Recombinant Expression of Sry3 Raises Blood Pressure Indices in *Rattus norvegicus*

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Recombinant Expression of Sry3 Raises Blood Pressure Indices in *Rattus norvegicus*

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Thesis

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DEDICATION

For James and Suzanne Felter, who gave me life then filled it with love and laughter. Their constant nurturing of my curiosity has surely paved the path in my quest for knowledge.
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CHAPTER I
INTRODUCTION

Hypertension is a disease that effects one-in-three American adults and cost an estimated 73.4 billion dollars in 2009 (Lloyd-Jones et al., 2009). Essential hypertension is a complex disease that often leads to serious health complications such as coronary heart disease, stroke, heart failure, and kidney disease (August and Oparil, 1999). There is gender variation in hypertension, middle-aged men of all ethnic groups have higher mean systolic and diastolic blood pressures than women of the same age and male-linked hypertension confers an elevated risk factor for cardiovascular mortality (Kannel, 1996). The Framingham study found that hypertensive men had a death rate double that of hypertensive women (Kannel, 1996).

The Y-chromosome of mammals contains a gene that is responsible for the differentiation of the male phenotype (Koopman et al., 1990). In 1986, Vergnaud et al. discovered this region of the Y-chromosome responsible for male sex differentiation in mammals. It was later discovered, by gene mapping in humans, that the gene responsible for creating the male phenotype was located on the short arm of the Y-chromosome. The short arm of the mammalian Y chromosome contains the element
responsible for testis formation (Vergnaud et al., 1986). After extensive study of fetal expression (Koopman et al., 1990) it was hypothesized that the specific testis-determining locus is located on the short arm of the Y chromosome within the sex-determining region Y (Sry). Sry is highly conserved among mammals and has a tightly controlled expression pattern that corresponds well with testis-determination (Koopman et al., 1990). Further support for the role of Sry in testis-determination came with the discovery of XY human females with a four nucleotide mutation in the Sry gene leading to gonadal dysgenesis, these females had an otherwise normal Y chromosome (Jäger et al., 1990).

The same year that Sry was named as the testis determination factor, a different research lab discovered a Y-chromosome link to hypertension in the rat (Ely and Turner, 1990). The spontaneously hypertensive rat (SHR) has long been studied as an animal model for human essential hypertension and when male SHR’s were selectively crossbred for several generations with normotensive female Wistar Kyoto rats (WKY), the resulting SHR/y male offspring were hypertensive. Ely et al., hypothesized that there was potentially a hypertensive allele located on the Y chromosome of the SHR rat responsible for the increase in blood pressure seen with genetic crosses. At the time, only 14 loci on the Y chromosome were mapped but ten years later Sry was named as a candidate gene for Y-linked hypertension (Ely et al., 2000). The locus is located within a locus on the Y chromosome of the spontaneously hypertensive rat (SHR) that is associated with a 20-25 mmHG increase in blood pressure (Ely et al., 2000). Many
mammals have just one copy of Sry but *Rattus norvegicus* has multiple functional Sry copies that encode full-length proteins (Turner et al., 2007). While six of the loci are found in both WKY and SHR/y males, one loci, Sry3, is exclusively found in SHR/y males (Farkas, 2008).

At least two of the Sry loci raise blood pressure when exogenously delivered to WKY kidneys, Sry1 and Sry3 (Chiarappa, 2010). Sry1 increases blood pressure and tyrosine hydroxylase activity when injected and electroporated into the left kidney of normotensive, male Wistar Kyoto rats (Ely et al., 2009). Alpha receptor blockade with prazosin reversed the blood pressure effects seen after Sry1 delivery, further supporting that Sry1 facilitates a blood pressure increase through sympathetic nervous system activation. Sry3 delivery is associated with an increase in blood pressure but there are no changes in adrenal medulla tyrosine hydroxylase content or plasma catecholamine levels, Sry3 does not seem to utilize a sympathetic nervous system (SNS) pathway to increase blood pressure (Milsted et al., 2009). The blood pressure rise seen with Sry3 delivery seems to be due to changes within the renin-angiotensin system (RAS) rather than heightened SNS activity (Milsted et al., 2009).

It is has been shown that Sry3 alters the RAS activity, however, there is not a complete understanding of how Sry3 facilitates the increase in blood pressure (Chiarappa, 2010). Interference with the RAS, specifically Ang II signaling by blocking the AT₁ receptor may help to identify the mechanism that Sry3 utilizes to increase blood
pressure. Further examination of Sry3 and male-linked hypertension in an animal model will help to determine possible mechanisms of action and further scientific understanding of human male essential hypertension.
CHAPTER II

BACKGROUND OF THE STUDY

The Spontaneously Hypertensive Rat (SHR) is a popular animal model for genetic examination of essential hypertension (Igase et al., 2005). The SHR strain was originally derived through breeding of a hypertensive Wistar male to a Wistar female with slightly elevated blood pressure (Okamoto and Aoki, 1963). SHR and WKY rats were originally obtained from Harlan Sprague Dawley (Indianapolis, IN) and since 1981 have been bred at The University of Akron. The SHR/y strain is a borderline hypertensive, consomic strain developed by crossing a WKY female with an SHR male (Ely et al. 2002). Then an F1 generation male is backcrossed to a WKY female, this continues for 19 generations (Turner et al., 1991). Breeding out to generation F19 produces male offspring with a SHR Y chromosome and WKY autosomes that have significantly elevated blood pressure. Generation F19 male offspring with a WKY Y chromosome and SHR autosomes have significantly lower blood pressure than SHR males (Turner et al., 1999). The Y chromosome of the Spontaneously Hypertensive Rat (SHR) that is associated with a 20-25 mmHG increase in blood pressure and Sry has been identified as a locus capable of modulating blood pressure (Ely et al., 1993; Ely et al., 2007). Though Sry is primarily
known for its role in fetal gonadal development, Sry mRNA has been found in testis,
adrenal glands, and kidneys of adult SHR and WKY rats (Turner et al., 2007). This
continued transcription of Sry suggests that the Sry protein has a continuing role as a
transcription factor in adult tissues. The Sry protein contains a highly conserved HMG
box consisting of three alpha helices (Underwood, 2008). The 2\textsuperscript{nd} helix interacts with
DNA in the minor groove and allows the 1\textsuperscript{st} and 3\textsuperscript{rd} helices to act as cantilevers,
facilitating a 65-80° bend in the target DNA; for this fact, Sry is considered to be an
architectural transcription factor (Werner et al., 1996). The DNA bend induced by Sry
may allow interaction between the DNA and other proteins, acting as co-factors in a
complex to regulate gene transcription (Turner et al., 2007).

