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REGULATION OF MULTIPLE RENIN-ANGIOTENSIN SYSTEM GENES BY SRY

Amy Milsted[a], **Adam C. Underwood**[b], **Jeff Dunmire**[a], **Helen L. DelPuerto**[c], **Almir S. Martins**[d], **Daniel L. Ely**[a], and **Monte E. Turner**[a]

[a]Department of Biology and Program in Integrated Bioscience, University of Akron, Akron, OH, 44325-3908

[b]Division of Mathematics and Sciences, Walsh University, North Canton, OH, 44720

[c]Federal University of Minas Gerais, General Pathology Dept, UFMG 31270-010 Belo Horizonte, MG Brazil

[d]Dept. of Physiology and Biophysics, UFMG 31270-010 Belo Horizonte, MG Brazil

Abstract

We demonstrated that the Sry gene complex on the SHR Y chromosome is a candidate locus for hypertension that accounts for the SHR Y chromosome blood pressure effect. All rat strains examined to date share 6 Sry loci, and a seventh Sry locus (Sry3) appears to be unique to SHR males. Previously, we showed that Sry1 increased activity of the tyrosine hydroxylase promoter in transfected PC12 cells, and Sry1 delivered to adrenal gland of WKY rats increased blood pressure and sympathetic nervous system activity. The objective of this study was to determine whether renin-angiotensin system genes participate in Sry-mediated effects. Sry expression vectors were co-transfected into CHO cells with luciferase reporter constructs containing promoters of angiotensinogen (Agt −1430/+22), renin (Ren −1050/−1), ACE (ACE −1677/+21) and ACE2 (ACE2 −1091/+83). Sry1, Sry2 and Sry3 differentially up-regulated activity of the promoters of angiotensinogen, renin and ACE genes, and down-regulated ACE2 promoter activity. The largest effect was seen with Sry3, which increased activity of angiotensinogen promoter by 1.7 fold, renin promoter by 1.3 fold, ACE promoter by 2.6 fold, and decreased activity of ACE2 promoter by 0.5 fold. The effect of Sry1 on promoter activity was significantly less than Sry3. Sry2 activated promoters at a significantly lower level than Sry1. The result of either an additive effect of Sry regulation of multiple genes in the renin-angiotensin system or alterations in expression of a single gene could favor increased levels of Ang II and decreased levels of Ang-(1-7). These actions of Sry could result in increased blood pressure in males and contribute to gender differences in blood pressure.

Keywords

hypertension; Y chromosome; Sry genes; renin-angiotensin system

Corresponding author: Amy Milsted, Ph.D. Dept. of Biology University of Akron 302 Buchtel Commons Akron, OH 44325-3908 Tel 330-972-7976 Fax 330-972-8445 milsted@uakron.edu .

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Introduction

There are consistent gender differences in hypertension, with a greater proportion of males affected than females in most mammalian populations [1,2]. Our earlier studies demonstrated that a portion of the gender differences in blood pressure (BP) in the SHR rat mapped to the SHR Y chromosome [3]. In rats, males with the SHR Y chromosome have higher blood pressure than females, or males with a different Y chromosome. Consistent with these results, several human population studies have confirmed a Y chromosome effect on blood pressure [4,5].

We have demonstrated that the Sry gene complex is a candidate hypertension locus responsible for the hypertensive phenotype of the SHR Y chromosome [6]. Sry is a transcription factor encoded by a gene or genes on the Y chromosome. In human, mouse and most other eutherian mammals except some rodents, a single locus is present that serves as the testis-determining factor [7]. The laboratory rat *Rattus norvegicus* and a few other rodents have multiple Sry loci. On the Y chromosome of a single male *R. norvegicus* rat with a SHR/Akr Y chromosome, we identified 7 Sry loci encoding full-length Sry proteins [8]. Six of these loci are identical to those in the WKY rat, while one locus (Sry3) is unique to the hypertensive SHR/Akr Y chromosome [6]. When we analyzed expression of transcripts from each locus, we determined that all loci are expressed and that different Sry loci are expressed at different levels in different tissues and at different ages [6,8]. All loci appear to be functional, since the coding sequence of each can be expressed to produce a Sry protein.

