weight. The basis of this technique is the exposure of denatured proteins to the detergent sodium dodecylsulfate SDS, which binds stoichiometrically to the polypeptide backbone and carries a large negative charge (Twyman R, 2004). The use of this ionic detergent SDS, give proteins the same charge to mass ratio, which allow the proteins to move at the same rate in an electric field. ß-mercaptoethanol is added to break disulfide bonds. Proteins are then electrophoresed in a polyacrylamide gel. Proteins with less mass travel more quickly than proteins with a greater mass.

1. One Dimensional Gel Electrophoresis: Separation by molecular weight

12% polyacrylamide mini gel plates were clamped into place in a Mini Protean 3 cell chamber (BIO- RAD). The 1 mm well comb was removed from mini gel plates. The first lane was loaded with 10 µl of ProSieve Color Protein marker (Lonza). The second lane was loaded with 20 µl of treated protein sample. The treated protein sample consisted of 20 µl of sample and 50 µl of 4X SDS. The cell chamber was filled with SDS buffer, also known as electrode buffer. The electrode buffer was made by the addition of (6.05 gr. Tris, 28.84 gr. Glycine, 2.00 gr. SDS and 2000 ml ddH_20). Both sides of the cell chamber were filled with electrode buffer to allow them to be conductive. The cell cap has a

negative (black cord) and a positive (red cord) electrode that connect to the EC570 (E-C Apparatus Corporation) power supply. The negative electrode attached to the bottom half and the positive electrode attached to the upper half of power supply. The mini gels were run at a constant current of 0.026 amps for one hour. When the mini gels were finished running, the glass plates were pried apart and the gels were either placed into coomassie blue to be stained, or used in western blotting.

2. Coomassie Blue Staining

Coomassie Brilliant Blue is an organic dye that is used to stain protein bands in polyacrylamide gels. Coomassie Brilliant Blue stain solution is composed of 0.2% Coomassie Brilliant Blue R-250, 45% methanol, and 10% acetic acid. The gels were stained overnight. The gels were destained in a high destained solution of 45% methanol and 10% acetic acid for two hours with frequent changes of destaining solution. The stained gels were dried between two sheets of dialysis membrane for further analysis.

C. Two Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis is a powerful and widely used method for the analysis of complex protein mixtures from tissue samples. 2DGE is a technique that separates proteins in two independent properties. The first-dimension is isoelectric focusing (IEF), which separates proteins according to their isoelectric points (pI in which the net charges are zero). The second-

dimension is SDS-PAGE, which separates proteins according to their molecular weights (MW) (Twyman R, 2004).

1. 1st Dimension: Passive Rehydration and Isoelectric Focusing

Upon completion of the Bradford assay, the proper concentration of sample load was determined based upon 100 µg of protein for 7 cm immobilized pH gradient gel strips. Ampholytes were precasted into the gel strip to establish the pH gradient in the gel, resulting in migration of the proteins to their isolelectic pH (pI). The present pH gradient in the gel strip eliminates problems such as gradient drift. Carrier ampholytes were used to improve protein solubility and to ensure uniform conductivity during Isoelectric Focusing (IEF). The proteins of interest isoelectric point ranges between 4.0- 6.0, so Ready IPG 4-7 pH strips were utilized. 125 µl of the protein sample was loaded into the rehydration/equilibration tray and spiked with 1.3 µl of 4-7 pH biolyte carrier ampholytes. IPG gel strips were placed gel side down in the rehydration/equilibration tray on top of protein sample. 2-3 ml of mineral oil was overlaid on top of the strips to prevent evaporation during the rehydration process.

The IPG strips rehydrated overnight (approximately 18 hours) on an orbital shaker. After 18 hours of rehydration, the IPG strips were blotted on a moistened Kim wipe to remove excess mineral oil. Before the rehydrated IPG strips were placed on the IEF tray, electrode wicks were dipped in ddH_20 and blotted on paper towel to remove excess water. The electrode wicks were placed

across the electrodes in the IEF tray. The rehydrated IPG strips were placed in the IEF tray on top of the electrode wicks. If any air bubbles were present, they were removed. The IPG strips were overlaid with mineral oil and placed into the Protean IEF cell machine. Focusing conditions were set linearly to 40,000 volt-hours with a maximum current of 50 μA per strip and a default temp of 20° Celsius. The IPG strips were held at a 500 Volts upon completion of isoelectric focusing. The entire 1st dimension process required approximately 24 hours.

