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HYPOTENSIVE EFFECT ASSOCIATED WITH A PHOSPHOLIPASE C-δ1 GENE MUTATION IN THE SPONTANEOUSLY HYPERTENSIVE RAT

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SUMMARY: To identify the genes responsible for blood pressure in the spontaneously hypertensive rat strain, we performed a cosegregation analysis between the genotype and blood pressure in a set of male F2 rats obtained by crossmating SHR with Wistar-Kyoto rats, a parental normotensive strain. Our investigation revealed that the phospholipase C-δ1 polymorphism, which resulted in missense mutation, cosegregates with the lower blood pressure in SHR, and that PLC-δ1 gene is located on chromosome 8. On the other hand, we found the lack of cosegregation between blood pressure and the nerve growth factor receptor gene, which is linked to a hypertensinogenic gene locus (denoted as BP/SP-1) on chromosome 10. We propose that PLC-δ1 gene itself or closely linked gene on chromosome 8 is a new candidate with the hypotensive effect, and that BP-SP1 locus does not directly contribute to blood pressure elevation in original SHR.

Recent advances in molecular biology have allowed the studies on hypertension by cosegregation analysis (1-4). Using F2 progeny of the spontaneously hypertensive rats (SHR) crossmated with its normotensive control rats, several groups have reported that specific markers of renin (5) and kallikrein (6) genes cosegregate with blood pressure. Furthermore, recent

Abbreviations: SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat; PLC-δ1, phospholipase C-δ1; PLC, phosphoinositide-specific phospholipase C; NGFRR, rat nerve growth factor receptor.

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studies using chromosomal mapping proposed that two genetic loci, BP/SP-1 on chromosome 10 with hypertensinogenic effect and BP/SP-2 on chromosome X with hypotensinogenic effect, cosegregated with blood pressure in SHRSP (7-9). However, biometric analysis suggests that the blood pressure is a polygenic trait and that the previously reported genes cannot explain all the variations in blood pressure, and it was not evident that these loci associate with blood pressure in the original SHR strain.

Recently, we discovered novel point mutations in the phospholipase C-δ1 (PLC-δ1) gene in hypertensive rats (SHR and SHRSP), but not in control normotensive rats, which cause amino acid substitutions in a putative catalytic X domain (10). Phosphoinositide-specific phospholipase C (PLC) is a key enzyme that regulates the signal transduction system and intracellular calcium handling (11). It is widely agreed that both intracellular calcium and PLC activity increase in SHR (12-14). It has been hypothesized that elevated PLC activity facilitates augmentation of cytosolic calcium concentration, enhancing vascular smooth muscle tension and thus inducing hypertension.

In this study, we used a set of F_2 rats obtained by crossmating the original SHR with WKY. Using a new set of F_2 progeny, we investigated the cosegregation of directly measured blood pressure with the PLC-δ1 mutation polymorphism, and that with previously reported NGFRR using a microsatellite marker, which is mapped as being most closely linked to the locus of BP/SP-1 gene (7).

MATERIALS AND METHODS

Design of Genetic Crosses

Crosses were made between 3 male SHR (191.5±1.9mmHg) and 3 female WKY rats (128.3±0.8mmHg) in the Laboratory Animal Science and Toxicology Laboratories, Sankyo Co., Ltd., Shizuoka, Japan. We confirmed that these substrains were genetically inbred by fingerprint analysis (15). The F_1 hybrid was intercrossed for the study of F2 segregating generation.

Animal Maintenance

All rats were housed under identical environmental conditions at the temperature of 24±1°C, humidity of 55±5% and a 12 hour dark-light cycle and were fed by commercial pellet CE-2 (Japan Clea Co. Ltd.).

Blood Pressure Measurements

A catheter was implanted into the abdominal aorta through a femoral artery under light ether anesthesia at 15 weeks of age. Systolic and diastolic blood pressure and heart rate were measured three times at a 24 hour interval after catheterization, while the animals were quite at rest and in a freely mobile state. The measurements were carried out using a pressure transducer (TP-400T), monitors (AT-601G and AT-641G) and a recorder (RTA-1300), all from Nihon Koden Co. Ltd. During the measurements, animals were shielded from the observer.

Genetic Typing by PCR

The genomic DNA samples were extracted from rat livers (16). Primers of PLC-δ1 were designed to amplify the region flanking the mutation. The forward primer (5'-3') was CCTGGAGAACCACTGTAGCC, and the reverse primer (5'-3') was CTCCCAGCTTCTTCCCTTTCA. Sample DNA was amplified in 50mM Tris, pH8.5, 1mM $MgCl_2$, 20mM KCl, 500μg/ml BSA, 200μM dNTP and 10μM of each primer. Samples were denatured for 60sec at 94°C and then cycled 30 times through the following steps: 15sec at 94°C, 15sec at 55°C, and then 45sec at 72°C. All PCR were performed in a 10μl reaction volume enclosed into glass capillary tubes using the Air Thermo-Cycler (Idaho Technology Co.). PCR products digested by *Xho*I were electrophoresed on 2% agarose gel. The presence of *Xho*I site in PLC-δ1 gene divided F_2 progeny into following 3 groups: homozygote with mutation, +/+, heterozygous mutation, +/-, and homozygote without mutation, -/- (Fig. 1).