Our previous studies both in cultured cells and in rats showed that Sry1 activates the sympathetic nervous system (SNS). In PC12 cells, Sry1 increased tyrosine hydroxylase (Th) promoter activity; this activation was reduced but not eliminated when the AP-1 site in the promoter was mutated [9]. In WKY rats with normal BP, delivery of Sry1 or Sry3 genes by electroporation increased blood pressure by approximately 15mm Hg by 21 days after gene delivery [10]. Elevated BP was maintained for about another 2 weeks, then gradually returned to baseline levels. The BP response did not fully reach the total response associated with the presence of the SHR Y chromosome, compared to that seen in a rat which has the SHR Y chromosome in a WKY background (our SHR/y consomic strain), suggesting that the magnitude of the response of Th to Sry1 was not by itself sufficient to fully explain the cause of the Y chromosome BP effects. Delivery of exogenous Sry is likely to affect multiple systems that influence BP in the rat. Following electroporation of Sry1, blood pressure is elevated, Th is activated and NE levels are elevated [10]. These results of are consistent with Sry1 increasing SNS activity, resulting in higher blood pressure.

The maintenance of multiple highly related Sry loci that generate functional proteins, together with the differential expression of the Sry loci in male rats of all ages, supports the hypothesis that Sry functions in systems other than its role in testis determination. In an effort to identify other genes that might respond to Sry and whose actions are known to affect blood pressure, we examined genes of the renin-angiotensin system. We and many others have demonstrated that altered expression of these genes is associated with changes in blood pressure [11-13]. Further, examination of promoter sequences of angiotensinogen (Agt), renin, ACE and ACE2 reveals the presence of consensus Sry-binding sites in each [14], consistent with the potential of Sry to bind these genes and affect their expression. Because the renin-angiotensin system is also known to play a critical role in BP regulation, we hypothesized that a component of the BP rise in rats after electroporation with Sry vectors was due to the actions of Sry on the renin-angiotensin system. Small changes in expression patterns of Agt, renin, ACE and ACE2, the major participants of the classical RAS, could serve to shift the relative amounts of production of the vasoconstriction or pro-hypertension peptide Ang II compared to the vasodilation or

anti-hypertension peptide Ang-(1-7). If this change in expression occurred in response to Sry, it could provide another mechanism for gender-related differences in blood pressure.

In the studies described here we delivered mammalian expression vectors containing Sry1, Sry2 or Sry3 coding sequences by cotransfection into cultured cells together with target renin-angiotensinogen system gene promoters to examine whether these genes altered activity of promoters of the Agt, renin, ACE and ACE2 genes.

Methods

Growth and Cotransfection of CHO Cells

Chinese Hamster Ovary cells (CHO-K1, ATCC #CCL-61) were cultured on 100mm plates (Nunc™) to approximately 50% confluence in HAM's F12K medium (Sigma) supplemented with 10mM HEPES and 10% fetal bovine serum (Atlanta Biologicals) in a humidified atmosphere at 37°C and 5% CO_2.

Prior to transfection, ~ 25,000 cells (6.6×10^3 cells/cm^2) were seeded to 24 well cassettes (COSTAR) and incubated overnight. Each well was transiently cotransfected with 50 ng effector plasmid, 500 ng firefly luciferase reporter (either Agt(−1430/+22), renin (−1050/−1), ACE(−1677/+21) or ACE2(1091/83+)), and 500 pg of control construct, phRL-null Renilla, (Promega). After a 4hr incubation, complexes were removed and fresh complete medium was applied. After 24 hrs cells were processed for luciferase activity using the reagents and protocol provided in The Dual-Luciferase® Reporter (DRL™) Assay System (Promega). Luciferase activity of all reporters was measured on a Turner Biosystems $20/20^n$ luminometer. Firefly Renilla luciferase ratios were used to calculate the activity of each reporter in the presence of an Sry effector construct relative to reporter activity obtained from CHO transfected with an empty vector, pEF1/CTL, the negative control. All samples were run in triplicate in at least 3 different experiments.

