THE EFFECTS OF EXOGENOUS SRY1 AND SRY3 ON THE RAT KIDNEY

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THE EFFECTS OF EXOGENOUS SRY1 AND SRY3 ON THE RAT KIDNEY

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Thesis

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For my grandparents, Frank & Louise Chiarappa, whose great sacrifice paved the way for my endless opportunity.
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CHAPTER I

INTRODUCTION

There exists on the Y-chromosome of mammals a gene that is responsible for the differentiation of testis. This region of the Y-chromosome was pinpointed in 1986 by Vergnaud et. al. By utilizing deletion mapping in humans it was discovered that the gene responsible for creating the male phenotype was located on the short arm of the Y-chromosome. This region on the Y-chromosome was shown to contain the element responsible for testis formation and thus was named the testis determining gene or factor (TDF) (Vergnaud, 1986). The actual gene on the Y-chromosome was later named Sry (Sex determining region of the Y-chromosome) and was originally associated with, and named for, male gonadal development. This is due to male differentiation occurring after expression of Sry in the genital ridge. The testis determining factor (TDF) was later defined as the protein produced from transcription of the Sry gene (Koopman, 1990).

Since Sry was discovered almost 20 years ago, relatively little has been discovered about the exact mechanisms by which it acts. There has been enough work done, however, to replace the now inadequate name of “testis determining factor”. This is due to the many diverse functions of Sry throughout the entire body; not only in
development, but throughout adulthood as well. The protein expressed by Sry is now called, accurately, the SRY protein.

At about the same time Sry was discovered, Y-chromosome linked hypertension in the rat was also being revealed (Ely et al. 1990). However, a decade passed before Sry was even suggested as a candidate for the Y-linked hypertension (Ely et al. 2000). Most mammals have only one copy of Sry while the rat has multiple functional copies. The determination of multiple copies in humans is currently under investigation. Only two years ago were all six full length copies of Sry and their ancestral lineage described in the SHR rat, a strain of the common lab rat *Rattus norvegicus* (Turner et al. 2007). The SHR rat is the logical choice when determining the causes of Y-linked hypertension because the defining characteristic of this strain is hypertension partially associated with the Y-chromosome.

The problem then, is which copies of Sry cause hypertension and by what mechanism do they act? We know certain strains of rat are hypertensive due to their Y-chromosome. We know Sry is associated with hypertension and located on the Y-chromosome. We also know there are 7 Sry loci in the SHR strain and only 6 loci in the WKY strain, and the 6 loci in common are identical; SHR contains the locus Sry3, which is not found in the normotensive WKY strain (Turner et al. 2009). Additionally, Sry1 has been shown to elevate blood pressure and sympathetic nervous system (SNS) indices when delivered to the kidney of WKY rats (Ely et al. 2009). Hence, Sry1 and Sry3 are the most likely candidates for the Y-chromosome linked hypertension found in rats. It is
likely that they act via different mechanisms; and there might be an interaction or relationship between the two causing elevated blood pressure beyond what would be seen if only one copy was expressed or delivered exogenously.

The importance of studying the Sry gene, and its functions, is multifaceted. First and foremost, we still do not have a complete understanding of how and why Sry alters blood pressure and other physiological factors. Therefore, the scientific body of knowledge will be increased by identifying the mechanisms by which different copies of Sry cause hypertension. Secondly, by identifying the functions of different copies in different tissues, physiological effects other than modification of blood pressure might be revealed. Thirdly, if it becomes possible to identify which loci of Sry cause hypertension and by which mechanisms, it would also be possible to screen individuals at risk for developing Sry-related hypertension (Y-linked hypertension) or diagnose the exact cause of existing hypertension should it be Y-linked. Finally, it might then be possible to target and turn on/off which copies of Sry get expressed, thereby controlling blood pressure via gene therapy.

Therefore, the specific goal of this study is to determine the physiological effects of Sry1 and Sry3 on the rat kidney as it relates to blood pressure; this includes measurement of urine sodium, urine catecholamines, and glomerular filtration rate in the whole animal, along with catecholamine release and total catecholamine content of the renal nerve in the isolated perfused kidney. It is hypothesized that when Sry3 is delivered to the kidney, catecholamines in the kidney and urine will not change but,
sodium will retained compared to controls, as it is suspected that Sry3 acts via the renin-angiotensin system; additionally, when Sry1+3 combination is delivered, it is hypothesized that catecholamines will increase in both the kidney and urine, and sodium will still be retained as the action of Sry1 is through the sympathetic nervous system.
CHAPTER II

