Mapping of a *Dde* I RELP of the α_2 -Adrenoceptor Gene and the Correlation of Genotype with Blood Pressure in the F₂ Rats of SHR-WKY Cross

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To examine a pathogenetic role of α_2 -adrenoceptor (α_2 -AR) in genetically hypertensive rats, we compared the α_2 -AR gene of spontaneously hypertensive rats (SHR) and its normotensive control Wistar-Kyoto (WKY) rats by restriction fragment length polymorphism (RFLP) analysis and did a genetic cosegregation study. After screening with 52 restriction endonucleases (RE), an RFLP was found by digestion of the genomic DNA of SHR and WKY rat with *Dde* I RE and using a human platelet α_2 -AR probe, the gene (ADRA2R) which is localized in chromosome 10. Restriction mapping analysis revealed that the RFLP is located in the 5' region of the α_2 -AR gene. This RFLP was used as a marker in a genetic cosegregation study to examine the correlation of genotype with blood pressure in F₂ rats of SHR and WKY rat cross. The distribution of the genotypes based on the RFLP conformed to a 1:2:1 ratio in male F₂ rats as expected for Mendelian inheritance, but not in female F₂ rats. The number of female F₂ rats with double SHR alleles was decreased about 68% from expected. Blood pressure of the male or female F₂ rats homozygous for the SHR α_2 -adrenoceptor alleles was not significantly higher than the F₂ rats heterozygous or homozygous for the WKY alleles with either indirect or direct blood pressure measurement. The additional finding was that males had higher blood pressure than females. This study ruled out the cosegregation of α_2 -adrenoceptor RFLP with higher blood pressure in the F₂ rats of SHR and WKY cross. However, Since the abnormality of the renal α_2 -adrenoceptor in genetically hypertensive rats is one of altered regulation, and the RFLP we found is located in the 5' region of the gene where functionally important sequences are located which regulate gene expression, this RFLP may still be of interest in genetically hypertensive rats. (*Hypertens Res* 1992; 15: 143-148)

Key Words: α_2 -adrenoceptor, SHR, RFLP

A pathogenetic role of altered α_2 -adrenoceptors in essential hypertension has been suggested in both human and animal studies. Renal α_2 -adrenoceptor density is increased in genetically hypertensive rats and this increase precedes the development of high-blood pressure and does not occur in animal models of acquired hypertension (1-8). High dietary sodium chloride augments both the blood pressure and the already increased renal α_2 -adrenoceptor density in most genetically hypertensive rats (7, 9). A genetically determined increase in renal α_2 -adrenoceptor density could raise renal vascular resistance, increase Na⁺ and water reabsorption and play a role in the pathogenesis of genetic hypertension (10-11). Cerebral α_2 -adrenoceptors are also increased in spontaneously hypertensive rats (SHR)(12) but not in deoxycorticosterone acetate salt hypertensive rats (8).

In humans, Brodde *et al.* (13) found increased platelet α_2 -adrenoceptor density in 40 male hypertensive patients compared to normotensive patients. Moreover, the aggregation of platelets in response to 10 μ M epinephrine (acting via α_2 -adrenoceptors) was enhanced in the hypertensive patients suggesting functional significance of the increased density of α_2 -adrenoceptors. Van Hooft (14) studied platelet α_2 -adrenoceptor densities in children with two normotensive parents, with one hypertensive parent and with two hypertensive parents. He found the lowest receptor number in those with normotensive parents and the highest in those with two hypertensive parents. Thus, genetic factors appear to participate in the control of α_2 -adrenoceptor regulation, and genetically hypertensive patients and animals have greater platelet, cerebral and renal α_2 -adrenoceptor densities than normotensive controls.

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In this study, we have compared the α_2 -adrenoceptor genes of SHR and Wistar-Kyoto (WKY) rats by restriction fragment length polymorphism (RFLP) analysis using human α_2 -adrenoceptor probes. An α_2 -adrenoceptor RFLP was found between the genome of SHR and WKY rats using *Dde* I restriction endonuclease and a human α_2 -adrenoceptor genomic probe, the gene for which is localized in chromosome 10 (ADRA2R) (15). This RFLP was then used as a marker for examining the correlation of the α_2 -adrenoceptor RFLP with blood pressure in the F₂ generation derived from crossing SHR and WKY rats.