Sry Effector Plasmids

Protein coding regions of Sry1 (GenBank: AY157669), Sry2 (GenBank: AY157670), and Sry3 (GenBank: AY157672) were subcloned into the expression vector pcDNA3.1(-) (Invitrogen™) or pEF1/*Myc*-His (Invitrogen™) using Sry sequences originally amplified from a male SHRy rat. (2 refs) The control plasmid, pEF1/CTL, contains no inserted DNA. Effector plasmids pEF1/Sry1, pEF1/Sry2 or pEF1/Sry3, as well as the control plasmid pEF1/STL containing no insert were used for relative comparison to the Sry effectors.

Renin-Angiotensin System Gene Promoter/Luciferase Reporter Constructs

The primers listed in Table 1 were used to amplify promoter sequences of Agt, renin, ACE and ACE2, using SHR/y genomic DNA as template. Amplicons were digested with the indicated restriction endonucleases and cloned into pGL3 Basic vectors (Promega). The reporter plasmid phRL-null (Promega), a promoter-less vector containing a synthetic *Renilla* luciferase gene, was used as the internal control for each sample. The phRL-null vector provided a consistent basal level of *Renilla* luciferase expression in CHO-K1 cells and was validated for use in sample normalization.

Statistical analysis

Data reported represent means ± SEM of 3-4 trials conducted in triplicate with each Sry effector construct. Statistical analysis was performed by using Student's t-test. Analyses were run on SigmaStat software (Jandel Scientific, San Rafael, CA) with significance assumed at $p<0.05$.

Results

Sry regulation of Agt, renin, ACE and ACE2 promoter activity

Cotransfections with Sry3 effector plasmid and promoters of rat Agt, renin, ACE and ACE2 genes show that promoter activity of Agt, renin and ACE promoters is increased while activity of ACE2 is decreased (Figure 1). Sry3 only is shown in Figure 1. Sry3 increased activity of the Agt promoter by1.6-fold ($p<.001$), increased activity of the renin promoter by 1.3-fold ($p<.05$), increased activity of the ACE promoter by 2.5-fold and decreased activity of the ACE2 promoter to 0.5-fold compared to the control. Sry3 is the SHR/Akr-unique Sry locus and transfection of the Sry3 effector plasmid has the greatest effect on transcriptional activity.

Individual Sry proteins have differential effects on promoter activity

Effects of Sry1 and Sry2 on promoter activity are similar to Sry3, but of smaller magnitude (Figure 2). Sry1 is intermediate and Sry2 consistently shows the smallest effect. Compared to the control plasmid, Agt (−1430/+22) promoter activity was increased by Sry1 ($p<.001$), Sry2 ($p<.001$) and Sry3 effector plasmids ($p<.001$) (Figure 2A). Compared to the control plasmid renin (−1050/−1) promoter activity was increased by Sry1 ($p<.05$) and Sry3 ($p<.05$) plasmids but not by Sry2 (Figure 2B). Activity of the ACE (−1677/+21) promoter was significantly increased by Sry1 ($p<.001$) and Sry3 ($p<.001$) and to a lesser extent by Sry2 ($p<.001$) (Figure 2C). All Sry effector plasmids significantly decreased activity of the ACE2 promoter (−1091/+83) (Sry1, $p<.01$; Sry2, $p<.05$; Sry3, $p<.001$) (Figure 2D).

On each promoter, Sry3 elicited the largest change in promoter activity, followed by Sry1, with Sry2 producing the smallest effect. These results showed that promoter activity of Agt, renin and ACE was increased by all Sry expression constructs while ACE2 promoter activity was decreased by all Sry constructs. In addition, there were statistically significant differences among the effects of Sry1, Sry2 and Sry3 on promoter activity, except for the ACE2 promoter.