BACKGROUND OF THE STUDY

Sry has been identified as the Y chromosome locus responsible for testis determination in many mammalian species (Koopman et al. 1990). It encodes a transcription factor including a 79-80 amino acid high mobility group (HMG) box DNA binding domain (Underwood, 2008). The HMG box is highly conserved across species however regions outside the HMG box are prone to rapid mutation as Sry is not involved in genetic recombination (Graves, 2002). Sry has been implicated as the cause of hypertension in the spontaneously hypertensive rat ( SHR) and is responsible for the blood pressure disparity between genders (Ely et al. 1993; Ely et al. 2007). Sry has been shown to increase blood pressure in Wistar-Kyoto Rats (WKY) with the SHR Y chromosome (SHR/y rat) (Ely et al. 2007). The rat Y chromosome is unique, in that it contains multiple functional Sry loci, whereas the human Y chromosome has not been studied for multiple copies. The 6 loci found on the SHR Y chromosome have been identified as Sry1, Sry2, Sry3, Sry3B, Sry3B1, and Sry3C (Turner et al. 2007). Of all transcripts, Sry2 has been found to be present in the largest quantities in all tissues and the remaining loci are found in different relative amounts depending on the specific
tissue (Turner, 2007). It is these differences in the relative amounts of each factor that could be responsible for hypertension in the SHR rat.

There have been differences noted between sexes in the human and animal model related to blood pressure. Males tend to have higher blood pressure and higher sensitivity to changes in blood pressure. It was found that in young adult SHR rats blood pressure (mean arterial pressure) is higher in males versus females and the sex difference can be eliminated by the inhibition of the renin-angiotensin system (RAS) (Yanes et al. 2006). It was also shown that male rats given AngII and high salt had increased renal injury, whereas females were protected from this effect; this injury could result in the exacerbation of hypertension caused by AngII and the high salt diet (Sartori-Valinotti et al. 2008). A possible culprit responsible for this sex related difference is testosterone. It has been suggested that hypertension in SHR rats is mediated through the RAS and this effect is shown to be exacerbated by testosterone (Reckelhoff et al. 2000). Androgen receptor antagonism also attenuates hypertension in male SHR (Reckelhoff et al. 1999). It was reported that the renal nerves play an important role in regulating blood pressure in SHR rats, but they are not responsible for the sex difference in blood pressure (Iliescu et al. 2006).

Here, the focus will be on the kidney, as it plays an integral role in the modulation of water balance, electrolytes, vessel tone, and ultimately blood pressure. Blood pressure in the kidney is regulated within seconds via neural receptors and regulated longer term (hours to days) via hormones (Guyton, 1991). Blood pressure is
controlled by the central nervous system (CNS) acting on the kidneys through the renal nerves as well as through the renin-angiotensin system primarily through the many effects of angiotensin II (AngII). AngII upregulates SNS activity, tubular Na resorption, and arteriolar vasoconstriction. Many parts of the kidney are known to be innervated including the glomeruli, the vessels, and certain parts of the tubules (DiBona, 2005). Changes in the frequency of the signals in the renal nerves can effect homeostatic regulation of renal blood flow, glomerular filtration rate, renal tubular epithelial cell solute and water transport, and hormonal release (DiBona, 2005). This innervation allows for the kidney to coordinate circulation, filtration, reabsorption, excretion, and renin secretion (DiBona, 2002).

The effects of Sry1 and Sry3 on renal function will be examined in an attempt to identify the mechanism by which each copy of Sry alters blood pressure. It has been shown that Sry1 acts via the sympathetic nervous system (SNS) pathway by upregulating tyrosine hydroxylase (TH) and increasing resting plasma norepinephrine (NE) levels (Ely et al. 2007; Ely et al. 2009) leading to increased blood pressure. These Sry1 blood pressure effects were negated when SNS blockers were introduced (Ely et al., 2000). Additionally, there was found to be an elevated stress response associated with Sry1 (Ely et al. 2009). We know that Sry3 does not act through the SNS and preliminary data suggests that Sry3 might be acting via the renin-angiotensin system (RAS). An important component of hypertension is stress and therefore, particular attention was paid to how different copies of Sry might be involved in a stress-specific hypertensive response. A
A variety of techniques were used in an attempt to tease apart differences in renal function that Sry might induce. Sry1 and Sry3 will be examined individually as well as simultaneously in an effort to observe any interaction between the two.
CHAPTER III

METHODS

Electroporation and Surgery

In all cases throughout these experiments, male WKY rats of similar age were used. The rats were sedated with Isoflurane gas anesthetic. A 5cm incision was made on the midline of the abdomen and the left kidney was located. The kidney received 100uL containing 50ug of DNA. 10 received Sry3, 10 received Sry1+3, and the remaining 7 served as controls. The injection was done with one 50uL delivery to the lower portion of the kidney and the other 50uL to the upper portion. Both injections were given in the medulla. The 50 ug of DNA consisted of the expression construct Sry1/pcDNA 3.1, a combination of Sry1/pcDNA 3.1 and Sry3/pcDNA 3.1, or control vector (pcDNA 3.1 only). The control vector contained sequences necessary for transcription but no exogenous DNA was added to the vector. Tweezer electrodes (BTX Tweezertrodes Model #522) connected to an electro-stimulator (ElectroCellManipulator™, ECM® 830, BTX, a division of Genentronics), were placed on
opposite sides of the kidney and 20 pulses were administered at 200 volts, each lasting 20 msec, at 1000 Hz. The animals were then sutured, given 2500 units (0.5mL) of penicillin via intramuscular injection, supplied with Tylenol water (25mL in 400mL H₂O) ad libitum, Pro-lab 3000 food, and allowed to recover for 7 days.

