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Comparative Analysis of Renin-Angiotensin System (RAS)-Related Gene Expression Between Hypertensive and Normotensive Rats

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Background: The renal renin-angiotensin system (RAS) is physiologically important for blood pressure regulation. Altered regulation of RAS-related genes has been observed in an animal model of hypertension (spontaneously hypertensive rats – SHR). The current understanding of certain RAS-related gene expression differences between Wistar-Kyoto rats (WKY) and SHR is either limited or has not been compared. The purpose of this study was to compare the regulation of key RAS-related genes in the kidneys of adult WKY and SHR.

Material/Methods: Coronal sections were dissected through the hilus of kidneys from 16-week-old male WKY and SHR. RT-PCR analysis was performed for *Ace*, *Ace2*, *Agt*, *Agtr1a*, *Agtr1b*, *Agtr2*, *Atp6ap2* (PRR), *Mas1*, *Ren*, *Rnls*, and *Slc12a3* (NCC).

Results: Increased mRNA expression was observed for *Ace*, *Ace2*, *Agt*, *Agtr1a*, *Agtr1b*, and *Atp6ap2* in SHR compared to WKY. *Mas1*, *Ren*, *Slc12a3*, and *Rnls* showed no difference in expression between animal types.

Conclusions: This study shows that the upregulation of several key RAS-related genes in the kidney may account for the increased blood pressure of adult SHR.

MeSH Keywords: **Kidney • Rats, Inbred SHR • Rats, Inbred WKY • Receptor, Angiotensin, Type 1 • Receptor, Angiotensin, Type 2 • Renin-Angiotensin System**

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Background

The kidney is an important regulator of blood pressure and can be involved in the pathogenesis of hypertension. Renal genes involved in hypertension are responsible for functions such as sodium resorption, renin-angiotensin system (RAS) activity, and catecholamine regulation [1–3]. Hypertension can result from altered gene expression during different time points of development; however, the exact changes that occur during adult life that contribute to hypertension are unclear [2,4].

In this study, renal gene expression was compared between adult hypertensive rats (spontaneously hypertensive rats – SHRs) and normotensive control rats (Wistar-Kyoto rats – WKYs). The genes studied are involved in renal physiology and mechanisms for blood pressure regulation. Most of these genes are related to the RAS and consisted of angiotensin-converting enzymes 1 and 2 (ACE and ACE2; *Ace* and *Ace2*), angiotensinogen (AGT; *Agt*), angiotensin type-1a, type-1b, and type-2 receptors (AT1Ra, AT1Rb, and AT2R; *Agtr1a*, *Agtr1b*, and *Agtr2*), (pro) renin receptor (PRR; *Atp6ap2*), Mas receptor (*Mas*; *Mas1*), and renin (*Ren*). Two additional genes that were investigated were the Na⁺/Cl⁻ cotransporter gene (NCC; *Slc12a3*) and renalase (*Rnls*). Although altered expression of RAS-related genes has been previously linked to hypertension, information regarding several important genes is still limited and several genes have yet to be studied in the adult SHR kidney [2]. Further, NCC was selected because there are no previous studies that have analyzed its mRNA expression in SHRs. Renalase, an enzyme which lowers the activity of catecholamines (i.e., adrenaline, noradrenaline, and dopamine), was selected because it was shown to be significantly upregulated in the kidneys of critically hypertensive rats (systolic blood pressure >180 mmHg) [5]. This finding also represents the only study to date examining the regulation of renalase in SHRs. Adrenaline and noradrenaline stimulate adrenergic receptors, create the general effect of vasoconstriction throughout the circulatory system, and play a pivotal role in blood pressure homeostasis and hypertension [6]. Because renalase may regulate the activity of catecholamines, it is proposed to be a new candidate gene for hypertension [3]. The purpose of our study was to determine if there are any novel alterations in gene expression that could be responsible for increased blood pressure in SHRs.

Material and Methods

Animals, blood pressure measurements, and tissue collection

Male SHRs and WKYs were purchased from Taconic Farms (Germantown, NY, USA). All protocols regarding the handling of animals have been previously described [7]. Animals were

cared for in accordance with the Canadian Council on Animal Care guidelines, and were approved by the Laurentian University Animal Care Committee. Blood Pressure was determined using a non-invasive BP monitor (Coda6, Kent Scientific, CT, USA) as previously described [7]. The kidneys (n=6; 16 weeks of age) were collected and stored at –80°C until use.