2. Equilibration

Prior to running the second dimension, the IPG strips were equilibrated in SDS containing buffers I and II. This two step equilibration ensures that cysteines are reduced and alkylated, which minimizes or eliminates vertical streaking that may be visible after staining of the second dimension gels. Equilibration buffer I is composed of 6 M urea, 0.375 M Tris-HCI, ph 8.8, 2% SDS, 20% glycerol, 2% (w/v) DTT. Equilibration buffer II is composed of 6 M urea, 0.375 M Tris- HCI, ph 8.8, 2% SDS, 20% glycerol and 2.5% (w/v) iodoacetamide. Equilibration buffer I contains DTT which reduces sulfhydryl groups, while equilibration buffer II contains iodoacetamide, which alkylates the reduced sulfhydryl groups. The IPG strips were placed into an equilibration tray that was filled with buffer I, and then placed on the orbital shaker for 15 minutes. The same process was performed with buffer II.

3. 2nd Dimension: SDS

12% acrylamide gels were prepared as previously described. The resolving gels were poured to a level 1.5 cm lower than the top of the shorter (7 cm) glass plate. After the IPG strips were equilibrated, the strips were ready for 2nd dimension. Before applying the strips to the mini gel plates, an agarose overlay (5% agarose) was heated until fully liquefied and pitetted over the gel strips, while inserting the gel strip onto the mini gel plate. Timing was essential as to not allow the agarose to solidify before the gel strips was inserted.

The mini gel plates were inserted into an electrophoresis cell (Mini Protean 3 Bio-Rad). The IPG strips were then placed into the mini gel plates and topped off with agarose to prevent any leakage. The mini gel plates were then topped off with SDS buffer, also known as electrode buffer into the cell. Both sides of the cell were filled with electrode buffer to allow them to be conductive. The cell cap has a negative (black cord) and a positive (red cord) electrode that connects to the EC570 power supply. The negative electrode attached to the upper half and the positive electrode attached to the bottom half. When the dye front created by the Bromphenol Blue present in the agarose overlay had reached the bottom portion of the gels, electrophoresis was complete. The gels were run at a constant current of 0.015amps for two hours. When the gels were finished running, the glass plates were pried apart and the gels were placed into coomassie blue overnight to be stained. The next day, gels were destained in high destain for

3-4 hours with frequent changes and rinsed in ddH_20. The gels were then scanned for further analysis.

D. Western Blotting

Western blotting is performed to identify and to locate proteins based on their ability to bind to specific antibodies. Western blot analysis detects the protein of interest from other complex proteins on the membrane. Western blotting provides information about the size of the protein and the expression amount of the protein. In order to complete a Western Blot, the following steps were performed; Tissue preparation, SDS-PAGE, Electrophortic transfer of proteins, Blocking buffer, Application of primary antibodies, which were Rabbit polyclonal to Myosin light chain (Abcam), Rabbit polyclonal Myosin light chain phosphatase (Abcam), and Rabbit polyclonal to Rho kinase alpha (Abcam), Application of secondary antibody, which was Goat Anti Rabbit IgG Horseradish (Abcam), Chemiluminescent detection, and Gel analysis. Tissue preparation and SDS- PAGE were completed as stated previously.

1. Electrophortic transfer of proteins

First, the two Scotch Brite pads and the PVDF membrane soaked in methanol. The Scotch Brite pads were transfered into the Mini Protean II Tube cell that contained an inch of transfer buffer. Two pieces of filter paper were cut to the size of the Scotch Brite pads and placed into the transfer buffer to soak. The transfer buffer consisted of 4OOmls of 10X transfer buffer, 800 ml of

methanol, and 2800 ml of ddH_20. 10X transfer buffer consisted of glycine, 12 gr. TRIS, 800 ml methanol and 3200 ml ddH_20. The membrane holder was opened and a Scotch Brite pad was placed on top of the black portion of the holder. Next, one piece of filter paper was placed on top of the Scotch pad. The finished electrophoresis one dimensional gel was carefully placed on top of the filter paper, ensuring that there were no air bubbles between the layers. The methanol was removed from the PVDF membrane and was rinsed three times with ddH_20. The PVDF membrane was then placed on top of the gel. A second piece of filter paper was placed over the membrane. The second Scotch Brite pad was then placed over the filter paper. The membrane holder was carefully locked and was placed into a Mini Protean II blotting transfer cell. The transfer cell was filled with transfer buffer and the positive and negative electrodes were connected to the EC570 power supply. After blotting for two hours, at a constant voltage of 100, the proteins were transferred over to a PVDF membrane from an acrylamide gel.