The animals were placed in metabolic cages 4 times throughout the study. Each time they were placed in a metabolic cage, they remained there for 24±1 hours. The first placement in the cages occurred on day 0, before gene injections, which allowed the animals to become acclimated to the cages and additionally served as a baseline for subsequent urine analysis. The remaining placements in the cages occurred on days 7, 14, and 21. 1 mL of 1N HCl was added to each collection tube as well as a few drops of mineral oil to prevent urine evaporation. The animals were provided food and water ad libitum while in the metabolic cages. Urine was collected at the end of each time interval, the volume was noted, and the samples were immediately frozen in a -80⁰C freezer for later analysis. Urinary sodium was analyzed using flame photometry while epinephrine (Epi), norepinephrine (NE), and dopamine (DA) were measured using high pressure liquid chromatography (HPLC) with electrochemical detection (as described in Ely et al. 2007). Additionally, creatinine clearance rate (Cₘ) was also measured as an approximation of the glomerular filtration rate (GFR) of the kidneys. On day 21, after urine had been collected, the kidneys were removed and perfused using the technique in the “Perfused Kidney Analysis” section below. The kidneys were frozen in a -80⁰C
freezer after perfusion, eventually homogenized, and kidney Epi, NE, DA, and tyrosine hydroxylase (TH) was measured via HPLC with electrochemical detection (Ely, 2007).

Catecholamine and Tyrosine Hydroxylase Analysis

Renal TH and catecholamines were measured by HPLC with electrochemical detection (Waters 2465, Milford, MA) (Ely et al. 2007). For TH analysis kidneys were homogenized in 500 μl of 0.25 M sucrose. The homogenate (100 μl) was added to both a blank tube and a reaction tube. The chemical reaction was based on procedures developed by Nagatsu et. al. and modified by Hooper et. al. and Kumai et. al. followed by extraction using the same method as for catecholamines.

Perfused Kidney Analysis

The animals were divided into two groups: Some animals would have their kidneys removed and perfused 8 days post gene delivery (Sry1 only) and others would be perfused 21 days post gene delivery (Sry1, Sry3, and Sry1+3). The 21 day group contained 50 animals with 30 each receiving Sry1, Sry3, or Sry1+3 (20 serving as controls) and the 8 day group contained 22 animals (11 serving as controls). The rats
were sedated with 0.5mL to 0.8mL sodium pentothal. An incision was made on the midline of the abdomen and the left kidney was located. The kidney received a 100uL injection containing 50ug of DNA and was electroporated as previously described. Each animal received either Sry1/pc3.1 or control vector as illustrated above.

Depending on the group, at 8 or 21 days post gene injection, the kidneys were removed for analysis. The animals were given 1000 units of heparin (1.0mL) and sedated with 0.5-1.0mL sodium pentothal. The abdominal cavity was opened and the left kidney located. Adipose and connective tissue were dissected away from the kidney, the renal artery, and the aorta in close proximity to the renal artery so that the renal nerve was exposed (Figure 1). The kidney was removed and a 21-gauge blunted needle was secured in the aorta with suture string. The other end of the cut aortic section was closed with suture string, allowing fluid to only flow into the kidney via the renal artery. A syringe was attached to the needle so that oxygenated Krebs solution could be immediately delivered to the kidney (Figure 2). In each case the ischemic time of the kidney was less than 2 minutes. Once the kidney had been nearly blanched (clear Krebs solution replacing blood giving the kidney a beige coloration), which took about 10mL, the syringe was detached from the needle. The needle with the attached kidney was then suspended from the perfusion apparatus (Figure 3). The apparatus delivered oxygenated Krebs solution to the kidney at a rate of 6mL/min via pulsatile pump. The Krebs solution was kept heated for delivery to the kidney at a constant physiological temperature (37±1°C) and a lamp was placed directly next to the suspended kidney to
keep it warm and aid in visualization of the renal nerve. The kidney was allowed to
equilibrates and reach a constant baseline pressure (about 40-45mmHg). The renal
nerve was then stimulated with an electrode in one minute intervals. The voltages
delivered via the electrode were 0, 4, 8, and 16 volts. The pressure was recorded
constantly on a Gould physiograph and the typical pressure increase can be seen from
the tracing in Figure 4. The procedure for perfusing the kidney was adapted from Jones
Figure 1. Left kidney with renal artery exposed. Left kidney exposed with fat & connective tissue dissected away. Note placement of suture string on abdominal aorta; when kidney was removed, the blunted needle was placed where the lower string is tied off allowing delivery of Kreb’s to the kidney via the renal artery. In the Doppler procedure the cuff was placed around the renal artery directly next to the kidney.
Canulating Aorta in Proximity to Renal Artery