Homogenization of tissue, RNA extraction, and reverse transcription PCR (RT-PCR)

Coronal sections approximately 2 mm thick were cut along the hilus of each kidney. Samples were placed in TRIZOL reagent (Sigma-Aldrich Corp., MO, USA), homogenized with the QIAGEN TissueLyser (Newtown, PA, USA), and RNA was extracted by following the manufacturer's instructions. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). RNA samples were treated with DNase as per the manufacturer's instructions (Sigma-Aldrich), and reverse transcribed using the M-MLV reverse transcriptase kit (Promega, Madison, WI, USA). PCRs were performed using the GoTaq flexi DNA polymerase (Promega), appropriate primers, and amplification conditions (Table 1). PCR products were resolved on a 2% TAE gel and imaged with the BioRad UV ChemiDoc UV analyzer. Densitometry was performed using Quantity One software (BioRad). Specific gene expression was normalized to rat 28s rRNA (*Rn28s1*) and fold changes in the SHR were calculated with respect to WKYs. Statistical analyses were performed using GraphPad Prism software (La Jolla, CA, USA). Differences between SHRs and WKYs were analyzed using an unpaired *t* test.

Results

At 16 weeks of age, average systolic, diastolic, and mean arterial blood pressures were higher in SHRs compared to WKYs, as previously described [7]. Renal gene expression analysis revealed that mRNA levels of several genes related to the RAS were significantly higher in SHRs when compared to WKYs. Figure 1 shows that mRNA levels in SHRs were significantly increased for AT1Ra (1.71-fold; *P*<0.0001), ACE (1.27-fold; *P*<0.0001), PRR (1.12-fold; *P*=0.0003), ACE2 (1.16-fold; *P*=0.0017), AT1Rb (1.97-fold; *P*=0.0044), and AGT (1.21-fold; *P*=0.022). AT2R expression could not be detected in either WKYs or SHRs. Further, 2 RAS genes (*Mas* and *renin*) showed no difference in expression between WKYs and SHRs (Figure 2). Neither renalase nor NCC showed significant differences (Figure 3).

Discussion

All genes that were found to be upregulated in the SHR belonged to the RAS (Figure 1). Previous studies have shown

Table 1. Primer specifications and conditions used for conventional reverse transcription PCR.

Gene name and NCBI accession number	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Amplicon size (bp)	Annealing temperature (°C)
Rn28s1 NR_046246	AGGGATAACTGGCTTGTGGC	TAAACCCAGCTCACGTTCCC	143	58
Ace NM_012544.1	GCCCCCTGTACAAGTGTGAT*	TAGGAAGAGCAGCACCCACT*	347	61
Ace2 NM_001012006.1	CAGGAAGCTGAAGACCTGTCT	TTCAACTGTTTGTCTTGTCTG	251	56
Agtr1a NM_030985.4	CAAAGCTTGCTGGCAATGTA	TCCAGCTCTGACTTGTCTT	228	59
AT1Ra	CTGCCACATTCCTGAGTTAAC	ATCACCACCAAGCTGTTTCC	302	??
Agtr1b NM_031009.2	GAGTGACAGAGACCAGACCAGAC	ATCACCACCAAGCTGTTTCC	307	63
Agtr2 NM_012494.3	TAATCTCAACGCAACTGGCACC	GCCAAAAGGAGTAAGTCAGCCA	222	59
Mas1 NM_012757.2	GGCGGTCATCATCTTCATAGC	CTTCTTCTACTGCTGCCAC	313	59
Slc12a3 NM_019345.3	ATGTTCTGCTTACCTGGTGG	GTGCTCACAAGTCCACAAG	251	57
Atp6ap2 NM_001007091.1	CCGTGGCACCATGGCTGTGCT	GCAAGCCCTGGCCAAGACAG	204	65
Agt NM_134432.2	TTCAGGCCAAGACCTCCC*	CCAGCCGGGAGGTGCAGT*	309	63
Rnls NM_001014167.1	GATAACAAGTGGGAAGTCTC	CATAAAAGAGGCCAGAGC	187	54
Ren NM_012642.4	CACTTGTGCTCTGGACCT	GGGGTACCAATGCCGATCTC	250	63

* Primer pairs for *Ace* and *Agt* were from previous papers ([8,19], respectively).