2. Blocking Buffer for PVDF

After the proteins were transferred to the PVDF membranes, the membranes were placed in blocking buffer for two hours. This prevented non-specific binding of antibodies to the PVDF membranes. The buffer consisted of 10 grams powdered non-fat dry milk and 100 ml of TBS-T (Tris buffered saline) and 2% Tween 20 detergent.

3. Primary Antibodies Application

After blocking, the PVDF membranes were placed in primary antibody 1:1000 concentration with 1% blocking buffer (previously describe). The primary antibodies were either Rabbit polyclonal to Myosin light chain, Rabbit polyclonal to Myosin light chain hHS-M21 phosphatase, or Rabbit polyclonal to Rho kinase alpha (Abcam). After one hour of allowing the PVDF membranes to soak in the primary antibody at room temperature, the membranes were then placed in the refrigerator overnight.

4. Secondary Antibodies Application

Before applying the secondary antibody to the membranes, the membranes were removed from the refrigerator and set out at room temperature for an hour. After an hour, the membranes were rinsed three times, ten minutes each in TBS-T. The membranes were placed in Goat Anti Rabbit IgG Horseradish Peroxidase (HRP) conjugate secondary antibody (Abcam) for two hours at a 1:3000 concetration with 1% blocking buffer (previously describe). After membranes were done soaking in secondary, they were rinsed 3 times, ten minutes each in TBS-T and one time in TBS.

5. Chemiluminescent Detection

The detection of bound secondary antibodies was performed by the addition of a chemiluminescent substrate that would react with HRP labeled in the secondary antibody. Immun-Star HRP Chemiluminescent Substrate Kit (BIO-

RAD #1705040) consists of Luminol/Enhancer Buffer and Peroxide Buffer. The membrane soaked in 5mls of Luminol/Enhancer Buffer and 5 ml of Peroxide Buffer (combined) for five minutes. In the darkroom, the membrane was then placed in between two sheets of plastic. The Kodak autoradiography film was first placed over the membrane for a 30 seconds exposure. Depending on the chemiluminescent signal two or five minutes exposures were completed. When the developing process was completed, the photos were dried and scanned latter for analysis.

6. Gel Scanning and Analysis

Images of gels and blots were digitally scanned and computer stored for analysis. The developed x-ray films were scanned using an Epson perfection 4490 photo scanner, a Macintosh Quadra 800 computer, and Adobe Photoshop 3.0 software. The blots were scanned using the following perimeters: a gray-scale mode, a reflective original, scaled to 100%, high sharpness, general preferences, and optimum quality. The molecular weight for each western blot was manually estimated.

III. Results

The Bradford Protein assay was performed by using BSA concentration standards. The concentrations were determined by comparing samples to a standard curve of increasing concentrations of BSA, an example is seen in Table 1. Both sets of values were recorded for the construction of the linear Standard BSA curve, represented in Figure 1. The equation of the line from the graph was $Y = 0.0087X + 0.058$ and the R^2 value was 0.9912. The R^2 value was greater than 0.95, which was accepted in determining the unknown protein sample concentrations.

Unknown protein sample concentrations for15 µl and 20 µl determined by Standard BSA curve values are indicated in Table 2. Table 2 indicates the calculated sample load volumes for 7 cm IPG strips in µl based on a total volume of 125 µl. The calculated concentration loading volume samples for the 7cm strips were not sufficient enough for adequate results. Possibly due to the nature of the corpus cavernosum tissue and the cell lysis method that was performed. 125 µl of protein sample was loaded to each 7 cm IPG strip.

Figure 2 is a two dimensional gel that shows defined protein spots of the corpus cavernosum. It has successfully separated proteins by their iso-electric point and molecular weight. In the 1st dimension, the 2D gel strip was passively hydrated at 40,000 V-hours using 100 µg of protein on a 7 cm IPG strip with a pH range 4-7. The 2nd dimension was performed on a 12% polyacrylamide gel using

a BIO RAD Mini PROTEAN 3 cell with a running power of 15 mA. The gel was stained in coomassie brilliant blue. Figure 2, the pH range is increasing from left to right and the molecular weight is decreasing from top to bottom. Actin is identified at a pI of 5.00 and a molecular weight of 43,000kDa.