Methods

RFLP Analysis

a. Animals. Pairs of SHR and WKY rats were purchased from Sasco Inc. (Omaha, NE, USA), Charles River Breeding Laboratories (Wilmington, MA) and Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). One SHR-stroke prone (SHRSP) rat and a WKY rat were purchased from Taconic Farms (Germantown, NY, USA). The rats were killed by decapitation. The livers were removed and frozen in liquid nitrogen and stored in -80°C for later genomic DNA preparation.

b. Preparation of genomic DNA. Genomic DNA of rats was prepared by using a 340 A Nucleic Acid Extractor from Applied Biosystems, Inc. (Foster, CA, USA). Rat liver (0.1-0.3g) was minced with a sterile razor blade and transferred to a 15 ml Dounce homogenizer. The tissue was homogenized in 10 ml of cold phosphate buffered saline (PBS) and centrifuged at $1500 \times g$ at 4°C for 15 min. The crude nuclear pellet was resuspended in 1.5 ml of PBS and delivered to a 14 ml reaction vessel. After completing each extraction, the precipitate cartridge was opened gently and the filter was transferred into a microcentrifuge tube with TE (10 mM Tris-HCl, 1 mM EDTA) buffer. The microcentrifuge tube was then placed in a $55-65^\circ\text{C}$ heating block for 24h to dissolve the DNA. The DNA was quantitated spectrophotometrically.

c. Southern blot analysis. Ten micrograms of genomic DNA from each rat was digested overnight with 25 units of restriction endonuclease purchased from New England Biolabs, Inc (Beverly, MA, USA) under conditions recommended by the manufacturer. DNA restriction products were separated by electrophoresis on 1% agarose gel and transferred to nylon membranes according to the procedure described by Southern (16) as modified by Smith (17). The filter membrane was baked for 2 h at 80°C . Prehybridization was performed in a solution containing $6 \times \text{SSPE}$ ($1 \times : 0.15 \text{ M NaCl}$, $0.012 \text{ M NaH}_2\text{PO}_4$, 1 mM EDTA), $10 \times \text{Denhardt's solution}$, 1% sodium dodecyl sulfate (SDS) and $50 \mu\text{g/ml}$ salmon sperm DNA at 42°C . After 4-6 h, the prehybridization solution was changed to the hybridization solution which contained $6 \times \text{SSPE}$, $5 \times \text{Denhardt's solution}$, 0.5% SDS, $100 \mu\text{g/ml}$ salmon sperm DNA, 50% deionized formamide, 10% dextran sulfate and denatured [α - ^{32}P]dCTP labeled

DNA probes at $1-2 \times 10^6$ cpm/ml. Two human α_2 -adrenoceptor probes were used in this study. One is a human kidney cDNA probe (ADRA2R2L). This probe contains 1.24 kb coding sequence for ADRA2R2L gene (#110-1383) and 240 bp of the 3' untranslated region (18). The other probe is a human platelet genomic probe (ADRA2R). This 0.95 kb probe starts at base #151 in the coding region of the ADRA2R gene and ends at base #1099 (15). Since α_2 -adrenoceptor genes are intronless, both probes contain most of the coding regions. Hybridization was allowed to proceed for 18-24 h at 42°C . The filters were washed twice in $3 \times \text{SSC}$ ($1 \times 0.15 \text{ M NaCl}$, $0.015 \text{ M Na}_3\text{-citrate}$), 0.1% SDS at 55°C for 30 min followed by twice in $0.1 \times \text{SSC}$, 0.1% SDS at 55°C for 30 min. Filters were exposed to Kodak film at -70°C with intensifying screens for about 18 h.

d. Restriction mapping. In order to determine which part of the DNA probe was responsible for the RFLP, the ADRA2R DNA probe was further dissected into 3 fragments using *Acc* I and *Bgl* II restriction endonucleases as illustrated in Fig. 2A. Those fragments were separately radiolabeled and used as probes for Southern blot analysis.