Figure 2. Canulating aorta in proximity to renal artery. Abdominal aorta being cannulated with a 21-gauge blunted needle. The syringe contains oxygenated Kreb’s solution.
Figure 3. Kidney hanging from perfusion apparatus. Kidney being perfused via pulsatile pump. The pressure transducer (blue, top center) relays pressure to the physiograph (see Fig. 5). The condenser tube (top left) heats Krebs solution to physiological temperature before it reaches kidney. The light facilitates visualization of the renal nerve and provides additional warmth.
Collecting Sample with Electrode on Renal Nerve.

Figure 4. Collecting sample with electrode on renal nerve. Kidney being perfused with electrode on renal nerve. An LDH sample was being taken at the end of the experiment to assess damage to kidney. Note complete blanching of kidney as Krebs solution replaces blood.
Physiograph Pressure Recording

Figure 5. Physiograph pressure recording. A pressure tracing from the Gould physiograph. As the renal nerve is being stimulated by the electrode (see Fig. 4), NE is released causing vasoconstriction and a concomitant rise in (blood) pressure. Each stimulation period (4, 8, and 16 volts) lasted one minute and a 2mL sample is collected during that time. Samples were also collected for each control period and twice for LDH.

The perfusate was collected and analyzed for catecholamines (CATS). Lactate dehydrogenase (LDH) was also collected at the beginning and end of the perfusion as a marker for cellular damage. The collection procedures for the kidney perfusate CATS consisted of delivering 4V, 8V, and 16V to the renal nerve stepwise for one minute with one minute control periods in between each stimulation. After perfusion, the kidneys were frozen in a -80°C freezer, homogenized and analyzed for EPI, NE, DA, and TH.
Doppler Probe Monitoring of Renal Blood Flow

Six animals were used for renal Doppler blood velocity analysis. The rats were sedated with 0.5mL to 0.8mL Sodium Pentothal. A 5cm incision was made 3cm right of the midline of the abdomen and the left kidney was located. The kidney received a 100uL injection containing 50ug of Sry1 or control vector. The kidney was electroporated and the animal sutured, given 5000 units of penicillin and Tylenol water as described previously.

The Doppler unit was implanted 19 days post gene injection. The animals were anesthetized with Sodium Penethol and again a 5cm incision was made right of the abdominal midline. The renal artery was separated from the renal vein and a 10mm section of artery was isolated as close to the kidney as possible. The Doppler probe (0.5mm Craig Hartley, Houston, TX was placed around the isolated portion of the renal artery and secured with two sutures around the outside of the probe. The probe fit around the artery in the same way foam insulation fits around a copper pipe; a slit along one side can be opened then slid around the pipe for a secure fit. Think of the two sutures then as tying two pieces of string around the foam insulation to keep it from sliding, rotating, or opening at the slit. The two electrical leads from the probe were looped inside the body cavity to provide slack before being routed under the skin and out dorsally through the animals neck. A 1cm incision was made in the skin on the back of the animal and another on the neck posterior to the ears. The leads were routed out
of the body cavity opening and under the skin to the opening on the back and then to the opening on the neck. Approximately 3-4 cm of the lead was exposed from the neck which was then sutured in place; two more sutures were used to close the incision. At this time the body cavity was sutured shut, with the last suture including the leads to secure them, the skin was then sealed with staples. A single staple closed the small opening made on the back of the animal. Antibiotic ointment was applied to the wounds and the animals were given 5000 units of Penicillin and Tylenol water (10% solution) and allowed to heal for one day.

Starting 20 days post injection, Doppler readings were recorded. The animals were sedated with 0.4-0.8cc of Sodium Brevital (50mg/kg, I.P.). Electrical leads were attached to the leads protruding from the animals’ necks. These leads were connected to a Doppler signal amplifier and blood flow could be heard. The onomatopoeia for blood flow through the renal artery is “wish-shoe” in the same way that a beating heart sounds like “lub-dub”. In addition to hearing the flow, a readout of blood flow velocity was given in units of cm/s. The range in flow velocity was collected for one minute while the animal was unconscious and again while the animal was awake to create a baseline value. The flow velocity always increased once the animal regained consciousness and did not vary between groups. A baseline was established for all animals as a point from which to compare changes in flow velocity. The high and low values for flow during the one minute awake period were averaged from all animals and
all recordings; a value of 4.0cm/s resulted which served as the baseline value in all subsequent calculations.

After the animals were awake and walking around their cage, they were stressed with a tail pinch. They were pinched with the thumb and index finger at the base of the tail for 10 second intervals. The animal was allowed to settle down and once blood flow velocity returned to baseline they were stressed again; this was repeated 3 times. The results during stress were therefore calculated as the average of those 3 successive tail pinches.