The genotyping of NGFRR gene was determined according to the same protocol of Hilbert et al. (7). The differences of the number of CA repeats divided F_2 progeny into 3 groups: SHR/SHR, SHR/WKY and WKY/WKY.

Data Analysis

Analysis of variance (ANOVA) was conducted utilizing the program package, Stat View (Abacus Concepts, Inc.).

Chromosomal assignment

Eighteen rat × mouse somatic cell hybrids (YS01-18) were produced by fusing thymus cell of an ACI/N male rat with mouse myeloma, Sp2/O-Ag14 cells. This panel was used to assign PLC-δ1 locus to a rat chromosome. Distribution of rat chromosomes in this hybrid panel was reported previously (17, 18). Using primers designed to amplify the region flanking the mutation, we amplified a rat specific PLC-δ1 sequence in hybrid clones. Amplifications of rat specific PLC-δ1 sequence in the hybrid clones were scored according to whether they were amplified or not and were compared to the segregation patterns of individual rat chromosomes in the clone panel.

RESULTS

We previously reported two missense mutations and several silent mutations in the PLC-δ1 gene of SHR and SHRSP (10). One missense mutation (Thr to Ser) located in X domain was proposed to affect the activity of the enzyme by changing the conformation or the phosphorylation pattern of the domain. This mutation in PLC-δ1 gene can clearly be detected by digestion of the polymerase chain reaction (PCR) products with *Xho*I. The presence of *Xho*I site in PLC-δ1 gene divided F_2 progeny into following 3 groups: homozygote with mutation, +/+, heterozygous mutation, +/-, homozygote without mutation, -/- (Fig. 1). As shown in Table 1 and Fig. 2, we found that the PLC-δ1 polymorphism as determined above significantly cosegregated with lower systolic ($p<0.01$) and mean ($p<0.05$) blood pressure in SHR. Though the BP/SP-1 gene was proposed to have a dominant effect in the F_2 population of SHRSP, the PLC-δ1 mutation allele was revealed to have a co-dominant

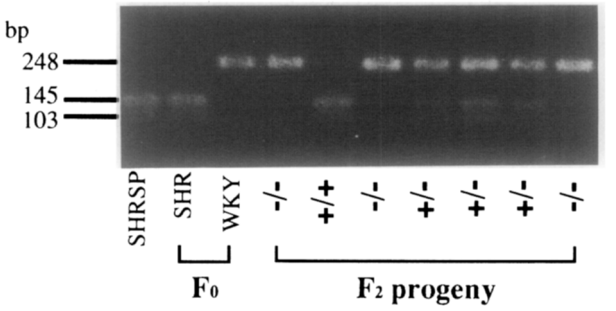

Figure 1. Genotypes of SHR, WKY and F2 progeny determined by PCR-RFLPs for the presence of *Xho*I site in the PLC-δ1 gene.
+ symbol indicates that this allele has *Xho*I site, the SHR specific mutation site causing a Thr to Ser amino acid substitution (10). - symbol indicates that the allele is not digested with *Xho*I. The SHR strain is homozygous for *Xho*I restriction site and gives rise to fragments of 145bp and 103bp. The PCR product from WKY gives only the 248bp fragment. The F2 progeny was either homozygous for the SHR allele, +/+, heterozygous for the SHR and WKY alleles, +/-, or homozygous for the WKY allele, -/-.

inheritance in SHR. Furthermore the rat × mouse somatic cell hybrid panel assigns the PLC-δ1 locus to the chromosome 8 with highest concordance of segregation patterns (Table 2).

On the other hand, we carried out a cosegregation analysis to find out whether the genotype of the NGFRR gene associated with blood pressure in our F2 progeny. However, we failed to find a significant cosegregation between them (Table 1).

Table 1. Statistics and analysis of variance on blood pressure measurements(mean±S.D. mmHg) by genotype in the F2 population

	PLC-δ1			
Genotype:	+/+	+/-	-/-	ANOVA
Phenotype	n=33	n=51	n=22	
Systolic BP	153.8±11.8	158.9±11.4	164.3±13.1	**$F_{2,103}=5.1$**
Diastolic BP	112.5±9.5	113.9±7.2	117.8±9.3	$F_{2,103}=2.7$
Mean BP	126.3±9.8	128.9±7.9	133.3±10	**$F_{2,103}=4.0$**

The genotype in the PLC-δ1 indicates the presence of the missense mutation. Statistics for $p<0.05$ are indicated in bold, and for $p<0.01$ are double underlined.