Sry has a role as a transcription factor involved in testes determination, and has
been implicated in behavioral aggression, blood pressure regulation and many other
functions (Ely et al., 2009). There are Sry binding sites located on sex determination
genes, such as P450 aromatase and anti-mullerian hormone (Haqq et al., 1993). Sry has
also been found to interact with proteins like the androgen receptor through the HMG
box, repressing androgen receptor transactivation (Yuan et al., 2001). The protein KRAB-
0 and Sry interact, this particular interaction has implications for gene silencing (Mitchell
et al., 2002). Sry also interacts with Wilm’s tumor gene 1 through the HMG box,
enhancing activation of WT-1 (Matsuzawa-Watanabe et al., 2003).
The *Rattus norvegicus* Y chromosome contains multiple functional Sry loci that encode full-length proteins. Most mammals have only one identified copy of Sry, but eight out of nine vole species examined also contained multiple different copies of the Sry HMG box (Bullejos et al., 1999). The seven indentified loci in *Rattus norvegicus* are Sry1, Sry2, Sry3aA, Sry3B, Sry3B1, and Sry3C (Turner et al., 2009). The various loci are expressed in all tissues examined but their relative amounts vary in the specific tissues studied (Turner et al., 2007). Sry2 is the major copy expressed in both testes and adrenal gland and real time PCR found total Sry transcript level in the testes to be 13 times greater than those in adrenal glands (Turner et al., 2007). The Sry3 copy is present on the hypertensive SHR/y Y chromosome but not on the normotensive WKY Y chromosome (Farkas, 2008). The other Sry proteins show divergence in the HMG box region. Any amino acid differences in the Sry coding region could potentially change binding of the Sry protein to target DNA and other proteins (Farkas, 2008). One such difference is between the Sry1 protein and the Sry3 protein, in which amino acid 37 (in the HMG box) is a glutamine (Q, uncharged polar) in Sry3 and a histadine (H, positively charged) in Sry1 (Underwood, 2008).

The effect of Sry on blood pressure seems to be via two different pathways, the sympathetic nervous system (SNS) and the renin angiotensin system (RAS), two powerful regulators of blood pressure. Numerous experiments were performed in which 50µg of plasmid vector, containing various copies of Sry, were injected and electroporated into the left kidney of normotensive WKY rats (Ely et al., 2009). Delivery
of exogenous Sry1 led to significantly increased blood pressure versus empty vector from 7-21 days post gene delivery, the blood pressure returned to normal by 28 days (Ely et al., 2009). In the same study, delivery of Sry2 had no blood pressure effect compared to empty vector, interesting due to the high percentage of Sry2 found in various tissues through fragment analysis. Following Sry1 delivery, the blood pressure response to air stress also significantly increased (Ely et al., 2009). This increase is due to a rise in sympathetic nervous system activity, demonstrated by a reversal of effect when given the alpha-blocker, prazosin (Ely et al., 2009). The same study also found that Sry1 delivery increases renal tyrosine hydroxylase activity after 21 days. Tyrosine hydroxylase catalyzes the conversion of tyrosine to L-Dopa and is the rate-limiting step in the synthesis of catecholamines such as dopamine (DA), norepinephrine (NE) and epinephrine (EPI). Catecholamines are the major neurotransmitters employed by the sympathetic nervous system. Indeed, Sry1 delivery does result in increased renal NE and DA content after 35 days. Just 7 days after Sry1 delivery, urine NE and DA content significantly increased. Plasma renin levels had no change with Sry1 delivery, supporting a role for Sry1 in SNS-mediated blood pressure increases but not for RAS-mediated blood pressure changes.

Despite the fact that in vivo studies showed that Sry1 does not have a discernable effect on the RAS, there are Sry consensus binding sites on the promoter regions of many RAS genes, including angiotensinogen (agt), renin, angiotensin-converting enzyme (ACE), and ACE2 (Milsted et al., 2010). The RAS is integral in
regulating blood pressure and minor changes in RAS gene expression can lead to altered blood pressure (Sealey et al., 1986). The afferent arterioles of the kidney contain juxtaglomerular cells that secrete renin into circulation (Sealey et al., 1986). Angiotensinogen is secreted into circulation by the liver, and renin, a protease, cleaves agt into Angiotensin I (Ang I). Agt is relatively abundant in the plasma, and renin is the limiting factor in the production of Ang I. ACE, a carboxypeptidase, converts Ang I into Ang II, a powerful vasoconstrictor (Ichihara et al., 2004). ACE 2 alternatively converts Ang I into Ang-(1-9), but Ang-(1-9) is present at extremely low levels in the plasma (Reudelhuber TL, 2005). ACE 2 also converts Ang II into Ang-(1-7), a vasodilator (Ferreira et al., 2005). The equilibrium between Ang II and Ang-(1-7) can greatly affect blood pressure, greater Ang II production would increase blood pressure while more Ang-(1-7) would decrease blood pressure.

Electrophoretic mobility shift assays showed that Sry1, Sry2, and Sry3 proteins bound to ACE and ACE2 oligonucleotides (Scott, 2009). Additionally, reporter assays were performed to examine the interaction between Sry and genes of the RAS. When Sry plasmids were co-transfected with promoters for renin, agt, ACE, and ACE2, Sry1 and Sry3 were found to increase promoter activity for renin. Sry1, Sry2 and Sry3 were all found to increase promoter activity for agt and ACE; Sry1, Sry2 and Sry3 all decreased promoter activity for ACE2 (Milsted et al., 2010). The up-regulation of agt and renin by Sry would lead to increased production of Ang II, the down-regulation of ACE2 by Sry
would lead to decreased production of Ang-(1-7) (Figure 1). The shift in balance between Ang II and Ang-(1-7) could lead to increased blood pressure.

Studies performed in vivo show that Sry3 increases blood pressure when exogenously delivered (Milsted et al., 2009). Sry3 (50µg) was delivered to the left kidney of normotensive, telemetred WKY rats. After gene delivery (14 days), the systolic blood pressures of the Sry3 rats were significantly elevated over rats given empty vector. Plasma renin activity also increased at 14 days post gene delivery with Sry3 treatment but not Sry1 or empty vector treatment (unpublished results). The animals were terminated after 21 days and the kidneys that had been electroporated with Sry3 had significantly increased Ang II content compared to both empty vector and Sry1 kidneys (unpublished results). These studies illustrate that Sry3 does raise blood pressure when exogenously delivered, but not through its action upon the SNS, Sry3 likely acts upon the RAS to mediate its actions.