Statistical Analysis

Data analysis was performed and graphs were created using SigmaStat and SigmaPlot (Systat Software Inc. Richmond, CA). Statistical outliers were removed using the Dean and Dixon Outlier Procedure. In all data sets, ANOVAs were used to determine significance and if there were differences found, individual t-tests were performed to specify exactly which subsets of data were significant (i.e. if a certain day in a month long trial was significant). Data in the graphs are displayed as the mean ± standard error. All data in the graphs are consistently organized (i.e. controls are always white bars; Sry1 is always black, etc.). Any p-value below 0.05 was considered statistically significant, illustrated by an asterisk (*) in the graphs. Two asterisks (**) indicate a p-
value less than 0.01, and three asterisks (***) indicate a p-value less than 0.001. In all cases, comparisons are versus controls, unless otherwise noted.
CHAPTER IV

RESULTS

Urine Analysis

This section pertains only to Sry3 and Sry1+3 combination. Sodium output in the urine differed significantly between the groups. A 2-way ANOVA (treatment, day) showed the groups were significantly different ($F= 11.613, p<0.001$). There was a significant difference between the animals receiving Sry3 vs. Sry1+3 combo, but no difference between days 0, 7, 14, or 21. There was no significant interaction between day and treatment group. There were no differences between Sry3 and Sry1+3 combo on day 7. However, on day 14 Sry1+3 had significantly lower sodium output than both the control ($p=0.003$) and Sry3 ($p=0.019$). On day 21 both Sry3 and Sry1+3 animals had decreased sodium in their urine ($p=0.014$ and $p=0.003$ respectively). On days 14 and 21, animals receiving Sry1+3 had about a 2-fold lower sodium output. These results were calculated using the % change from the individual animal’s baseline obtained on day 0. Sodium output was calculated in mmol/hour/100 grams of body weight and the results are shown in Figure 6.
Urine volume also differed between the groups. A 2-way ANOVA (treatment, day) showed significant differences between Sry3 and Sry1+3 combo (F=18.688, p<0.001). There was no significant difference between the days and there was no interaction between day and treatment group. On all days Sry1+3 produced decreased urine output (p=0.003 day7, p<0.001 days 14 & 21). This difference was about 2-fold less than controls on all days. Sry3 alone produced a decrease in output but to a lesser extent. On days 7 and 21 there was about a 75% decrease in output (p=0.01 and p=0.008). On day 14 there was a decrease in urine output by Sry3; it was not statistically significant (p=0.077). The results are graphed in Figure 7 as a % change from the baseline of individual animals.

Creatinine clearance values followed the same trend as Na output and urine volume with both Sry3 and Sry1+3 causing a significant decrease compared to controls using a 2 way ANOVA (treatment, day) (F=16.733, p<0.001). There was no significant difference between the days and there was no interaction between day and treatment. On all days Sry1+3 caused a decrease in C\textsubscript{Cr} (p=0.003 day 7, p=0.007 day 14, p<0.001 day 21). Sry3 caused a decrease on day 21 only (p=0.001) but was close on day 7 (p=0.057). For clarity this data is shown in Figure 8 as the % change from baseline.

The amount of epinephrine in the urine was decreased by Sry1+3, but not by Sry3 alone. A 2-way ANOVA (treatment, day) showed that on all days Sry1+3 caused a significant decrease in the amount of Epi excreted in the urine (F=23.719, p<0.001). There was no significant difference between the days and there was no interaction.
between day and treatment. The decreased became less pronounced over time (p<0.001 day 7, p=0.001 day 14, p=0.041 day 21). The results were calculated as nanograms excreted per hour and shown in Figure 9.

There was no statistically significant difference in the body weights of the animals between groups. There was no difference in the NE or DA concentrations in the urine, nor were there any differences in NE, Epi, or DA output in the kidney perfusate. Additionally, there were no differences found in NE, Epi, or DA content in the kidney homogenate.
Sry3 and Sry1+3 Decrease Na Excretion

On day 14 Sry1+3 was significantly lower than both the control (p=0.003) and Sry3 (p=0.019). On day 21 both Sry3 and Sry1+3 animals had decreased sodium in their urine (p=0.014 and p=0.003 respectively). On days 14 and 21, animals receiving Sry1+3 had about a 100% lower sodium output.