Discussion

The main products of the renin-angiotensin system are Ang II and Ang-(1-7). In blood vessels the two peptides have opposing actions: Ang II causes vasoconstriction and Ang-(1-7) causes relaxation of blood vessels [15]. Ang peptides have multiple effects on other organs and systems as well, particularly in the kidney, as reviewed recently by Santos et al [16], Ingelfinger [17], and Raizada and Ferreira [18]. The relative balance of these two Ang peptides can tilt in favor of increased BP with more Ang II and decreased BP with relatively more Ang-(1-7). Any regulator of RAS genes has the potential to alter the balance of Ang II to Ang-(1-7) produced, which could ultimately alter BP.

Gender differences in RAS activity have been documented by many studies over the past 30 years, including those of Ellison et al. [19] and Chen et al. [20]. Male rats were shown to have higher levels of Agt mRNA and higher PRA. Thus there is ample evidence to support a somewhat more active RAS in males compared to females. A recent study of congenic mRen (2).Lewis and control Lewis rats by Pendergrass et al. describes higher levels of plasma Ang II in male congenics compared to male or female Lewis rats. Plasma Ang-(1-7) was higher in female in female congenics. Male congenic hypertensive mRen(2).Lewis rats also had higher levels of circulating renin and ACE, angiotensinogen, and renal cortical and medullary Ang II, and cortical ACE2 activity, all of which are indicators of an activated RAS [21].

The effects of this coordinate regulation would lead to increasing activity of promoters of genes of the RAS cascade involved in generating Ang II. By decreasing activity of ACE2, the enzyme primarily responsible for the generation of Ang-(1-7) from Ang I and the processing of Ang II to Ang-(1-7), the net effect of Sry protein regulation on the RAS is a shift in peptide

production to favor the production of Ang II and suppresses production of Ang-(1-7). Increased Ang II levels due to increased Sry expression would be expected to lead to increased BP.

Although the magnitude of the response of each RAS promoter activity is modest, the fact that Sry modulates activities of multiple RAS gene promoters could produce a significant additive effect. This would be the case whether the regulatory effects of the Sry proteins on the genes of the renin-angiotensin system were direct or indirect. Further studies will be required to determine if the effects of Sry are direct and involve Sry binding to cognate DNA sequences. Whatever the mechanism, the overall effect of Sry is to increase expression of genes involved in Ang II generation and to decrease expression of ACE2, the gene whose product has been shown to be associated with shifting the balance of Ang peptide generation from Ang II to Ang-(1-7) (Figure 3). More circulating Ang II, as well as more NE, raise BP, and since females lack Sry, the increased BP would be seen in males but not females.

Data from this study and our previous work [10,22] suggests possible mechanisms involving activation of the RAS and SNS that contributes to gender differences in hypertension. Sry activates the SNS by increasing tyrosine hydroxylase promoter activity, resulting in production of more NE. Sry also activates the renin-angiotensin system by increasing promoter activity of angiotensinogen, renin, ACE genes and suppressing activity of ACE2 promoters, resulting in generation of more Ang II and less Ang-(1-7). Several studies have shown that Sry loci are expressed in appropriate BP-regulating tissues (kidney, adrenal gland, brain) [23-25].

CHO cells were selected for use in these experiments because they are a commonly used model cell line for studies of this type, and because ovarian cells have been shown to express pro-renin, renin, Agt, ACE, Ang II, Ang II receptors and ACE2 [26,27]. The use of this responsive cell line is not meant to imply that ovarian cells are involved in a major way in BP regulation; it does indicate that in cells, even those not directly involved in BP regulation, Sry has the demonstrated ability to modulate expression of RAS genes.