There is a sex-based discrepancy in hypertension, males are prone to higher blood pressure and are more susceptible to changes in BP. Male SHR have higher mean arterial pressure than female SHR and inhibition of the RAS eliminates the sex difference in BP (Yanes et al., 2006). Ang II treatment leads to renal injury in SHR males, but SHR females were protected from renal injury (Sartori-Valinotti et al., 2008). In order to further elucidate the possible actions of Sry3 on the RAS, it is important to inhibit the RAS to observe an attenuation of Sry3-mediated blood pressure elevation. Ang II binds
to different receptors with very different physiological actions (Ferrario, 2006). The Angiotensin II Type 1 (AT$_1$) receptor mediates hypertensive effects such as vasoconstriction, excretion of aldosterone, and retention of salt and water (Igase et al., 2005). Ang II also binds to Angiotensin II Type 2 (AT$_2$) receptors, with a decreased affinity, these receptors mediate vasodilation, cell differentiation and apoptosis. Ang II can also bind to the AT$_3$ and AT$_4$ receptors, but these receptors are not well characterized and little is known about their actions (Miyazaki et al., 1996). Blockage of the AT$_1$ receptors would not only inhibit hypertensive effects of Ang II but would increase Ang II binding to AT$_2$ receptors, stimulating antihypertensive effects.

Olmesartan medoxomil [(5-methyl-2-oxo-1,3-dioxolen-4-yl)methoxy-4-(1-hydroxy-1-nethylethyl)-2-propyl-1-[4-[2-(tetra-5-y1)-phenyl]phenyl]methylimidizol-5-carboxylate] is a commercially available Ang II blocker; it selectively antagonizes AT$_1$ receptors without effecting AT$_2$ receptors (Koike et al., 2001; Pugsley, 2005). When orally administered to hypertensive rats, olmesartan successfully reduced blood pressure and renal vascular resistance (Koike et al., 2001). Due to its selective antagonism of AT$_1$ receptors, olmesartan is a good candidate to inhibit the RAS mediated effects of Sry3 treatment.
CHAPTER III

METHODS

Hypothesis

Delivery of Sry3 to normotensive rats will increase blood pressure through the renin angiotensin system pathway. Blockage of the AT$_1$ receptor with olmesartan medoxomil will reverse the blood pressure effect in Sry3 treated animals and will also decrease the blood pressure of empty vector treated animals.

Animals

All animal protocols were performed in compliance with the regulations set forth by the National Institutes of Health and were approved by The University of Akron’s Institutional Animal Care and Use Committee. All experiments were performed on 12 week old, normotensive Wistar Kyoto rats. A total of 10 animals were used for three experimental groups. There were 3 empty vector animals and 7 Sry3 treated animals. All of the empty vector animals and 4 of the Sry3 animals received drug treatment with olmesartan medoxomil. Animals received a standard 12 hour light/dark cycle and were given standard rat chow (22.5% protein, 52% carbohydrate and 6% fat by weight, Prolab
3000, Agway, Syracuse, NY) and water ad libitum. Rats were individually housed in polycarbonate cages (48cm x 27cm x 20cm) with heat-treated bedding (Sani Chips, R.J. Murphy, Rochelle Park, NJ). Cage changes were performed once a week, scheduled so as not to interfere with blood or urine sampling. All surgeries were performed aseptically as recommended by the AAALAC.

Timeline

Animals were implanted with telemetry devices and baseline measurements of systolic pressure, diastolic pressure, heart rate and activity were collected. All animals were monitored for telemetry measurements throughout the study, at 30-minute intervals, except for the 24-hour periods that animals spent in metabolic cages. Animals were allowed to recover from telemetry surgery for one week before a baseline metabolic cage study was done. All animals were injected and electroporated with PEF1(-) or PEF1/Sry3 into the left kidney, which is day 1 of the study. On day 6, the first 24-hour urine was taken followed by a first plasma sample on day 8. On day 14, animals were given SHAM or olmesartan treated drinking water. The drug was given for 1 week total and on day 17 a second 24-hour urine was taken followed by a second plasma on day 19. Drug treatment was then stopped and time was given to allow the drug to leave the system. On day 25, a final 24-hour urine was collected followed by a final plasma sample on day 27.
Radiotelemetry

Radiotelemetry was utilized to directly measure arterial pressure, heart rate and activity. Radiotelemetry is a particularly useful innovation because it takes blood pressure measurements without restraining or inflicting stress upon the animal. The small, internal telemetry device (weight: 7.6g, volume: 4.4ml, model: PAC40; Data Sciences International, St. Paul, MN) digitally takes measurements and sends them to an external receiver (RPC-1, Data Sciences international, St. Paul, MN). The receiver translates the reading to the data collection system (Dataquest A.R.T., Data Sciences International, St. Paul., MN), which normalizes the data based on the ambient pressure. The biocompatible telemetry device body contains a sensor, electronics that receive, amplify and transmit the signal, and a silver cell battery (Mills et al., 2000). Attached to the body is a flexible, fluid-filled catheter with a compliant end containing an anti-thrombogenic substance that blocks aortic blood flow from entering the catheter (Mills et al., 2000). Each individual telemetry transmitter was turned on 24-hours prior to implantation and the offset was calibrated while the transmitter stabilized in sterile saline. Maximum allowable unadjusted offset was ± 1 mmHg.

Adult WKY males were anesthetized in a chamber with 3% isoflurane delivered by vaporizer. Once suitably anesthetized, as determined by stimulus reaction to a toe-pinches, the rat was removed from the chamber and administered isoflurane via a
facemask. Anesthesia level was maintained for the length of the procedure through monitoring of respiratory rate.

The entire abdomen was shaved and swabbed with iodine and the rat was placed upon a heating mat to maintain body temperature. On the ventral abdomen, a 3.5-centimeter midline incision was made, below the umbilicus and above the pubic symphysis, with a 21-blade scalpel. Half of a piece of gauze was immersed in sterile saline and used to reposition the intestines to the far side of the peritoneal cavity. The cavity was further opened with a retractor (Kent Scientific Corporation, Torrington, CT) and the descending aorta was located. A 1.0 cotton suture was carefully wound between the aorta and vena cava, just posterior to the renal artery. The suture was slowly elevated and aortic blood flow was temporarily occluded. At a point at least 10mm anterior to the iliac bifurcation, the aorta was punctured with a 21-gauge hypodermic needle (tip bent 90°) and the catheter of the telemetry device was inserted into the aorta. The area around the inserted catheter was swabbed dry and sealed with an instant adhesive. The 1.0 cotton ligature was released and a surgical patch was glued to the catheter entry site. The patch served to shield and secure the entry site, as well as to promote growth of protective connective tissue. The suture, gauze and retractors were removed and the intestines were gently moved back in place. 0.5cc of sterile saline was delivered to the peritoneal cavity and the telemetry device was carefully placed inside. The device was secured to the peritoneal wall with 3.0 silk suture, the
abdominal muscle was sutured closed and the dermis was secured with auto clips (Kent Scientific, Torrington, CT). Each rat was given 2500 units of penicillin IM to prevent post-surgical infection and supplied with Tylenol water (25mL in 400mL H₂O) ad libitum as an analgesic. The Tylenol water treatment was given the day before all surgeries and continued for three days post-op. Animals were allowed to recover for one week before use in experiments and during this time, baseline blood pressures were established, with telemetry sampling every 30 minutes for systolic pressure, diastolic pressure, heart rate, and activity.