All values graphed as mean ± SEM. *= p<0.05, **= p<0.01.
Sry3 and Sry1+3 Decrease Urine Output

Figure 7. Sry3 and Sry1+3 decrease urine output. Sry1+3 produced decreased urine output (p=0.003 day7, p<0.001 days 14 & 21). This difference was about 100% less than controls on all days. Sry3 alone produced a decrease in output, but to a lesser extent. On days 7 and 21 there was about a 75% decrease in urine output (p=0.01 and p=0.008) due to Sry3 alone. All values graphed as mean ± SEM. **= p<0.01, ***= p<0.01.
Figure 8. GFR decreased by Sry3 and Sry1+3. Creatinine clearance was measured as a way of estimating the glomerular filtration rate (GFR). On all days Sry1+3 caused a decrease in C\textsubscript{Cr} (p=0.003 day 7, p=0.007 day 14, p<0.001 day 21). Sry3 caused a significant decrease on day 21 only (p=0.001), but was close on day 7 (p=0.057). All values graphed as mean ± SEM. ** = p<0.01, *** = p<0.01.
Figure 9. Urine epinephrine decreased by Sry1+3 combo. Sry1+3 caused a significant decrease in the amount of epinephrine excreted in the urine. The decrease became less pronounced over time (p<0.001 day 7, p=0.001 day 14, p=0.041 day 21). Sry3 had no effect. All values graphed as mean ± SEM. *= p<0.05, **= p<0.01, ***= p<0.001.
Perfused Kidney

This section pertains only to Sry1. LDH levels ranged from 21 to 169U/L which fell within the normal range (136-196 U/L) indicating no tissue damage. LDH values had a mean of 38.93U/L for the first perfusate collection and 112.64U/L for the final collection with an overall mean of 81.84U/L for all samples. There was no Epi or DA in any of the collected perfusates. The NE perfusate content of the Sry1 animals was compared to the NE content of the empty vector animals and the results have been graphed in Figure 9.

The NE output in the empty vector groups were significantly different in the two 16V trials at 8 and 21 days (p=0.006). These two groups had been hypothesized to be equal, as exemplified in all of the 8V trials.

T-tests and a 2-way ANOVA were performed and there was a significant increase in NE output during stimulation between the Sry1 and control animals at the 16V stimulation level 8 days post injection (p=0.02). It should be noted that there was no difference between treatments at the 16V level at 21 days post injection. There was no significant difference when comparing the groups at the 4V stimulation level. When looking at the 8 day group and comparing the SRY vs. Control animals at 8V and 16V, the p-values were 0.819 and 0.020 respectively. When looking at the 21 day group and comparing the SRY vs. Control animals, there was no significant difference at 8V or 16V (p-values were 0.728 and 0.620 respectively).
Now discussing only the 8 day trial; while the NE output upon stimulation was increased by Sry1 (p=0.02), there was no significant difference in either total NE in the kidney homogenate (p=0.0547) or the fractional release of NE (p=0.665). Although it should be noted that NE in the kidney homogenate might be considered significantly increased by Sry1 with a p-value of 0.0547.
Sry1 Increases NE Release from the Kidney

Figure 10. Sry1 increases NE release from the kidney. Sry1 caused a significant increase in the amount of norepinephrine released from the kidney upon stimulation of the renal nerve (p=0.02). This difference was only noted at 16 volts, 8 days post gene delivery. All values graphed as mean ± SEM. ** = p<0.01.
It was found that in all animals there was a decrease in flow velocity during stress. This was to be expected because when an animal is stressed, the SNS is activated resulting in decreased blood flow to the kidneys and other non-essential organs momentarily. However, there was a more significant decrease in the animals that received Sry1. A 2-way ANOVA (treatment, day) showed this decrease compared to controls (F=50.152, p<0.001). The ANOVA showed there was not only a treatment effect, but a day effect as well. It showed Sry1’s effect on the kidney dissipated over time (F=3.899, p<0.001). It is important to note there was no significant change over time in the control group.

The difference in blood velocity decrease was dramatic, almost a 2-fold decrease due to Sry1, up to about 25 days post gene injection. The difference became less dramatic and at about 32-35 days, there appeared to be no difference between the Sry1 and the control groups. Figure 11 shows the results calculated as a percent decrease in blood flow velocity during stress compared to the average standard baseline. The standard baseline was calculated as the average of all baseline readings before each tail pinch stress was delivered.
Sry1 Decreases Renal Blood Velocity After Stress

Days Post Gene Injection

Renal Blood Velocity (% Change from Baseline)

-300
-250
-200
-150
-100
-50
0

Sry1
Control

Figure 11. Sry1 decreases renal blood flow velocity after stress. Sry1 caused a significant decrease in blood flow velocity in the renal artery upon stress (p<0.001 for days 21-31). Blood flow velocity was measured using a Doppler probe & stress was induced via a tail pinch. Values are graphed as % change from baseline before stress. All values graphed as the mean for the 3 day range. **= p<0.01.
Table 1. Perfused kidney raw data. Comparison of the average values of samples collected while perfusing the kidney at different voltages. LDH was collected before the application of voltage and again after all samples had been collected. DA and Epi were never detected in any perfused kidney samples.