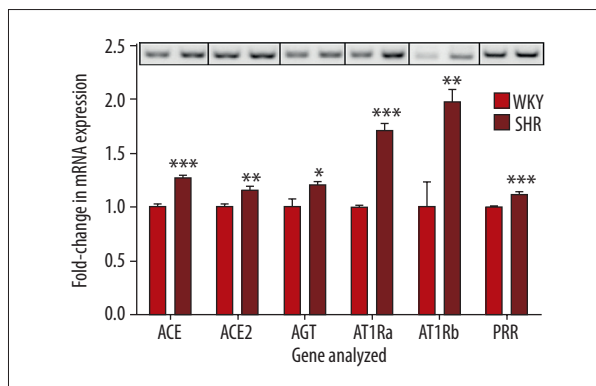


Figure 1. Elevated expression of renal RAS genes in SHRs compared to WKYs. RT-PCR analysis of transcripts for ACE, ACE2, AGT, AT1Ra, AT1Rb, and PRR (n=6). Data are normalized to the 28s gene and fold changes between WKYs and SHRs are expressed as mean \pm SEM. Upper panels show representative gel bands. Significant differences between strains are denoted by * $p \leq 0.05$; ** $p \leq 0.01$, and *** $p \leq 0.001$.

similar results for ACE, PRR, Mas, renin, and renalase [5,8–11]. Our data for the expression of AGT, ACE2, AT1Ra, and AT2R are inconsistent with several previous reports suggesting that the regulation of these genes may depend on factors such as age, sex, stress hormones, epigenetics, sodium intake, and/or complex feedback mechanisms [4,9,10,12].

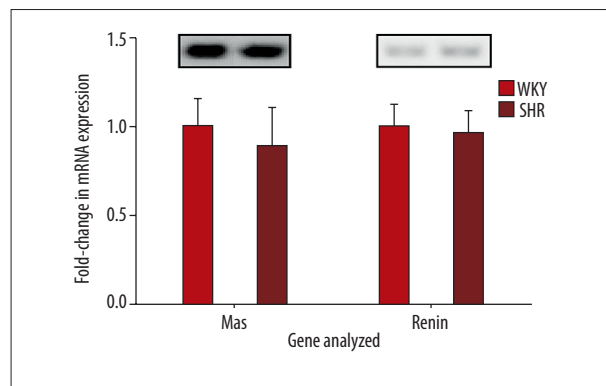


Figure 2. Renal RAS genes that remain unchanged between WKYs and SHRs. RT-PCR analysis of transcripts for Mas and renin (n=6). Data are normalized to the 28s gene and fold changes between WKYs and SHRs are expressed as mean \pm SEM. Upper panels show representative gel bands.

In previous studies, the expression of AGT mRNA has been observed to be either similar, upregulated, or downregulated in SHRs compared to WKYs [4,9,11]. These findings suggest that RAS activation, which is initiated by AGT, is likely regulated by a complex mechanism. Factors that may play a role in this mechanism include age, sex hormones, and cortisol [4,11,13]. However, the increase in AGT mRNA reported here is consistent with the evidence that it is commonly upregulated in the SHR kidney during adulthood.

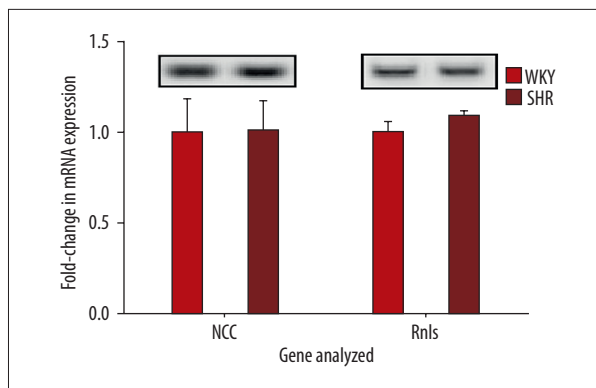


Figure 3. Transcript analysis of renal NCC and renalase in WKYs and SHRs. RT-PCR analysis of transcripts for renal NCC and renalase (n=6). Data are normalized to the 28s gene and fold changes between WKYs and SHRs are expressed as mean \pm SEM. Upper panels show representative gel bands.

At 16 weeks of age, ACE and PRR showed 1.27- and 1.12-fold increases in the SHR, respectively. Previous studies have reported much higher expression of these 2 genes in 18-week-old SHRs, which may be explained by different experimental methods (e.g., the use of qPCR and a different sample area for tissue collection) or differences in epigenetic modifications (e.g., H3Ac and H3K4me3 histone modifications) [8,9]. Upregulation of ACE, demonstrated in this and other studies of hypertension, support the functional importance of its known vasoconstrictive effects [2]. Regarding PRR, this receptor has been previously associated with an increase in blood pressure when binding renin or the renin precursor, (pro)renin [14]. Although renin expression is unchanged between WKYs and SHRs, upregulation of PRR may still independently increase the activity of this receptor [11]. However, when PRR is overexpressed in mice, hypertensive effects are not observed [14]. Although it has been reported that PRR mRNA is increased in the kidneys of SHRs and diabetic rats, this increase may be caused by its regulation by AT1R activity [9,15]. The increase in PRR expression suggests that this receptor may play a physiological role in blood pressure regulation in SHRs, but its cellular mechanism remains to be elucidated.