Purification of PEF1/Sry3

The protein-coding region of Sry3 (GenBank: AY157672) had previously been subcloned into the PEF1 expression vector (Invitrogen) containing a carboxy terminal c-Myc epitope and histidine tag under the control of a CMV promoter. Clones of this construct had previously been transformed into E. coli cultures (Underwood A, 2008). The transformed E. coli cells were streaked onto LB/ampicillin plates (75μg/ml) and incubated at 37°C for 24 hours. A single colony was chosen and inoculated into 5ml of liquid LB/amp at 37°C for 6 hours while shaking at 225 rpm. 500μl of the starter culture was transferred into 500ml of LB/amp and grown at 37°C for 16 hours while shaking at 225 rpm. The plasmid was then purified (Endofree Plasmid Mega Kit, Qiagen) and a restriction digestion was performed to ensure proper construct ligation. A 10 μl
digestion was set up containing: 1µg plasmid, 1 µl restriction enzyme, 1 µl 1X loading buffer and nuclease-free water (to 10 µl total volume). The mix was incubated at 37° for 1 hour then 2 µl of 10X loading buffer was added to the digest and the mixture was loaded onto a 1% agarose gel (TBE: 90mM Tris, 90mM boric acid, 2mM EDTA) containing 0.2µg/ml of ethidium bromide. The agarose gel was electrophoresed at 120V for 30 minutes and detected by ultraviolet light (Eastman Kodak, Rochester, NY). The verified plasmids were diluted to a final concentration of 0.5µg/ul in sterile saline.

Recombinant Gene Delivery

Purified PEF1/Sry3 (50µg/100µl) was delivered to 8 WKY males and PEF1(-) (50µg/100µl) was delivered to 4 WKY rats. All pre- and post-operative conditions for electroporation surgery matched those for telemetry implantation. The incision was made laterally, 2cm to the left of the telemetry incision. The left kidney was located and 25µg (in 50µl TE) of Sry3 or empty vector was injected into the medulla with a 28 gauge needle in both the upper quadrant and lower quadrant of the kidney (for a total plasmid delivery of 50µg/100µl TE). A swab was held at the injection site for 1 minute to prevent bleeding and backflow of the plasmid. The kidney was stimulated with an electrostimulator (ElectroCellManipulator, ECM 830 Electroporation Protocol, BTX, a division of Genentronics, San Diego, CA) connected to tweezer-style electrodes (BTX Tweezertrodes Model #522) that were placed on opposite sides of the kidney. Twenty
pulses were administered at 200 volts, each lasting 20 msec, at 1000 Hz. The animals were then given 0.5cc sterile saline, closed as previously described in the telemetry methods, given 2500 units (0.5mL) of penicillin via intramuscular injection and supplied with Tylenol water (25mL in 400mL H2O) ad libitum. Animals were monitored while recovering for seven days post-surgically.

Western Blot Analysis

100µl of methylene blue dye was injected and electroporated into the left kidney of a two WKY male rats to determine the possible area of delivery of the plasmid. The kidneys were removed after fifteen minutes and 24 hours, respectively, and cut in half to visualize the methylene blue dye.

To verify the presence of the recombinant protein in the kidney a Western blot was performed to verify the presence of recombinant protein in the kidney. 50µg/100µl of the purified PEF1/Sry1 or empty vector was injected and electroporated into the medulla of the left kidney of two WKY rats, as previously described. Kidneys, testes, liver, lung, heart, adrenal glands were collected at 14 days following electroporation. These tissues were immediately frozen on dry ice.

The tissues were homogenized in a tissue lysis buffer consisting of 1X CellLytic B (Sigma), 1X protease inhibitor cocktail with no EDTA (Sigma), 1mMPMSF, 5 U DNase I,
25 mM NaF, 25 mM McIl, and Phosphate buffered imidazole. Lysates were centrifuged at 13,000G for 5 minutes and the supernatant was transferred to a 15mL tube containing 6mL PBI and incubated on a rotator for 1 hour at room temperature. The beads were collected by centrifugation at 500G for 5 minutes. Following 4 rinses with PBI, the his-tagged proteins were eluted with 1 mL of buffer EB containing 20mM Na$_2$HPO$_4$, 500mM NaCl, 500mM Imidizole, pH 7.4.

The his-tagged protein elutions were run on Precise® Protein Gels (Sigma) in Tris-Hepes-SDS running buffer and transferred onto PDVF membrane by semi-dry transfer at 15V for 1 hour. Membranes were stained using the SNAP I.D. system (Millipore, Billerica, MA) blocked for with 1% bovine serum albumin in PBS with 0.1% Tween-20 for 20 seconds under vacuum conditions at room temperature. Membranes were incubated with the blocking solution containing goat anti-cMyc (1:1000) for 10 minutes at room temperature and then actively driven through the membrane. The membranes were washed 4 times with PBS, 20 seconds under vacuum, and then incubated with Bethyl donkey anti-goat IgG, HRP conjugate (1:3000) in PBS for 10 minutes at room temperature. The 2° antibody was actively driven through the membrane and rinsed 4 times with PBS. The membranes were incubated with 2mL Luminol and 2mL hydrogen peroxide (Thermo Super Signal West Pico Chemiluminescent Substrate) for 5 minutes before capturing bands on the Kodak Gel Logic 2200 documentation system.
Examination of Sry3 Effects and Ang II Blocker treatment

Following gene delivery, animals were monitored every thirty minutes for systolic pressure, diastolic pressure, heart rate and activity. Seven days after gene delivery, animals were placed individually into metabolic cages for a period of 24 hours. The rats received rat chow and water ad libitum. The water bottle initially contained 100 ml and the end volume was recorded to note the total volume of water drank in 24 hours. The urine collection tube contained 1ml of 1N HCl; the acid stabilized the urine catecholamine content at room temperature and does not interfere with urine sodium or creatinine clearance measurements. Blood pressure was not monitored for the 24 hours that the rats were in the metabolic cages. The urine was aliquoted into two tubes, the first tube was refrigerated at 4° C to be assayed for sodium content and creatinine clearance rate. The second tube was frozen at -80°C to measure urine catecholamine content by HPLC.