Crossbreeding Study and Blood Pressure Measurement

One pregnant SHR and one pregnant WKY rat were purchased from Sasco Inc. The pups were weaned at four weeks of age. All rats were housed under identical physical and environmental conditions and were fed Purina rat chow which contains 0.28% sodium chloride, and tap water *ad lib*. Four female WKY rats were bred with four male SHRs at 12 weeks of age. The above mating produced 13 female and 20 male rats classified as the F₁ generation of SHR-WKY crossbreeding. Thirteen pairs of F₁ rats were mated in brother/sister pairs to yield an F₂ population of 110 rats. Blood pressure of each of the F₂ rats was measured indirectly at the age of 12 weeks using a well standardized tail cuff sphygmomanometer with a pneumatic pulse transducer connected to a CMP-4B physiograph (Narco Biosystems, Houston, TX) after warming the rats under a heating lamp (approximately 37°C) for up to 30 min. In addition to the indirect blood pressure measurement, direct mean arterial blood pressure was obtained immediately prior to sacrifice by anesthetizing the rats with pentobarbital Na and cannulating their left femoral artery. The pulsatile signal was dampened to a mean pressure signal by injecting a small air bubble into the catheter, and the catheter was connected to a Microswitch piezoresistive pressure transducer (156PC15GWL). The transducer was connected to a signal conditioning box (Wake Forest University Biomedical Electronics) which has trim pots enabling calibration with a mercury manometer. The output from the signal conditioning box was sent to a computer containing a Metrabyte DAS-16 analog/digital card that was controlled by TLABLOG software (Quinn-Curtis). Mean arterial blood pressure was measured every 5 seconds for 10 min, and these data were stored in

files for subsequent averaging using Microsoft Excel. The liver and kidney were removed from rats, frozen in liquid nitrogen and stored in a -70°C freezer. All studies were conducted in accordance with the guidelines of the committee on animal research of Creighton University.

Statistical Analysis

ANOVA and chi-square analysis were used for statistical analyses to determine the relationships between blood pressure and genotypes and to examine the genotype distribution.

Results

An RFLP was found between the genome of SHR and WKY rats with *Dde* I digestion and a human platelet genomic probe (ADRA2R) which marked an SHR allele of 1.6 kb fragment and a WKY rat allele of 0.9 kb fragment with a common band of 1.3 kb in SHR and WKY rats (Fig. 1). No RFLP was found between the genome of SHR and WKY rats digested with fifty-two different restriction endonucleases and probed with ADRA2R2L.

To exclude the possibility that the difference observed was due to an aberration limited to animals from one source, the DNA of SHR and WKY rats from several commercial suppliers (see methods) were examined. All the SHR including SHRSP showed the same band pattern. The WKY rats from Sasco Inc., Harlan Sprague Dawley Inc. and Charles River Breeding Laboratories had the same band pattern, but the WKY rat from the Taconic Farms showed three bands: 1.6 kb, 1.3 kb and 0.9 kb (data not shown).

After dissecting the ADRA2R probe with *Bgl* II and *Acc* I restriction endonucleases, three fragments were generated (Fig. 2A). These were a 227 bp of the 5' fragment, 324 bp of the middle fragment and 397 bp of the 3' fragment. When these three DNA fragments were separately used to probe Southern blots of DNA from SHR and WKY rats, the 3' fragment (397 bp) identified a 1.3 kb DNA band in SHR and WKY rat. The middle fragment (324 bp) identified all three bands, the 1.6 kb, the 1.3 kb and the 0.9 kb, whereas the 5' fragment (227 bp) failed to hybridize with any DNA band (Fig. 2B). The failure of the hybridization of the 5' probe with a DNA band in the Southern blot might be due to the sequence difference between the human probe and the rat genome.