Figure 3 shows the results of an one dimension (SDS PAGE). Lane 1 shows the Lonza ProSieve prestained molecular weight standard on a coomassie stained 12% polyacrylamide gel. Lane 2 shows the total corpus cavernosum tissue protein distribution on a coomassie blue stained 12% polyacrylamide gel. Lane 2 also shows that there are proteins expressed in each molecular weight.

Figure 4 shows the results of a western blot analysis of corpus cavernosum tissue from Long Evans Rats. The protein of interest was Rho kinase. The primary antibody (Rabbit polyclonal to Rho kinase α) concentration was 1:1000 and the secondary antibody (Anti Rabbit Goat Peroxidase Conjugate) concentration was 1:3000. Lane 1 shows Lonza prestained molecular weight standards on a coomassie blue stained PVDF membrane. Lane 2 shows the results of western blot autoradiograph. Lane 2 shows that there is immunoreactivity observed in the 160-170kDa range. Lane 3 shows the total corpus cavernosum tissue protein distribution on a coomassie blue stained PVDF membrane.

Figure 5 shows the results of a western blot analysis of myosin light chain. The primary antibody (Rabbit polyclonal to Myosin light chain) concentration was 1:1000 and the secondary antibody (Anti Rabbit Goat Gig Peroxidase Conjugate) concentration was 1:3000. Lane 1 shows Lonza prestained molecular weight standards on a coomassie blue stained PVDF membrane. Lane 2 shows the results of western blot autoradiograph. Lane 2 shows that there is immunoreactivity observed in the 24kDa range. Lane 3 shows the total corpus cavernosum tissue protein distribution on a coomassie blue stained PVDF membrane.

Figure 6 shows the results of a western blot analysis of myosin light chain phosphatase. The primary antibody (Rabbit polyclonal to Myosin light chain hHS-M21 phosphatase) concentration was 1:1000 and the secondary antibody (Anti Rabbit Goat Peroxidase Conjugate) concentration was 1:3000. Lane 1 shows Lonza prestained molecular weight standards on a coomassie blue stained PVDF membrane. Lane 2 shows the results of western blot autoradiograph. Lane 2 shows that there is immunoreactivity observed in the 110-117kDa range. Lane 3 shows the total corpus cavernosum tissue protein distribution on a coomassie blue stained PVDF membrane.

Table 1: Example of Bradford Assay Standard Curve Concentrations and Absorbances

This table represents the concentrations of BSA in micrograms and absorbances at 595 nanometers of Ultra Violet visible light, which was used to construct a linear standard BSA curve to determine unknown protein concentrations of samples.

Table 1:

Example of Bradford Assay Standard Curve Concentrations and Absorbances

Standard BSA Concentration (micrograms)	Absorbance (nanometers)
10	0.148
15	0.187
20	0.218
25	0.283
30	0.328
35	0.351
40	0.406

Figure 1: Standard BSA Curve

This graph represents a standard BSA linear curve based upon the absorbance of standard BSA concentrations at 10, 15, 20, 25, 30, 35, and 40 µg versus the absorbance of the protein dye complex at 595 nm.

Figure 1.