A blood sample was taken from each rat, nine days after gene delivery. The rat was anesthetized as previously described, and the blood was drawn by puncturing the orbital sinus with a heparinized capillary tube (Thermo Fischer Scientific, Hampton, NH). Blood was collected (500µl) into K EDTA blood collection tubes (Thermo Fischer Scientific, Hampton, NH). The blood tubes were centrifuged at 3000 rpm for 10 minutes at 4° C and the plasma was drawn off into a fresh microfuge tube. The blood samples were stored at 4° C for future measurement of plasma renin activity.
At 14 days after gene delivery, the empty vector animals and half of the Sry3 animals received water treated with the Ang II blocker, olmesartan medoxomil (10mg/kg). The Ang II blocker was delivered in drinking water via a 0.1% NaHCO₃ + KHCO₃ solution in distilled H₂O. The dose was adjusted by rat based on daily water intake, as measured when the animals were in metabolic cages for baseline studies. Olmesartan medoxomil works by interfering with the ability of Ang II to bind to the AT₁ receptor, and has been shown to be a superior blocker of the progression of hypertension in SHR (Yokoyama et. al, 2005). A second 24-hour urine was taken three days after the start of olmesartan treatment, and a blood sample was taken after 5 days. Drug treatment was stopped after 6 days. Three days later, a final metabolic cage urine was collected and two days after that, a final blood was drawn.

Biochemical Analysis

The blood samples were analyzed for plasma renin activity by Gamma Coat Plasma Renin Activity I¹²⁵ RadioImmuno Assay Kit (Diasorin, Stillwater, MN). Plasma renin is indirectly measured by the generation of Ang I under tightly controlled conditions and uses rabbit anti-angiotensin I antibody that is later conjugated to ¹²⁵I-angiotensin I. The urine samples were analyzed for sodium content by flame photometry (Instrumentation Laboratory, Bedford, MA) according to the sodium flame photometry protocol outlined by the World Health Organization. The urine
catecholamine content was determined by HPLC (Waters 2465, Milford, MA) analysis. Urine samples (pre-treated with 1N HCL) were diluted 1:10 in mobile phase (35mM citric acid, 90mM sodium acetate, 690μM octyl sodium sulfate, 130μm EDTA, 10% methanol, pH 4.7). Urine catecholamines were extracted by mixing 100μl of diluted urine sample and 75μl of internal standard, 2400pg of 3,4-Dihydroxybenzlamine (DHBA, Sigma, St. Louis, MO) into 1ml of extraction buffer (1M Tris, 10mM sodium metabisulphite, 20mM EDTA, pH 8.7, Sigma, St. Louis, MO) with 25mg LC-alumina A (Supelco, Bellfonte, PA). The extraction mixture was vortexed vigorously for 10 minutes and the supernatant was removed. The LC-alumina A was rinsed 3 times with wash solution (0.2% Tris EDTA, pH 8.1, Sigma, St. Louis, MO), vortexing for 3 minutes each time before aspirating off the wash solution. After rinsing, 400μl of 100mM perchloric acid (Sigma, St. Louis, MO) was applied to the LC-alumina A and the solution was vortexed for 5 minutes. The resulting supernatant was placed into 700μl autosampler vials and injected (50μl) by an autosampler (Waters 717 Plus, Milford, MA) into the HPLC and the pump (Waters 1515, Milford, MA) was set at 1.4ml/minute. The sample was maintained at 30°C as it progressed through the guard column (LC-18, supelco, Bellfonte, PA) and then into the column for separation (Waters C18, 5μ, 4.6 x 150mm, Milford, MA) before electrochemical detection. The samples were normalized to an internal control that was added before sample extraction.
The urine catecholamine content was calculated with analysis with the Waters Breeze system software (Waters, Milford, MA). A curve was generated from the peak height ratio of the standards and the internal standard. Three curves total were generated for each individual run, one each for NE, EPI and DA. The assay is sensitive and can measure as little as 30pg/mL of each analyte.

Both the blood and urine were analyzed for creatinine clearance with Infinity™ creatinine liquid stable reagent (Thermo Electron Corporation, Boston, MA) and a clinical analyzer (Datachem, Indianapolis, IN) to estimate GFR and to test for kidney damage.

Statistical Analysis

Data analysis was completed with SigmaStat and SigmaPlot (Systat Software Inc. Richmond, CA) software. Significance was determined by ANOVAs and individual t-test and statistical outliers were defined and removed using the Dixon Outlier Procedure. The graphs display results as the data mean ± standard error. Statistical significance is defined as any p-value ≤ 0.05. All significant measures are noted with an asterisk (* denote p ≤ 0.05, ** denotes p ≤ 0.01, *** denotes p≤ 0.001) in the graphs. Most comparisons are versus controls, it is noted whenever this is not the case.
Methylene blue dye injection into the kidney was visualized after 15 minutes and after 24 hours. The methylene blue was present in the left kidney medulla 15 minutes post injection (Figure 1A). After 24 hours, the methylene blue was present in the left kidney, medulla and cortex (Figure 1B), and in the right kidney (not shown).

Figure 1. Methylene blue dye injection in the kidney.

(A) 15 minutes and (B) 24 hours after injection. 4X Magnification.
Heart, testes, lung, liver, adrenal glands and kidneys were collected after 14 days from a rat injected and electroporated with Sry3 in the left kidney. Western blot analysis found of his-tagged proteins found recombinant Sry3 protein in all tissues examined, the control animal left kidney homogenate contained no his-tagged protein (Figure 2).

Figure 2. Western blot detection of Sry/Myc protein. Exogenous expression of Sry protein 14 days after vector delivery and electroporation. Lane 1) Heart. Lane 2) Testis. Lane 3) Lung. Lane 4) Liver. Lane 5) Right Adrenal Gland. Lane 6) Left Adrenal Gland. Lane 7) Right Kidney. Lane 8) Left Kidney. Lane 9) Empty Vector (-) Left Kidney.

Telemetry Measurements

Telemetry measurements were analyzed for blood pressure, heart rate and activity. Sry3 treatment significantly elevated blood pressure compared to empty vector, this elevation was attenuated with olmesartan treatment. Olmesartan treatment
also lowered blood pressure in empty vector animals (Figure 3). Heart rate did not change with Sry3 or empty vector treatment. Olmesartan treatment increased heart rate within each group, however, the increase was not significant between groups (Figure 4). Animals’ activity was measured in arbitrary units and remained unchanged for all groups throughout the study (Figure 5).