Increased AT1R expression may be another mechanism that contributes to hypertension. Previous studies have reported upregulation of renal AT1Ra in a transgenerational animal model of hypertension [16]. Our study supports this finding in SHRs, while other studies with 18-week-old SHRs did not [9]. AT1Rb, which is not present in humans, was also upregulated in our study. Although studies using AT1Rb knockout mice suggest the redundancy of this receptor subtype, both AT1Rs appear to be responsible for kidney structure and function, and AT1Rb may also compensate for lowered AT1Ra [17]. Both AT1Ra and AT1Rb were elevated in our study, suggesting that AT1Rb is not solely expressed as a compensatory mechanism. Although

the hypertensive effects of AT1Ra are evident, the effects of upregulated AT1Rb have not been reported [2].

Interestingly, our study shows that AT2R was not expressed in either SHR or WKY kidneys. Binding of angiotensin (Ang) II to AT2R stimulates vasodilatory effects that are antagonistic to the effects of AT1Rs [12]. Previously, research on renal AT2R expression in rats and humans has shown that it is highly expressed throughout the kidney during fetal development [2,18]. However, it is well known that both humans and rats undergo a dramatic reduction in AT2R expression postnatally [2,18]. AT2R expression in humans and rats may still exist in the glomeruli, proximal tubules, or collecting ducts during adulthood, but it appears to be only activated by sodium depletion [12,18]. Controlled sodium intake in our study may be the reason for a lack of overall AT2R expression, and comparison of with the increased expression of AT1Rs suggests that the most available Ang II receptors in the adult kidney may be AT1Rs. Protein levels should be assessed to determine their ratio of AT1R/AT2R in areas of renal Ang II production and to determine if this ratio contributes to increased blood pressure.

The current study also provides evidence of a complex regulatory mechanism between ACE2 and the Mas receptor. The physiological function of ACE2 is to produce Ang (1-7) from Ang II, which then binds to Mas and stimulates vasodilation [10]. Previously, in studies analyzing 16-week-old SHRs, renal Mas expression was unchanged but ACE2 expression was 1.5-fold lower than in WKYs; higher levels of Ang (1-7) might be responsible for this decrease [10]. In contrast, the 1.16-fold significant increase in ACE2 mRNA shown in our study presents a new observation for this gene. However, if the ACE2 upregulation found in our study was stimulated by lower levels of Ang (1-7), Mas would likely have been upregulated by this same feedback mechanism. Because Mas was unchanged between SHRs and WKYs in our study and previous studies, Mas may be tightly controlled by another mechanism in addition to Ang (1-7) regulation [10]. Further, it is likely that increased ACE2 increases Ang (1-7) production, thereby compensating for increased Ang II. Nevertheless, their overall effects on blood pressure are largely dependent on the expression of Mas, AT2R, and AT1Rs. Results from our study show that the only receptors upregulated were AT1Rs; therefore, the effects of increased Ang II production in the adult kidney may still be largely vasoconstrictive.

Previous studies analyzing NCC in younger SHRs reported that although protein levels of NCC were unchanged, ACE activity may redistribute the localization of several sodium transporters, including the NCC [1]. NCC was chosen for our study to determine if older SHRs had an alternate regulation of this gene. Although, the level of NCC transcripts were unchanged, the localization of NCC may still be influenced by an increased expression of ACE, leading to differential sodium absorption and blood pressure regulation.

Renalase expression was also unchanged in the SHRs. Previous studies show that renalase expression is increased in SHR kidneys with systolic blood pressures >180 mmHg [5]. The SHRs of our study had an average systolic blood pressure of 188.3 mmHg [7]. This suggests that there may be unknown factors responsible for increased renalase expression, in addition to the severity of the blood pressure increase. The currently unknown regulatory mechanism for renalase may not decrease catecholamines in adult SHRs sufficiently to effectively reduce the hypertensive effects of the RAS.

Conclusions

The present study adds new information and better characterizes which genes are important contributors to hypertension.

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