Interactions of the RAS and SNS may serve to amplify the effects of Sry on BP. Ang II is known to potentiate many of the effects of NE. In rat juxtaglomerular cells, the NE-induced renin secretion rate was amplified in the presence of Ang II and the NE-induced intracellular calcium response was also potentiated by Ang II [28]. Ang II infusion in the isolated pig adrenal gland caused up to a 7 fold increase in epinephrine and norepinephrine (NE) [29]. Ang II in sub-pressor concentrations enhanced the contractions of rabbit isolated aortic strips to noradrenaline. Noradrenaline constrictor responses are also potentiated by Ang II, independent of any effect of Ang II on neuronal uptake of noradrenaline [30]. Ang II has been shown to influence BP by autonomic nervous system modulation, and in the brain, Ang II can induce sympatho-excitation via the rostral ventrolateral medulla [31]. Ang II stimulates norepinephrine transporter and tyrosine hydroxylase (TH) in the neurons. In ganglionic cells direct stimulation of angiotensin type 1 (AT_1) receptors leads to exocytotic junctional catecholamine release. In both the absence and presence of preganglionic sympathetic activity, this mechanism contributes significantly to Ang II-induced enhancement of catecholamine release [32]. Ang II causes both acute and chronic stimulation of NE uptake in neuronal cultures and it is blocked by losartan. These effects are potentiated in SHR compared to WKY [33]. Results of these studies support the notion that small changes in Ang peptide levels can produce detectable physiological responses in the SNS and in the whole animal.

In summary, our results from cotransfecting Sry effector plasmids with RAS gene promoter/reporter vectors support our hypothesis of the potential of Sry to regulate not only the SNS but also the RAS, in males, to produce the male-specific increased BP seen in the SHR/Akr strain. Since females lack the Sry loci, BP in females with the same genetic background will not increase to the same extent since the Sry-stimulated contribution to the RAS is absent.

We now have evidence that rat Sry proteins modulate expression of both the sympathetic nervous system, through Th, and the renin-angiotensin system, through angiotensinogen, renin, ACE and ACE2 genes. Small effects on multiple genes by Sry could be sufficient to account for higher blood pressure in males compared to females. Recently two studies have reported copy number variation of the SRY genes in human males, with as many as 16 copies present in some individuals [34,35]. Whether these multiple human SRY copies are functional remains to be determined, but the mere presence of multiple copies in humans lends additional support to our studies of the function of the multiple rat Sry loci. It is interesting that in our studies, Sry3, the locus unique to the hypertensive SHR/Akr Y chromosome, consistently caused the largest effects as a regulator of RAS gene transcription. If the end result of the effects of Sry on BP is simply an additive effect of the contribution of each of the Sry loci, then the presence of the Sry 3 locus in hypertensive male rats but not in normotensive males may be sufficient to raise BP into the hypertensive range.

Condensed Abstract

To determine whether Sry genes from the SHR Y chromosome regulate renin-angiotensin system genes, Sry expression vectors were co-transfected into CHO cells with reporter constructs containing promoters of rat angiotensinogen, renin, ACE and ACE2 genes. Sry1, Sry2 and Sry3 differentially up-regulated activity of angiotensinogen, renin and ACE genes, and down-regulated ACE2 promoter activity. The potential for an additive effect of Sry regulation of multiple genes in the renin-angiotensin pathway favors generation of vasoconstrictive Ang II and decreased levels of Ang-(1-7), and results in increased blood pressure in males. These actions could partially account for gender differences in blood pressure.

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Abbreviations and symbols

BP	blood pressure
SNS	sympathetic nervous system
Th	tyrosine hydroxylase
Agt	angiotensinogen
RAS	renin-angiotensin system