Figure 3. Systolic Blood Pressure Over Time in Treatment Groups.

A 2-way ANOVA determined that systolic blood pressure was significantly different between treatment groups (F=17.098, p<0.001). There was no significant difference in the baseline systolic blood pressures between the three groups (means, ± s.e.m.).
Sry3+olmesartan treated animals had a significantly higher systolic blood pressure than empty vector treated animals 7 days after gene delivery (p< 0.05 means, ± s.e.m.). Both Sry3 and Sry3+olmesartan treated animals had significantly higher systolic blood pressure than empty vector animals 14 days after gene delivery (p<0.05 means, ± s.e.m). Treatment with olmesartan significantly lowered blood pressure from baseline levels in both empty vector and Sry3 treated animals (p< 0.05 means, ±s.e.m.) and was significantly lower than Sry3 SHAM systolic blood pressure (p<0.05 means, ± s.e.m). There were no significant differences between systolic blood pressure post-drug delivery (means, ± s.e.m).

Figure 4. Heart Rate Over Time in Treatment Groups.
There was no significant difference in heart rate between the three groups at any time point (means, ± s.e.m.)

Figure 5. Activity Over Time in Treatment Groups.
There was no significant difference in activity between the three groups at any time point (means, ± s.e.m.)
Urine Analysis

Urine sodium excretion was determined in samples take before and after gene delivery and during and after drug treatment (Figure 6). Baseline levels between groups were significantly different, Sry3 animals had higher baseline sodium excretion. After electroporation, both Sry3 groups had higher sodium excretion. Sry3 and empty vector animals treated with olmesartan had lower sodium excretion than Sry+SHAM animals; after drug treatment there were no differences between groups. Creatinine clearances were not significantly different between the three groups at baseline, after gene treatment, during drug treatment or after drug treatment (Figure 7).

Urinary catecholamine levels were determined by HPLC. Dopamine levels were significantly higher in Sry3+olmesartan animals at all points in the study except for post gene delivery, when there was no differences between groups (Figure 8). Epinephrine levels were not significantly different between the three groups at any point, although the Sry3 treatment groups were slightly higher at baseline than empty vector animals (Figure 9). Urinary norepinephrine was not significantly different between treatment groups at baseline, post gene delivery or post drug treatment (Figure 10). Sry3 animals treated with olmesartan had significantly increased urinary norepinephrine.
Figure 6. Urine Sodium Excretion in Treatment Groups Before, During and After Drug Treatment. A 2-way ANOVA determined that urine sodium excretion was significantly different between treatment groups (F=6.0111, p<0.001). Baseline urine sodium excretion was significantly different between the Sry3 and empty vector animals, but not the Sry3+olmesartan and empty vector animals (p< 0.05 means, ± s.e.m.). After electroporation with Sry3, urinary sodium excretion was significantly higher compared to empty vector (p< 0.05 means, ± s.e.m.). During drug treatment, the animals treated with Sry3 and SHAM had significantly decreased urinary sodium excretion compared to empty vector animals treated with olmesartan (p< 0.05 means, ± s.e.m.) and Sry3 animals treated with olmesartan (p<0.05 means, ± s.e.m.). Empty vector animals treated with olmesartan and the Sry3 animals treated with olmesartan showed no significant difference (means, ± s.e.m.). There was no significant difference in any of the groups post-drug treatment (means, ± s.e.m).
Figure 7. Creatinine Clearance in Treatment Groups Before, During and After Drug Treatment. There was no significant difference in creatinine clearance between any of the treatment groups at any point during the experiment (means, ± s.e.m.).
Figure 8. Urine Dopamine in Treatment Groups Before, During and After Drug Treatment. A 2-way ANOVA determined that urinary dopamine was significantly different between treatment groups (F=7.429, p<0.01). Urinary dopamine was significantly higher in Sry3+olmesartan animals at baseline, compared to empty vector animals (p< 0.05 means, ± s.e.m.). There was no significant difference between treatment groups after gene delivery (means, ± s.e.m.). Urinary dopamine was significantly higher in Sry3 animals treated with olmesartan compared to empty vector animals treated with olmesartan (p< 0.05 means, ± s.e.m.) and Sry3 animals treated with SHAM Drinking water (p< 0.05 means, ± s.e.m.). Post-drug treatment, Sry3 animals previously treated with olmesartan had significantly increased urine dopamine compared to empty vector animals (p< 0.05 means, ± s.e.m.) and Sry3+ SHAM animals.
(p< 0.05 means, ± s.e.m.). There was no significant difference between Sry3 treated animals and empty vector animals at any time point (means, ± s.e.m.).

Figure 9. Urine Epinephrine in Treatment Groups Before, During and After Drug Treatment. A 2-way ANOVA determined that urinary epinephrine levels were not significantly different between any of the groups at any time point (means, ± s.e.m.).
Figure 10. Urine Norepinephrine in Treatment Groups Before and After Drug Treatment (means, ± s.e.m.). A 2-way ANOVA determined that there was a significant difference in urine norepinephrine between treatment groups (F=10.632, p<0.01). There was no significant difference in urinary norepinephrine between groups at baseline or post gene delivery. Urinary norepinephrine was significantly higher in Sry3 animals treated with olmesartan compared to both empty vector+olmesartan animals (p< 0.05 means, ± s.e.m.) and Sry3+SHAM animals (p< 0.05 means, ± s.e.m.). There were no significant differences between treatment groups post-drug treatment (means, ± s.e.m.).
Blood Analysis

Plasma renin activity was measured with an RIA kit. Sry3 animals had significantly higher PRA than empty vector animals, after gene delivery (Figure 15). Olmesartan treatment lowered PRA in Sry3 animals but not in empty vector animals. Post drug treatment, the three groups had no significant differences.

Figure 15. Plasma Renin Activity in Treatment Groups Before, During and After Drug Treatment (means, ± s.e.m.). A 2-way ANOVA determined that plasma renin activity was significantly different between treatment groups (F=4.355, p<0.05). Sry3 and Sry3+olmesartan treated animals had significantly higher plasma renin activity levels.
after gene delivery, as compared to controls (p< 0.05 means, ± s.e.m.). Olmesartan treatment significantly lowered Sry3 plasma renin activity compared to control animals treated with olmesartan (p< 0.05 means, ± s.e.m.) and Sry3+SHAM (p< 0.05 means, ± s.e.m.). There was no significant difference between the groups post-drug treatment (means, ± s.e.m.).
CHAPTER V
DISCUSSION

In order to determine if the gene injection would be limited to the renal medulla, 100µl of methylene blue was injected into the left kidney medulla and electroporated. The dye was visualized after 15 minutes and after 24 hours (Figure 6). The presence of the dye in the medulla of the left kidney after 15 minutes and after 24 hours demonstrates that the vector can remain in the injected kidney to some degree but it was also determined that the dye translocates to the contra-lateral kidney.