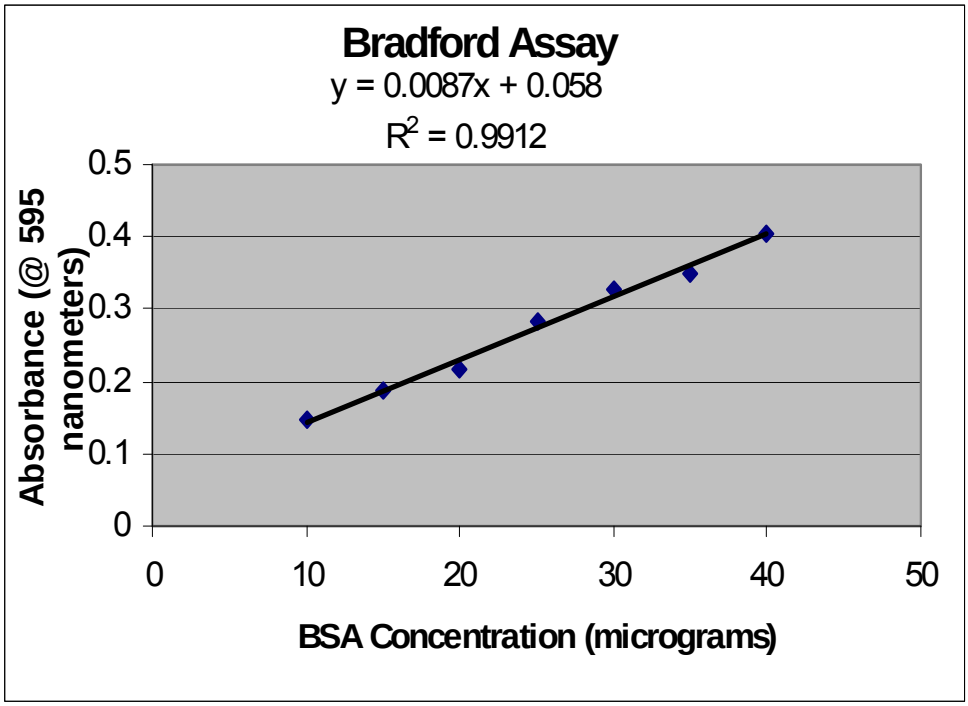

Bradford Assay
y = 0.0087x + 0.058
R2 = 0.9912
Absorbance (@ 595 nanometers)
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
BSA Concentration (micrograms)

Table 2: Protein Concentrations of gels

This table represents unknown sample protein concentrations in micrograms/microliters and absorbances at 595 nanometers determined by Standard BSA Curve values. The total load volume was 125 µl for 7 cm IPG strips. The calculated sample load volume and the actual sample load volume are indicated in the table.

Table 2.

Protein Concentrations of gels

Unknown Sample	Absorbance @ 595 nm	Concentration (micrograms)	Conentration (micrograms/ microliters)	Calculated sample load volume	Actual sample load volume
15ul	0.294	27.1	1.8	69.1	125.0
20ul	0.367	35.5	1.8	70.4	125.0

Figure 2: Two Dimensional Gel Electrophoresis

This is a 12% polyacrylamide coomassie blue stained gel that represents the total protein distribution in the corpus cavernosum sample prepared by two dimensional electrophoresis (IEF/SDS-PAGE).

Figure 2.

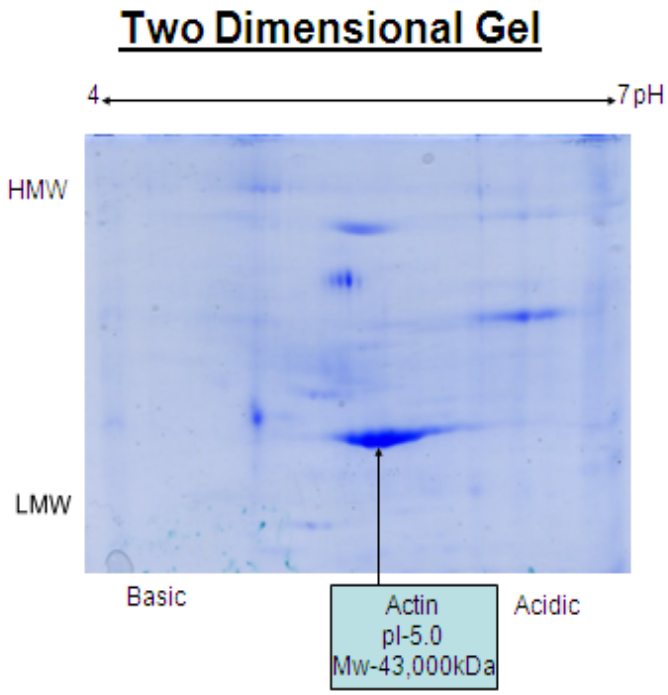

Two Dimensional Gel
4
7 pH
HMW
LMW
Basic
Actin
pI-5.0
Mw-43,000kDa
Acidic

Figure 3: Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis- 1D

This figure represents the results of a coomassie stained one dimensional gel electrophoresis on a 12% polyacrylamide gel. Lane 1 represents a Lonza ProSieve prestained molecular weight standard. Lane 2 represents the total protein distribution in a sample.

Figure 3.