Based on the above observations, the possible polymorphic site in the α_2 -adrenoceptor gene of SHR and WKY rat was deduced. In Fig. 3, the upper two lines represent the genomic DNA of SHR or WKY rat and the lower line represents the ADRA2R probe. *Bgl* II and *Acc* I sites are indicated. There is a *Dde* I site in the SHR and WKY rat genome, which is in the position in ADRA2R between *Bgl* II and *Acc* I sites. In the WKY rat genome there are two other *Dde* I sites in the 5' of the gene, separated from the middle *Dde* I site by 0.9 kb and 1.6 kb. In SHR there is only one *Dde* I site in the 5' of the gene, which is 1.6 kb away from

Fig. 1. Southern blot analysis of DNA from SHR (S) and WKY (W) rat digested with *Dde* I restriction endonuclease and probed with ADRA2R. Ten microgram of genomic DNA prepared from rats' liver was digested with *Dde* I, electrophoresed on 1% agarose gel, blotted onto a nylon filter membrane and hybridized with [α - ^{32}P] dCTP labeled ADRA2R. The end labeled, *Hind* III digested lambda DNA was used for size markers (STD).

Fig. 2. Schematic illustration of dissection ADRA2R probe with *Bgl* II and *Acc* I digestion (A) and Southern blot analysis of DNA from SHR and WKY digested with *Dde* I restriction endonuclease and hybridized with the dissected probe. The 3' fragment (397 bp) identified a 1.3 kb DNA band in SHR and WKY rat. The middle fragment (324 bp) identified all three bands, the 1.6 kb, the 1.3 kb and the 0.9 kb. The 5' fragment (227 bp) failed to hybridize with any DNA band (B).

Fig. 3. Schematic illustration of the possible polymorphic site in the SHR and WKY genome. The upper two lines represent the genomic DNA of SHR and WKY rat, with the *Dde I* restriction endonucleases sites indicated. The lower short line represents the ADRA2R probe. *Bgl II* and *Acc I* sites are indicated.

Table 1. Frequency of α_2 -Adrenoceptor and Renin Genotypes in the F₂ Rats Obtained from Crossing SHR and WKY Rats

Genotype*	α_2 -Adrenoceptor Gene			Renin Gene		
	Male	Female	Total	Male	Female	Total
WW	12	22	34	15	15	30
SW	34	24	58	27	26	53
SS	14	4	18	18	8	26

* WW, F₂ rats inheriting both α_2 -adrenoceptor/renin gene alleles from WKY rat; SW, F₂ rats inheriting one α_2 -adrenoceptor/renin gene allele from SHR, one from WKY rat; SS, F₂ rats inheriting both α_2 -adrenoceptor/renin gene alleles from SHR.

the middle *Dde I* site.

Data on α_2 -adrenoceptor genotype distribution in F₂ rats are shown in Table 1. The frequency of α_2 -adrenoceptor genotypes (WW:SW:SS 12:34:14) conformed to a 1:2:1 distribution in male F₂ rats as expected for Mendelian inheritance (chi-square analysis $P > 0.05$) but not in female (WW:SW:SS 22:24:4) F₂ rats (chi-square analysis $P < 0.05$). As an internal control, Southern blots of *Bgl I* digested genomic DNA from the same F₂ rats were probed with a rat renin gene. The probe detected a 7.6 kb band in SHR and an 8.0 kb band in WKY rat (19). The F₁ hybrid showed both bands (data not shown). The frequency of the renin genotypes did not differ in male or female from the 1:2:1 ratio expected for a Mendelian trait as evaluated with the chi-square test ($P > 0.05$) in both sexes (Table 1).

Blood pressure of the male F₂ rats homozygous for the SHR α_2 -adrenoceptor allele tended to be higher than male F₂ rats homozygous for the WKY α_2 -adrenoceptor allele with indirect systolic blood pressure measurement (ANOVA, $P > 0.05$). In fact, there was a stepwise trend of increasing indirect systolic blood pressure in males when compared to WW to SW to SS. These blood pressures were 122.1, 128.4 and 130.6 mmHg respectively. The

values for direct mean arterial blood pressure for male F₂ rats were 85.1 mmHg (WW), 83.0 mmHg (SW) and 86.3 mmHg (SS) respectively. The indirect systolic blood pressure of female F₂ rats was 113.9, 111.9 and 108.7 mmHg for the WW, SW and SS genotypes respectively. The mean arterial blood pressure for female F₂ rats was 62.1 mmHg (WW), 60.7 mmHg (SW) and 62.4 mmHg (SS) respectively (Fig. 4). ANOVA test showed that the blood pressure (indirect and direct) of male or female F₂ rats homozygous for the SHR α_2 -adrenoceptor alleles was not significantly higher than the F₂ rats heterozygous or homozygous for WKY α_2 -adrenoceptor alleles. An additional finding from this study was that male F₂ rats had significantly higher blood pressure ($P < 0.05$) than female F₂ rats with either systolic or mean arterial blood pressure measurement.