Using the Sry/Myc fusion proteins, recombinant protein was found in both kidneys, liver, lung, heart, testes, and both adrenal glands 14 days after electroporation (Figure 7). This suggests that the vector travels beyond the original injection site and that the recombinant protein is produced in multiple organs besides the injected kidney. Furthermore, the effects seen after gene delivery are due to protein expression not only in the kidney, but may also be due to recombinant protein expression throughout the body. This is important to note as there are numerous local renin-angiotensin systems
through out the body that could be affected by Sry3 delivery independent of the kidney renin-angiotensin system. The results of the western blot are not quantitative and are not indicative of comparative expression levels within the tissues examined.

Exogenous delivery of Sry3 to the left kidney significantly increased systolic blood pressure compared to empty vector controls (10-15mmHg; Figure 8). Seven days after gene delivery, Sry3 treated animals had slightly increased blood pressure and at fourteen days blood pressure was significantly higher in both Sry3 treated groups. Before drug treatment, heart rate was not significantly different in any of the groups but there was a trend of increased blood pressure with olmesartan treatment in both empty vector and Sry3 treated animals (Figure 9). Animal activity was not significant between any of the groups at any time point (Figure 10). Blood pressure is a product of both cardiac output and vascular resistance. Cardiac output is a function of stroke volume and heart rate. There was no difference in heart rate between empty vector control and Sry3 treated rats; this indicates that the increase in blood pressure is due to increased blood volume, increased vascular resistance, or a combination of both.

The increased blood pressure seen with Sry3 delivery is most likely not due to SNS activation. If the SNS were activated with Sry3 delivery, there would be increased levels of epinephrine that would cause increased heart rate via the B₁ adrenergic receptor. As previously mentioned, there was no significant change in heart rate following gene delivery. In addition, earlier studies showed that plasma catecholamine
levels and urinary catecholamine levels do not increase after Sry3 delivery (Chiarappa F, 2010). In the current study, urinary dopamine, epinephrine and norepinephrine content were not significantly different between groups after gene delivery (Figure 12, Figure 13, Figure 14). Baseline studies showed significantly different dopamine excretion in Sry3+olmesartan animals. This finding could be due to the stress of the urine collection in metabolic cages. Another study, in which empty vector and Sry3 were delivered to the adrenal medulla, showed no increase in blood pressure (Walch, 2010). The unchanged blood pressure following adrenal medulla injection suggests that Sry3 does not directly induce catecholamine synthesis in the adrenal medulla.

Though previous studies observed that Sry3 delivery significantly decreased glomerular filtration rate (GFR) using inulin clearance to estimate GFR (unpublished data), this study used creatinine clearance as an indicator of kidney damage and did not show any significant differences between any of the groups at any time point (Figure 11). Previous results also found that recombinant gene delivery and electroporation to the kidney resulted in no discernible gross tissue damage when histology was performed (Ely et al., 2009).

As previous studies have indicated, the increase in blood pressure after Sry3 delivery is likely through the renin-angiotensin system pathway (Chiarappa et al. 2010, Ely et. al, 2009). Indeed, the current study showed a significant increase in plasma renin activity after Sry3 delivery compared to empty vector control, supporting up regulation of the RAS as the primary facilitator of Sry3 increased blood pressure (Figure 15). The
increased PRA is the rate-limiting step in the RAS resulting in increased cleavage of agt into Ang I, which is then converted into Ang II by ACE. Though Ang II levels were not examined in the current study, previous studies found that Sry3 delivery does significantly increase renal Ang II content after 21 days (Milsted et. al, 2009).

It is important to note that circulating Ang I and Ang II have extremely short half-lives and it is not likely that circulating levels of these hormones could have large systemic effects before degradation (Atlas, 2007). In conscious rats, the half-life of Ang II was found to be 14.8 ± 2.5 seconds (Chapman et. al, 2006). Renin, on the other hand, is much more stable in circulation with a half-life of roughly 10 minutes (Oates et al., 2007). Furthermore, it was found that in humans, the half-life of renin was dramatically increased in hypertensive subjects, normal renin half-life was 10-15 minutes and the half-life in hypertensive subjects was 1 hour and 22 minutes (Skrabal et. al, 1974). Due to the longer half-life of renin, the increased PRA seen after Sry3 delivery would have a more significant effect than an increase in any downstream component of the RAS. PRA is indicative of circulating renin so increased PRA could initiate Ang II production in multiple systems throughout the body. Ang II then binds with a high affinity to the AT₁ receptor mediating vasoconstriction. The AT₁ receptor is a G protein-coupled receptor. Ang II binds to this receptor and activates phospholipases that produce inositol-1,4,5-triphosphate, which increases cytosolic Ca²⁺ concentration (Lemarie et. al, 2008). Increased Ca²⁺ activates myosin light chain kinase through
calmodulin, which augments interaction between actin and myosin resulting in contraction of vascular smooth muscle cells (Lemarie et. al, 2008). VSMC contraction increases vascular resistance and ultimately increases blood pressure.

As previously noted, blood pressure is a product of both vascular resistance and cardiac output. The activation of the RAS results in increased heart contractility and vascular resistance but the cardiac output can be further examined through blood volume, which is partially regulated by sodium excretion in the kidney. Urinary sodium excretion showed a significant decrease after gene delivery in control animals vs. Sry3 animals (Figure 10). Previous results from our laboratory showed that urinary sodium excretion significantly decreased after Sry3 delivery (Chiarappa et. al, 2010). Sry3+olmesartan animals had significantly higher sodium excretion at baseline and post-electroporation than either empty vector control or Sry3 animals (Figure 10). The difference in urine sodium excretion seen at baseline is likely due to the stress of the first encounter with the metabolic cages and the possible effect of such stress on the animals’ natural behaviors. Further examination of the first metabolic cage data shows that all animals had reduced water intake compared to later metabolic cage specimen collections. Additionally, empty vector animals had lower water intake than Sry3+olmesartan animals. A reduction in water intake would certainly effect urine sodium excretion. When examining only the urinary sodium excretion of Sry3+olmesartan treated animals, there is a significant decrease in sodium excretion following Sry3 delivery from 0.2 mmol/hr/100g BW at baseline before gene delivery to
0.1 mmol/hr/100g BW two weeks after Sry3 delivery and then to 0.07 mmol/hr/100g BW during drug treatment. This demonstrates that sodium reabsorption is significantly increased in Sry3 treated animals, which could result in water retention and increased blood volume. This sodium reabsorption likely occurs due to increased aldosterone. Aldosterone is a mineralocorticoid produced in the adrenal cortex that decreases sodium excretion, increases water reabsorption, and increases potassium excretion in the kidney (Atlas SA, 2007). Laredo et. al demonstrated that Ang II infusion into primary bovine adrenal cortex cells significantly increases aldosterone in a dose dependant manner (Laredo et. al, 1997).