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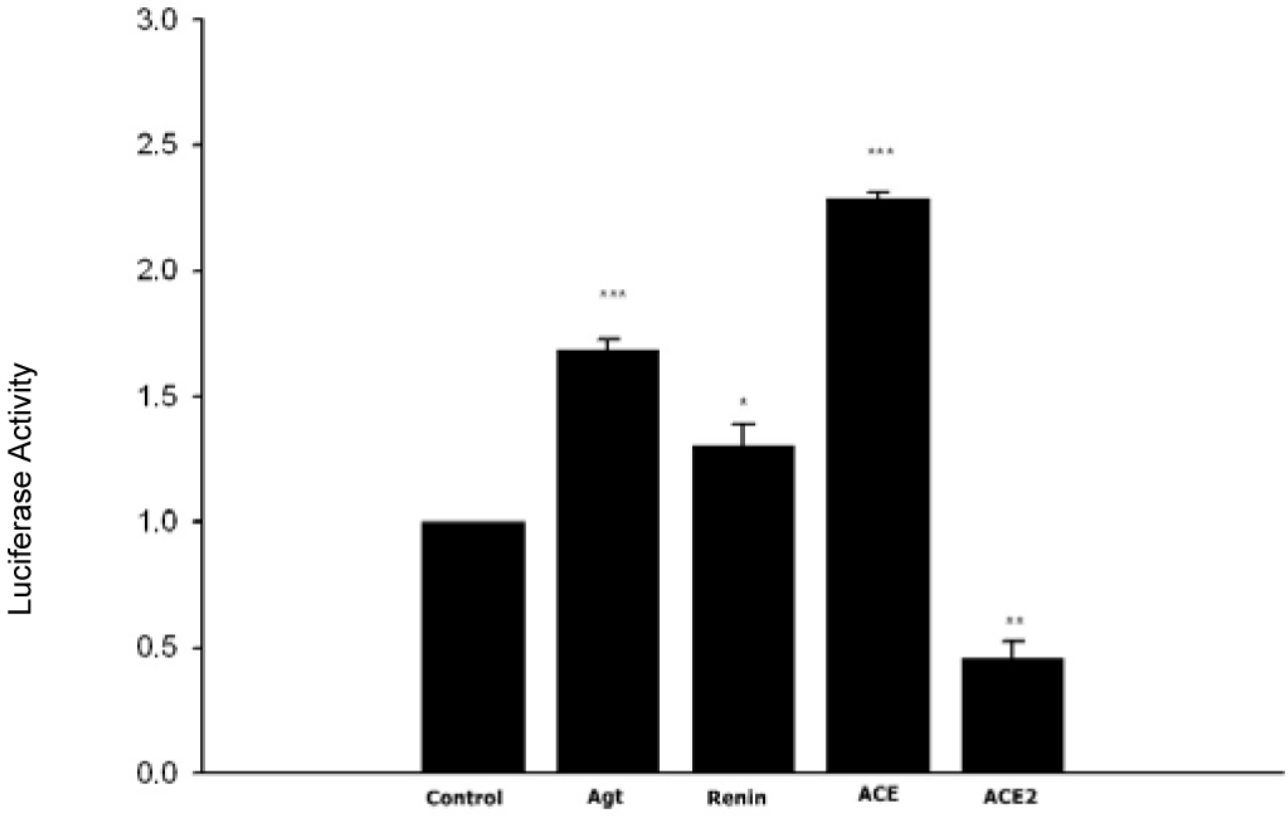

Figure 1.

Sry3 increases activity of renin, angiotensinogen and ACE promoters while decreasing activity of ACE2. *p=<.05 compared to control, **p<.001 compared to control, ***p<.001 compared to control (n=3-4).