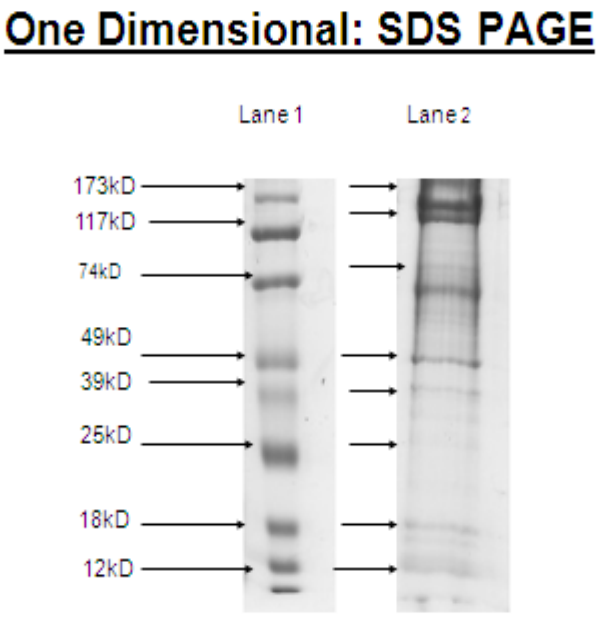

One Dimensional: SDS PAGE
Lane 1
Lane 2
173kD
117kD
74kD
49kD
39kD
25kD
18kD
12kD

Figure 4: Western Blot for Rho Kinase

This figure represents the results of a western blot autoradiograph performed on a 12% polyacrylamide gel. Lane 1 represents Lonza ProSieve prestained molecular weight standard. Lane 2 demonstrates immunoreactivity to Rho Kinase with molecular weight around 160-170kDa. Lane 3 represents a coomassie blue stained PVDF blot showing total protein distribution in the corpus cavernosum sample.

Figure 4.

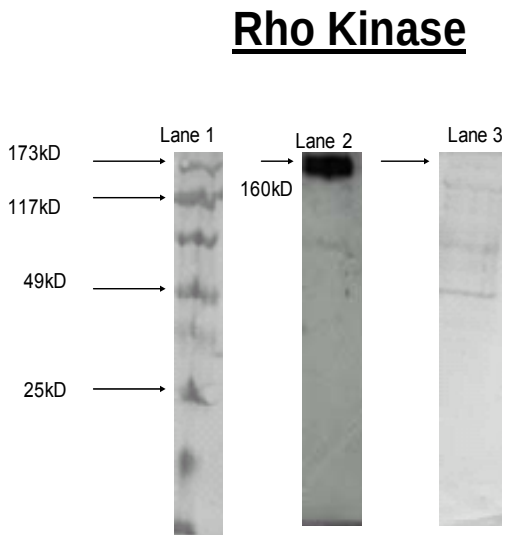


Figure 5: Western Blot for Myosin Light Chain

This figure represents the results of a western blot autoradiograph performed on a 12% polyacrylamide gel. Lane 1 represents Lonza ProSieve prestained molecular weight standard. Lane 2 shows immunoreactivity to myosin light chain with molecular weight around 24kDa. Lane 3 represents a coomassie blue stained PVDF blot showing total protein distribution in the corpus cavernosum sample.

Figure 5.

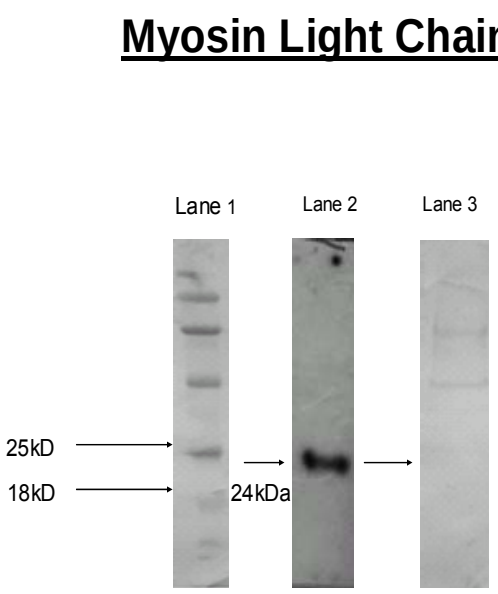
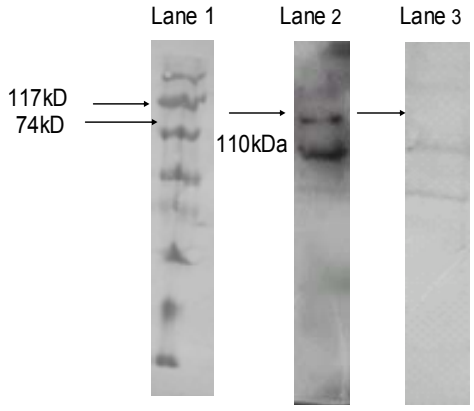