Therefore, the proposed mechanism is that Sry3 increases renin activity, which results in production of Ang II that binds to AT1 receptors mediating vasoconstriction and stimulating aldosterone release/sodium reabsorption, potentially increasing blood volume and ultimately increasing blood pressure. In order to further examine this pathway an AT1 receptor blocker, olmesartan, was administered.

Olmesartan treatment significantly decreased systolic blood pressure in both empty vector and Sry3 treated animals at day 17, compared to post-gene delivery. There was a decrease of 20-30 mmHg that occurred within 1 day of treatment and lasted until the drug treatment was ceased. The heart rate increased, though not significantly, and activity levels remained normal, indicating that the decreased blood pressure is largely due to decreased vascular resistance and probably not decreased blood volume. Interestingly, PRA was significantly lower in Sry3 treated animals treated
with olmesartan over control animals treated with olmesartan. A potential mechanism for this is that the increased PRA after Sry3 delivery led to increased Ang II, which negatively feeds back to inhibit renin production. The empty vector animals treated with olmesartan wouldn’t have the negative feedback signal from increased Ang II and baroreceptor signaling of low blood pressure would increase renin levels. Additionally, blockade of the AT\(_1\) receptor inhibits the negative feedback cycle of the RAS, resulting in increased renin and ang I (Atlas, 2007).

Urinary sodium excretion was significantly higher with olmesartan treatment in both empty vector and Sry3 animals compared to Sry3+SHAM animals. Ang II increases aldosterone through the AT\(_1\) receptor, so olmesartan treatment would block this effect and sodium excretion is higher than is seen in Sry3+SHAM animals. Urine DA and NE were also significantly higher in Sry3+olmesartan animals over empty vector+olmesartan animals. Increased Ang II production could stimulate the SNS, which could account for the increase in urinary catecholamines. However, the increased DA and NE are only seen in Sry3+olmesartan animals and not Sry3+SHAM animals. Additionally, the Sry3+olmesartan treated animals had higher urine DA at baseline and post-drug treatment.

Olmesartan binds to the AT\(_1\) receptor and blocks Ang II from binding, Ang II will then bind to the AT\(_2\) receptor, AT\(_3\) receptor, or AT\(_4\) receptor. Ang II has the highest binding affinity for the AT\(_2\) receptor, after the AT\(_1\) receptor (Miyazaki et. al, 1998). When Ang II binds the AT\(_2\) receptor it mediates vaso-relaxation and leads to a decrease in
blood pressure. It is expected that olmesartan treatment would lead to decreased blood pressure in Sry3 animals as Sry3 increases Ang II which binds to the AT\textsubscript{1} receptor and causes vasoconstriction, blocking the AT\textsubscript{1} receptor would abolish this effect. The available Ang II would then bind to the AT\textsubscript{1} receptor and result in vaso-relaxation.

The animals injected with empty vector also saw a significant blood pressure decrease during olmesartan treatment, despite the fact that they remained normotensive after gene delivery. It is important to note that the RAS actively regulates homeostatic control of blood pressure in all individuals, it is mis-regulation of the RAS that leads to the pathophysiology of hypertension (Ferrario, 2009). The empty vector treated animals would still have some Ang II available to bind to the AT\textsubscript{2} receptor when olmesartan treatment blocked the AT\textsubscript{1} receptor. The AT\textsubscript{2} receptor mediated vaso-relaxation is the most likely culprit for the decrease in blood pressure seen in empty vector animals treated with olmesartan. When healthy, human male volunteers were given olmesartan medoxomil, they had a decrease in pressor response that was dose-dependent on the amount of olmesartan delivered (Yoshida et. al, 2004). PRA increased in empty vector animals during olmesartan treatment, this is expected as blockade of the AT\textsubscript{1} receptor inhibits the negative feedback cycle of the RAS, resulting in increased renin and ang I (Atlas, 2007).
CHAPTER VI

CONCLUSIONS

The results of this study support the hypothesis that delivery of Sry3 to normotensive rats increases blood pressure through the renin angiotensin system pathway and that blockage of the AT₁ receptor with olmesartan medoxomil reverses the blood pressure effect in Sry3 treated animals and also decreases the blood pressure of empty vector treated animals. The study was designed to further examine the actions of Sry3 delivery upon blood pressure and renin-angiotensin system. Treatment with olmesartan did indeed reverse the blood pressure increase seen with Sry3 delivery, suggesting that the AT₁ receptor plays an extremely important role in mediating Sry3 induced blood pressure increase. Additionally, empty vector animals had greatly reduced blood pressure with olmesartan treatment, confirming that the AT₁ receptor is a critical mediator of blood pressure in all subjects, not just those with hypertensive indices.

There are multiple copies of Sry and different copies have been shown to have different actions upon the cardiovascular system. This study suggests Sry3 increases
blood pressure through the renin-angiotensin system, earlier studies show that Sry1 increases blood pressure through the sympathetic nervous system (Ely et al., 2010), Sry2 does not have a discernible effect on blood pressure (Ely et al., 2009). There are other copies of Sry that could have additional effects on blood pressure that have yet to be studied. These multiple copies are potentially expressed in tandem in tissues and exhibit their effects due to interaction with one another, making the current study a mere snapshot of the Sry story.

Though multiple copies have been shown to have expression in various tissues, Sry3 is unique to the borderline hypertensive SHR/y rat which has WKY autosomes and an SHR Y chromosome. This makes Sry3 of particular interest as a possible candidate gene in Y-linked hypertension. The current study indicates that the renin-angiotensin system and specifically the AT$_1$ receptor are potentially important targets in combating sex-specific hypertension and that olmesartan medoxomil is an appropriate drug to treat such hypertension. Hypertension is a complex, common disease that plagues the American medical system, costing billions of dollars each year, this cost is increased by difficulties in diagnosis and treatment of the disease (Alcocer et al., 2008). In order to correctly treat hypertension, it is imperative that the causes be examined in great detail. Studies such as the current one are important in investigating the possible causes and treatment in Y-linked hypertension.
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