Discussion

Previous studies have demonstrated that the density of renal α_2 -adrenoceptors is increased in several genetically hypertensive rat models and high salt feeding further increases the density of the α_2 -adrenoceptors, paralleled by an increment in blood

Fig. 4. Bar graph of mean arterial blood pressure (upper panel) and systolic blood pressure (bottom panel) of F_2 rats classified according to the α_2 -adrenoceptor genotypes. Systolic blood pressure was taken by tail-cuff method at 12 weeks of age, mean arterial blood pressure was taken by femoral artery cannulation prior to sacrificing the rats. Vertical bars and their brackets indicate group means $\pm$ SEM.

pressure (1-7). Recently, studies in our laboratory suggested that the increase in the density of renal α_2 -adrenoceptors in Dahl salt-sensitive (S) rats ingesting a high salt diet was due to proportional increases both in the high and low affinity binding sites (20). The percentage of high affinity binding sites did not change with strain, sodium content in the diets or whether it was measured by a full agonist or a partial agonist binding (20). Therefore, the extra α_2 -adrenoceptors produced in S rats are expected to be fully functional. In this study, restriction mapping analysis showed that the RFLP between the SHR and WKY rat appears to be located in the 5' region of the α_2 -adrenoceptor gene where functionally important sequences are located which control the gene expression and regulation. Thus, the differences in the renal α_2 -adrenoceptor density between SHR and WKY rats as reported previously (1-2), might be due to differences in the 5' region of the α_2 -adrenoceptor described herein.

The F_2 population derived from SHR and WKY rats cross, showed an atypical α_2 -adrenoceptor genotype distribution. Female F_2 rats did not follow Mendel's first law, i.e. segregation of alleles. There was a reduced number ($P < 0.05$) of the SS genotype (Table 1) in females. As an internal control, the renin gene was typed in the same F_2 rats. Though the number of F_2 female rats with two alleles of the renin gene from SHR was less than that of the F_2 male rats, the frequency of the renin genotype in

females did not differ from the 1:2:1 ratio expected for a Mendelian trait as evaluated with the chi-square test ($P > 0.05$). The mechanism of this atypical genotype distribution involving the α_2 -adrenoceptor gene in female F_2 rats is unknown. However, we do not think this selective deletion is by chance alone. We have found selective deletion of the SS α_2 -adrenoceptor genotype in the F_2 population of Dahl salt sensitive (S) and resistant (R) rat cross defined by another restriction endonuclease and an α_2 -adrenoceptor probe. That deletion involved both males and females (21). For these reasons, we suspect that the α_2 -adrenoceptor may be involved somehow in ontogeny.

Our present data fail to show the genetic cosegregation of α_2 -adrenoceptor gene RFLP with blood pressure in the F_2 rats of SHR and WKY cross. The blood pressure of male or female F_2 rats inheriting two SHR α_2 -adrenoceptor alleles was not significantly higher than that of the F_2 rats inheriting two WKY rat α_2 -adrenoceptor alleles with either indirect systolic blood pressure or direct mean arterial blood pressure measurement.

In summary, we found an α_2 -adrenoceptor RFLP between SHR and WKY. Restriction mapping analysis suggested that this RFLP is localized in the 5' of the gene. The two additional findings were that males had higher blood pressure than females and there was a selective deletion (by 68%) of females with double SHR alleles. This study failed to show the genetic cosegregation of α_2 -adrenoceptor gene RFLP with blood pressure in the F_2 rats of SHR and WKY cross. However, since this RFLP is located in the 5' region of the gene where functionally important sequences are located which regulate gene expression, the importance of this structural difference of α_2 -adrenoceptor between SHR and WKY rats can not be completely ruled out.

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