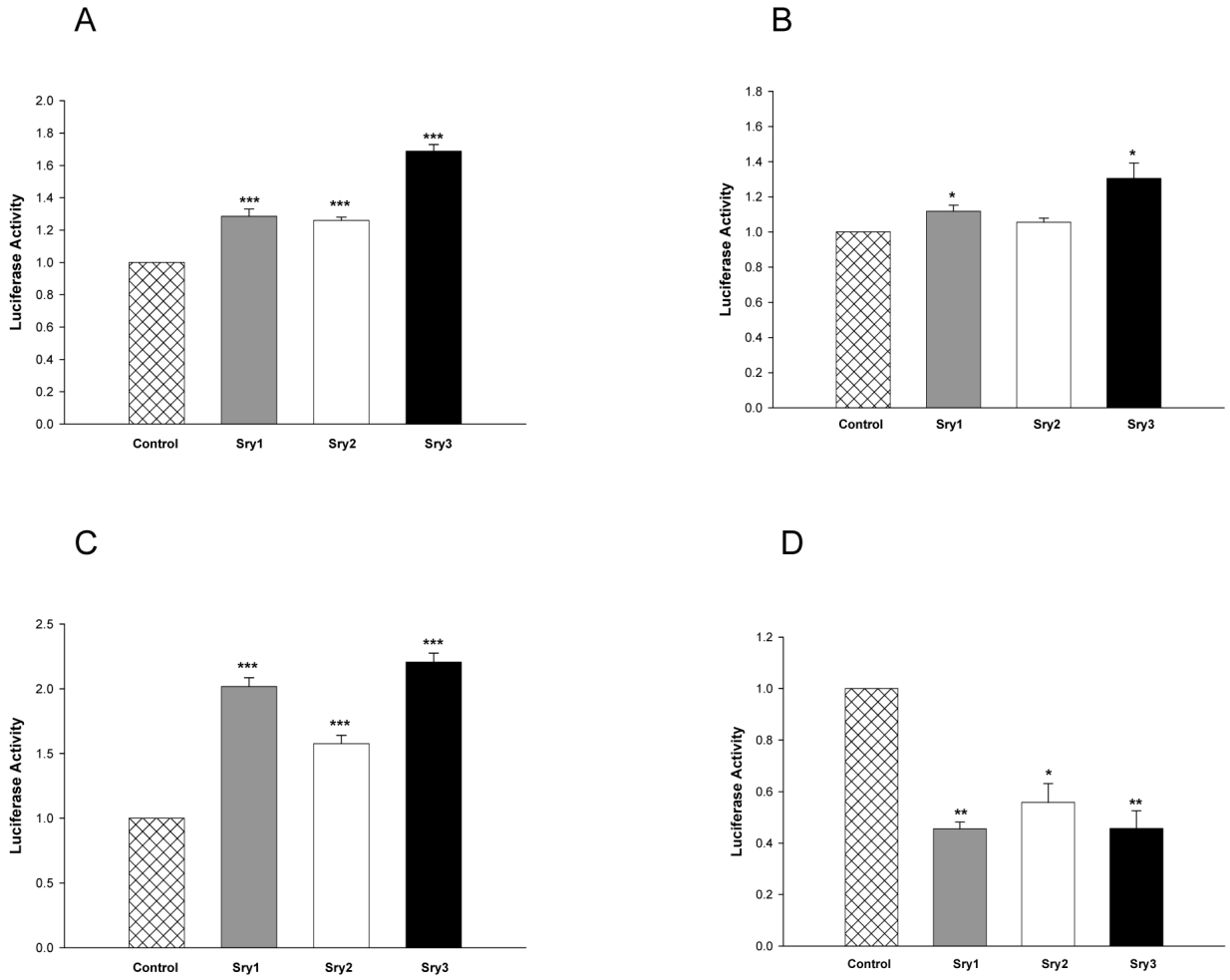

Figure 2.

Differential effects of Sry1, Sry2 and Sry3 on promoter activity of [A] angiotensinogen, Agt (−1430/+22) (n=4), [B] renin, Ren (−1050/−1) (n=4), [C] ACE, ACE (−1677/+21) (n=4) and [D] ACE2, ACE2 (−1091/+83) (n=3). *p=<.05 compared to control, **p<.001 compared to control, ***p<.001 compared to control. For Agt: Sry1 is significantly different from Sry2 (p<.001), Sry2 is significantly different from Sry3 (p<.001) and Sry1 is significantly different from Sry3 (p<.001). For Ren: Sry2 is significantly different from Sry3 (p<.05). For ACE: Sry1 is significantly different from Sry2 (p<.005) and Sry2 is significantly different from Sry3 (p<.001). For ACE2 there are no significant differences among the effects of Sry1, Sry2 and Sry3:

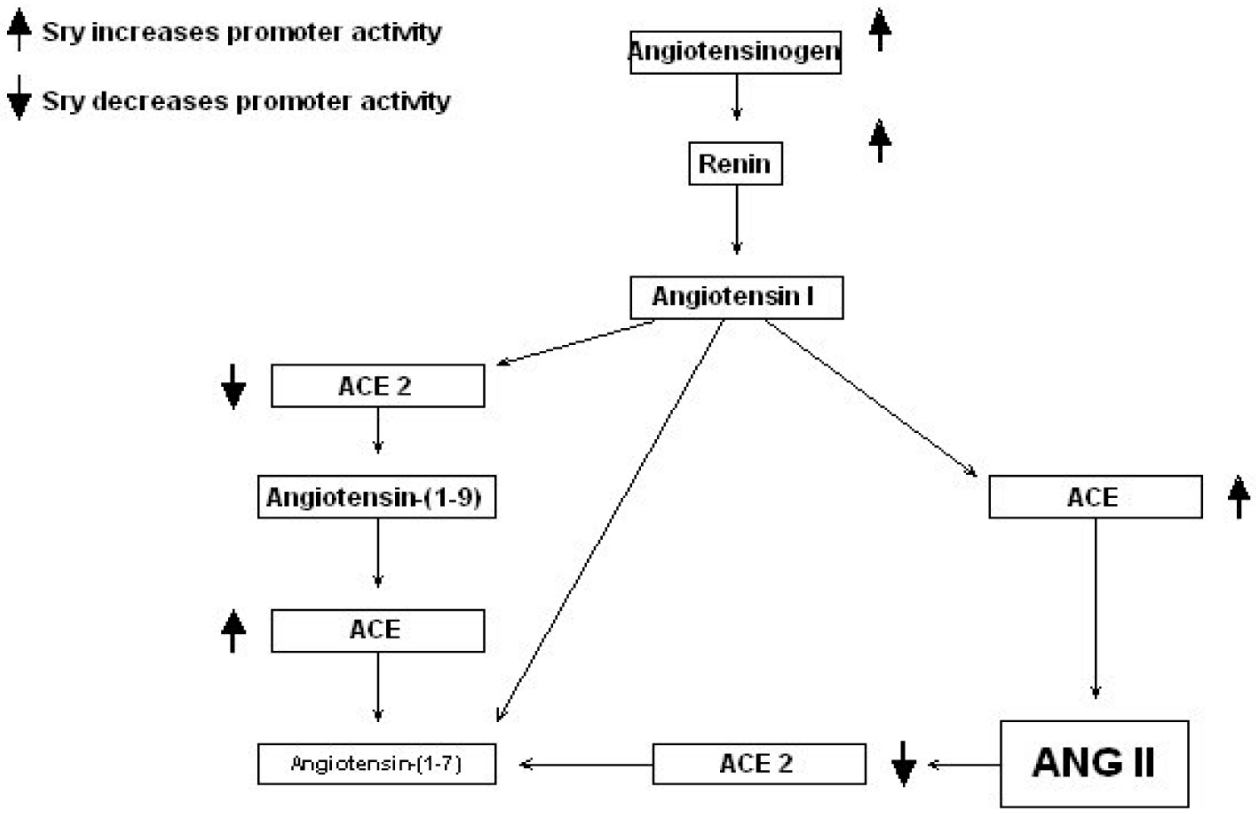

Figure 3.
The classical renin-angiotensin system, with arrows indicating promoter responses to Sry. The combined effects of Sry on genes of the renin angiotensin system would favor increased levels of Ang II and decreased levels of Ang-(1-7).

Table 1

Cloning promoters of rat renin, angiotensinogen, ACE and ACE2 genes

Gene	Primer name	Primer sequence
Renin	R-Renin Hind III	5′-CGT AAG CTT CAG TGA CGC TGG AGT C–3′
	L-Renin1000 Nhe I	5′–CCA AGC TAG CCC TGT CTT TAG ATA TCT GAG–3′
Agt	R-Agt Hind III	5′-CAC AAG CTT AGC CAA GAT GGA GCA AGG– 3′
	L-Agt1500 Nhe I	5′-CAA GCT AGC ATC ACT GGC CAG CTC CAT AG-3′
ACE	R-ACE CDS	5′-CAG CAG CAG CGA CAG CAT CAA GAG –3′
	L-ACE1200 Mlu	5′-CGA ACG CGT CTG AGT ACC CAG GCT ATC-3′
ACE2	ACE2Rt	5′-CTTTCCCCGTGCGCCAAGATCC-3′
	ACE2(933)Mlu	5′-CGA ACGCGTCACGATCTCATGCCTATGG-3′