Figure 6: Western Blot for Myosin Light Chain Phosphatase

This figure represents the results of a western blot autoradiograph performed on a 12% polyacrylamide gel. Lane 1 represents Lonza ProSieve prestained molecular weight standard. Lane 2 reports immunoreactivity to myosin light chain phosphatase with molecular weight around 110-117kDa. Lane 3 represents a coomassie blue stained PVDF blot showing total protein distribution in the corpus cavernosum sample.

Figure 6.


Myosin Light Chain Phosphatase
Lane 1
Lane 2
Lane 3
117kD
74kD
110kDa

V. Discussion

The main goal of this study was to identify proteins expressed in the resting (contracted) state of the corpus cavernosum using a proteomic approach. Myosin Light Chain Kinase, Myosin Light Chain Phosphatase and Rho Kinase were the three proteins that were investigated in this study.

There are many molecular mechanisms that function at the level of smooth muscle and are capable of regulating corpus cavernosum smooth muscle contraction. Figure 7 illustrates the two pathways that were investigated in this study, which were calcium dependent and calcium sensitization mechanisms. Ca^{2+} dependent mechanisms are initiated by norepinephrine binding to α-adrenergic receptors to activate smooth muscle contraction. Subsequent to this binding, the response of the cell is to increase phospholipase C activity through a G protein. Phospholipase C releases IP_3 and DAG from PIP_2. IP_3 activates receptors on sarcoplasmic reticulum and DAG activates PKC. Sarcoplasmic reticulum releases stored Ca^{2+} into the cell increasing cytosolic Ca^{2+}. Increased levels of cytosolic Ca^{2+}, bind to calmodulin, which in turn binds to and activates smooth muscle myosin light chain kinase, leading to smooth muscle contraction.

The contractile response is maintained by a Ca^{2+} sensitizing mechanism brought about by the inhibition of myosin phosphatase activity by PKC and Rho kinase. PKC mediates slow smooth muscle contraction with no increase in Ca^{2+} or even diminishing levels of Ca^{2+}. PKC regulates CPI-17, which promotes

inhibitory activity toward myosin light chain phosphatase (Fukata, 2001). The Rho kinase mechanism is initiated at the same time that phospholipase C is activated by G protein. This process involves the activation of the small GTP binding protein RhoA. RhoA increases Rho kinase activity, leading to inhibition of myosin phosphatase, promoting smooth muscle contraction.

Activated Rho Kinase has been proposed to increase the phosphorylation level of myosin either by directly phosphorylating the myosin (Amano et al, 1996) or indirectly by inhibiting the myosin binding subunit of phosphatase, which is responsible for dephosphorylating myosin (Kimura et al, 1996). Rho Kinase a is relatively large protein weighting about 160kDa-170kDa in the contraction state (Nakagawa et al, 1996). The western blot data for Rho Kinase (Figure 4) showed that there is immunoreactivity to a high molecular weight antigen around 160kDa-170kDa, which was expected.

The state of myosin light chain phosphorylation is further regulated by myosin light chain phosphatase, which removes the high energy phosphate from the light chain of myosin to promote smooth muscle relaxation (Somylo, 2000). There are three subunits of myosin light chain phosphatase: a 37kDa catalytic subunit, a 20kDa variable subunit, and a 110-130kDa myosin binding subunit (Ichikawa et al., 1996). The myosin binding subunit (110kDA-130kDa) in the contracted state is phosphorylated, inhibiting the enzymatic activity of myosin light chain phosphatase, allowing the light chain of myosin to remain

phosphorylated, thereby promoting contraction. The western blot data for myosin light chain phosphatase (Figure 6) showed that there is immunoreactivity to a high molecular weight antigen (around 110-117kDa), which was expected.