Perfused Kidney Raw Data

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NE @ 4V (pg/mL)</th>
<th>NE @ 8V (pg/mL)</th>
<th>NE @ 16V (pg/mL)</th>
<th>LDH before (U/L)</th>
<th>LDH after (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92.6</td>
<td>529.3333</td>
<td>978.6</td>
<td>50.2</td>
<td>104.3</td>
</tr>
<tr>
<td>Sry1</td>
<td>0</td>
<td>404.125</td>
<td>845</td>
<td>76.1</td>
<td>80.3</td>
</tr>
<tr>
<td>Sry3</td>
<td>37.6</td>
<td>622.4</td>
<td>990.9</td>
<td>71.5</td>
<td>69.6</td>
</tr>
<tr>
<td>Sry1+3</td>
<td>46.5</td>
<td>594.8</td>
<td>904.4</td>
<td>59.2</td>
<td>59.7</td>
</tr>
</tbody>
</table>

Table 2. Kidney homogenate raw data. Comparisons of the average values for catecholamines and tyrosine hydroxylase found in the whole kidney tissue (homogenized), after perfusion. Note DA and TH were elevated in the Sry1+3 group, but the results were not statistically significant.

Kidney Homogenate Raw Data

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DA (pg/g tissue)</th>
<th>NE (pg/g tissue)</th>
<th>Epi (pg/g tissue)</th>
<th>TH (fmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8195.4</td>
<td>106029.4</td>
<td>0</td>
<td>32.1</td>
</tr>
<tr>
<td>Sry1</td>
<td>8550.8</td>
<td>108694.4</td>
<td>0</td>
<td>32.88</td>
</tr>
<tr>
<td>Sry3</td>
<td>8579.7</td>
<td>106897.1</td>
<td>0</td>
<td>32.22</td>
</tr>
<tr>
<td>Sry1+3</td>
<td>9497.8</td>
<td>105509.3</td>
<td>0</td>
<td>36.45</td>
</tr>
</tbody>
</table>
CHAPTER V
DISCUSSION

As stated previously, there are 2 blood pressure modulating systems at work in the kidney; the SNS and the RAS. These two systems alter blood pressure via different mechanisms but they are intertwined and inherently interconnected. The RAS’s actions are carried out by AngII and most of the known effects are mediated through the angiotensin type 1 (AT$_1$) receptor; its actions include vasoconstriction, aldosterone and vasopressin release, salt and water retention, sympathetic activation, and oxidative stress (Yanes et al. 2006). One can see that if the RAS is activated, then the SNS system is also activated. Therefore, if Sry1 acts through the SNS and Sry3 acts through the RAS it might be inferred that the combination of the two could have more dramatic effects than either Sry1 or Sry3 alone. This combination effect was observed in this study with respect to Na excretion, urine volume, GFR, and urine epinephrine.

It is unclear why there was such a dramatic decrease in urine Epi with Sry1+3 but no difference with only Sry1 or Sry3. It might be due to more NE being utilized in the SNS response and thus a lesser amount would be available for conversion to Epi. However, there was no difference in the amount of NE in the urine.
In a disease state such as hypertension, abnormal regulation of sympathetic nerve activity contributes to the abnormalities of renal function (DiBona, 2002). Low-frequency renal nerve stimulation does not affect renal blood flow or GFR, was still found to cause a decrease in urinary sodium excretion (DiBona et al. 2004).

Norepinephrine is the neurotransmitter in the renal sympathetic nerves and NE has been shown to increase Na\(^+\)K\(^+\)ATPase activity in vitro, an effect that was reversed with the addition of α\(_{1B}\)-adrenoreceptor antagonists (DiBona, 2002). It was originally thought that antinatriuresis caused by renal sympathetic activity increasing renal tubular sodium resorption were artifacts from the stress of anesthesia and surgery (Smith, 1937; Smith 1951). It has now been demonstrated in conscious animals that increasing sympathetic nerve activity causes Na resorption (antinatriuresis) and conversely decreasing nerve activity causes Na excretion (natriuresis) (Miki et al. 1989; Miki et al. 1991).

Sodium plays a key role in the modulation of blood pressure and hypertension. Whether by SNS or RAS, Na is the key component to altering blood pressure in both systems. If sodium is resorbed in the tubules, then water will follow, which could increase blood pressure. In all forms of hypertension, pressure natriuresis is abnormal due to sodium excretion being constant while arterial pressure is increased and in long-term hypertension, changes in the glomeruli and arterioles might exacerbate hypertension by allowing pressure natriuresis to become increasingly shifted from normal (Hall et al. 1996). Renal excretory function might be compromised in the hypertensive state due to an increase in renal sympathetic nerve activity (RSNA).
Increased RSNA results in reduced renal excretory function with one stimuli being AngII (DiBona, 2002).

It has been shown that SRY delivery to the adrenal medulla increases tyrosine hydroxylase and plasma NE levels (Ely et al. 2007). In this study it was found however that while NE release from the kidney is increased, there was no effect of Sry1 on total kidney TH levels. There was a significant increase in NE output in the 8 day 16V trial due to Sry1. Since there was no difference in the 21 day 16V trial it can be concluded that the effects of the Sry1 have dissipated by 21 days. Sry1 increased total NE content of the kidney (p=0.0547). It has been noted that the upregualtion of tyrosine hydoxylase, the rate limiting enzyme for production of NE, occurs after ischemia to the kidney, resulting in increase NE release (Czyzyk-Krzeska, 1997). However, their study investigated results of ischemia or low oxygen levels over longer periods of time such as hours to days and since the ischemic time of the kidneys in this experiment was <2 minutes, I feel their study have no bearing on our results.