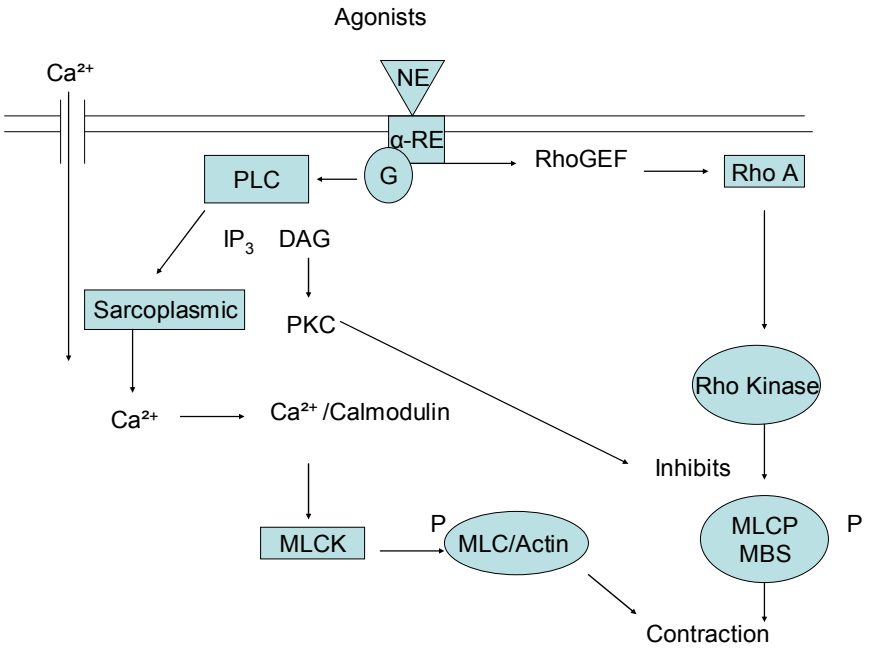
Smooth muscle cell contraction is regulated principally by receptor and mechanical activation of the contractile proteins myosin and actin. The actomyosin cycle begins with phosphorylation of myosin by myosin light chain kinase, leading to cross bridge cycling between the regulatory myosin light chain and actin (Kamm & Stull, 1985). For contraction to occur, myosin light chain kinase must phoshorylate the (20kDa -24kDa) light chain of myosin, enabling the molecular interaction of myosin with actin (Gallagher et al., 1997). The western blot data for myosin light chain (Figure 5) showed that there is immunoreactivity to a low molecular weight antigen around 24kDA, which was also expected.

The data that was obtained from this research supports and confirms published data that Rho kinase (Disanto, 2003), Myosin light chain, and Myosin light chain phosphatase (Webb, 2003) are present in the resting (contracted) state of the corpus cavernous. This research provides information that is based upon the involvement of three regulatory proteins in the penis. In the future, we will be able to compare these proteins, which are either active or inactive in a contracted state to proteins that are active or inactive in a relaxed state. We also plan to investigate changes in protein expression in aging and the effects of androgens in regulating protein expression in the corpus cavernosum. This information should

provide a better understanding of erectile dysfunction. This new information could also be helpful in formulating treatments to treat or cure erectile dysfunction by inhibiting or activating specific proteins

Figure 7

Smooth Muscle Contraction Pathways



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Tuesday, October 23, 2007

Dr. Robert Leipheimer
Department of Biology
UNIVERSITY

Re: IACUC Protocol # 10-07
Title: Regulation of smooth muscle contraction and relaxation in rats.
Approval date: October 15, 2007 Expiration date: October 15, 2010

Dear Dr. Leipheimer:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the aforementioned protocol you submitted for consideration titled **"Regulation of smooth muscle contraction and relaxation in rats"** and determined it should be unconditionally approved for the period of **October 15, 2007** through its expiration date of **October 15, 2010**.

This protocol is approved for a period of three years; however, it must be updated once a year via the submission of an Annual Review-Request to Use Animals form ***prior*** to its yearly anniversary date of **October 15, 2008** and **October 15, 2009**. You must adhere to the procedures described in your approved request; any modification of your project must first be authorized by the Institutional Animal Care and Use Committee.

Sincerely,

Dr. Peter J. Kasvinsky
Associate Provost for Research
Research Compliance Officer

PJK:dka

C: Dr. Walter Horne, Consulting Veterinarian, NEOUCOM
Dr. Robert Leipheimer, Chair IACUC, Chair Department of Biological Sciences
Dawn Amolsch, Animal Tech., Biological Sciences
Cheryl Coy, Grants and Sponsored