Sry1 causes a dramatic reduction in blood flow velocity to the kidney when injected into the kidney itself. Up to about 25 days post gene injection there was more than a 100% greater decrease in blood velocity in the animals receiving Sry1 compared to controls. This effect seems to dissipate over time, near 32 days post injection. It has been shown that plasma norepinephrine levels are increased after stress (Novak, 1990), and as stated earlier renal NE output is increased with the administration of Sry1. However, it is unclear how delivery of Sry1 to the kidney effects the renal artery, which
is upstream, to elicit a greater response on renal blood velocity during stress. I hypothesize that a significant portion of the gene spills over from the kidney into circulation and might have actions in other parts of the body.

It was recently discovered that Sry1 increases renal angiotensin II (AngII) content of the kidney slightly (p=0.047) and Sry3 increases renal AngII more than 2-fold (p=0.016) (Milsted, 2009). AngII causes resorption of Na in the proximal tubule and controls GFR by constricting the efferent arterioles (Hall, 1990). Since Sry3 was found to increase AngII it provides a mechanism by which Na was reabsorbed, and explains why GFR and urine volume were decreased. All of these effects were enhanced when Sry1 was combined with Sry3. This suggests an interaction between the SNS and the RAS system in the kidney. Renal sympathetic nerve activity was found to increase renin secretion rate (JG cells), decrease urinary sodium excretion (tubules), and renal blood flow (arterial vessels) (DiBona, 2005). So not only can the RAS affect the SNS but the SNS can affect the RAS with respect to renal function. If only one copy was given, say Sry1, the blood pressure effect incurred by activation of the SNS might be masked by the RAS. However, when both Sry1 and Sry3 are given simultaneously, both systems are likely activated and there is no mechanism to restore blood pressure to normal levels.
Doppler Limitations and Complications

The limitations to measuring blood flow via Doppler probe are numerous. First and foremost, there were only 6 animals in the study and data was collected from only 5 of them. On top of that, only 2 of the animals made it to the endpoint of 40 days post-injection, and data could not be collected on every day so there are gaps in the data. In addition to the small scale of the study there were numerous technical difficulties. There was no data for one animal because the Doppler implant was put on too tightly which cut off blood flow and the subsequent death of the kidney. In addition, only data from two rats were able to be collected out to 40 days post injection. Implant failure, broken leads, or infection caused 3 animals to be removed from the study early. The Doppler implant was not properly seated for the first 3 days of implant in 3 animals until tissue started to form around the probe. This issue was attempted to be resolved by wrapping umbilical tape around the probe after implant to secure it. This worked originally, but it was found the tape caused a severe increase in connective tissue surrounding the probe as well as the entire kidney and resulted in the slight atrophy in two of the kidneys. The data collection procedure could also be improved. The investigator had to observe the readout of the flow velocity while pinching the tail of the animal and simultaneously avoid being bitten while recording the values; the accuracy of this data collection could be dramatically improved by taking recordings on a computer or video recording the readout to be watched back.
CHAPTER VI

CONCLUSIONS

The findings of this study can be summarized as follows: 1. Sry1 increases renal NE release upon stimulation/stress and Sry3 or Sry1+3 do not; 2. Sry1 decreases renal blood flow velocity upon stimulation/stress; 3. Na excretion is decreased by Sry3 and further decreased by Sry1+3; 4. GFR is decreased by Sry3 and further decreased by Sry1+3; 5. Urine volume is decreased by Sry3 and further decreased by Sry1+3; 6. Epi in urine is decreased by Sry1+3, but not by either Sry1 or Sry3 alone.

This study was designed to investigate the effects of Sry1 and Sry3 on the kidney. Both Sry1 and Sry3 are known to act in the kidney, and the kidney is a main modulator of systemic blood pressure. The effects of Sry1 and Sry3 are important because they cause an increase in blood pressure and hypertensive indices. It has been shown that Sry1 acts via the SNS and Sry3 acts through the RAS. However, it is important to note that these two systems both act to control blood pressure and are intimately connected so it is faulty logic to conclude that Sry1 only effects the SNS and Sry3 only effects the RAS. Indeed, activation of one system will consequently affect the other.
It has been shown that there is an interaction between Sry1 and Sry3. This could allude to interactions between other copies of Sry, and those interactions are undoubtedly important in the overall function of the Sry gene. The interactions between different copies of Sry must be examined because all copies are expressed simultaneously in tissues.

Sry is a prime candidate for future investigation of Y-linked hypertension. It is still not understood what every copy of Sry does in the rat model and even less is understood about its actions in humans.
REFERENCES