	NGFRR			
Genotype:	SHR/SHR	SHR/WKY	WKY/WKY	ANOVA
Phenotype		n=32	n=41	n=33
Systolic BP	157.3±12.8	159.9±12.0	157.6±12.5	$F_{2,103}=0.5$
Diastolic BP	113.6±8.8	116.0±8.4	112.8±8.3	$F_{2,103}=1.5$
Mean BP	128.2±9.6	130.6±9.2	127.7±9.1	$F_{2,103}=1.1$

The NGFRR genotype was examined according to the reported protocol (7).

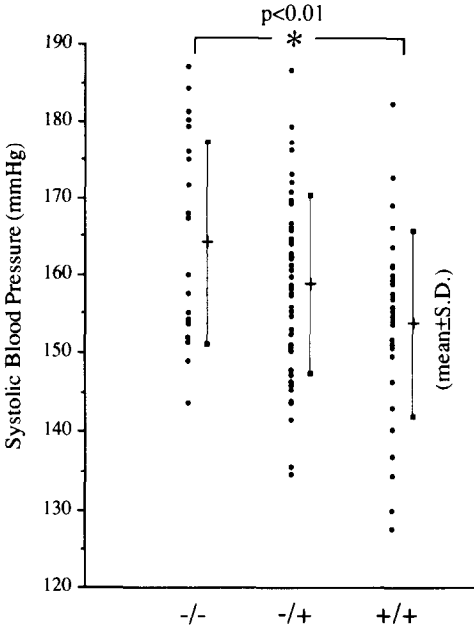

Figure 2. Distribution of the systolic blood pressure in F2 progeny classified according to the PLC-δ1 genotype.

The meaning of + and - symbols is as shown in Fig. 1. An asterisk indicates a statistical difference between +/+ and -/- ($p<0.01$). The systolic blood pressure of F2 progeny with homozygous SHR specific mutation was 10mmHg lower than that of F2 populations without the mutation.

DISCUSSION

We used the SHR and WKY rats in Sankyo Co. strain for genetic crosses. Crosses were made between male SHR and female WKY rats. F2 progeny was not influenced by the genes responsible for blood pressure on sex chromosome (for example, BP-SP2), because all F2 progeny used for this analysis were male rats with Y chromosome derived from male SHR and X chromosome derived from female WKY or male SHR in the same ratio. We measured blood pressure of all F2 rats directly without sodium loading, because SHR has been reported to be far less sensitive to salt loading compared to SHRSP (19).

The PLC superfamily comprises at least four sub-families each of which has isozymes (20). Little is known about the functional differences or interactions among these isozymes. Although the δ isoform of PLC is supposed to function in thrombin-mediated signal transduction (21), the exact function of PLC-δ1 has not yet been elucidated. Interestingly, Kato *et al.* recently found that both the PLC-δ1 mRNA level and the enzymatic activity in aorta increased in proportion to the extent of blood pressure elevation (22). It should be noted that contrary to our original expectation, the PLC-δ1 allele is inversely linked to the blood pressure values in the F2 progeny. Further investigation is needed

Table 2

(a) Distribution of rat chromosomes in hybrid clones

Rat chr. no.	Clone number (YS01-18) 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	+	-	+	-	+	-	-	-	-	-	-	-	+	-	-	-	+	-
2	+	(-)	-	-	(-)	+	-	-	-	(-)	-	-	-	-	-	-	-	-
3	+	+	+	+	+	-	-	-	+	+	+	-	+	-	+	+	+	-
4	+	+	-	+	-	-	-	+	+	-	-	-	±	-	-	+	+	+
5	+	+	+	+	+	-	+	+	+	-	-	-	+	-	+	+	-	-
6	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
7	-	-	-	+	-	+	-	-	+	+	-	-	+	+	+	-	+	+
8	-	-	-	±	-	+	-	-	+	-	±	-	-	+	+	-	±	+
9	-	-	-	(-)	+	+	-	±	(-)	-	-	-	±	±	+	-	±	+
10	+	-	-	-	-	-	-	±	-	-	-	-	-	-	-	-	-	+
11	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+
12	-	+	-	-	-	+	-	-	-	+	-	-	+	+	-	+	+	+
13	+	+	+	-	-	-	+	-	-	-	±	-	+	+	+	+	-	+
14	+	+	+	+	-	+	-	-	+	+	-	+	+	+	-	-	+	-
15	+	-	+	+	+	+	-	-	-	-	-	-	+	+	-	+	+	+
16	+	+	(-)	-	(-)	+	+	-	+	+	+	-	+	±	+	+	+	-
17	+	-	+	(-)	+	-	(-)	-	+	+	+	-	+	+	-	+	+	+
18	+	-	+	-	-	+	-	-	-	+	±	-	-	-	(-)	-	+	+
19	-	-	-	(-)	±	-	-	-	+	-	-	-	±	+	-	±	-	+
20	+	-	+	-	+	-	-	-	+	-	-	-	+	-	±	+	±	+
X	+	+	±	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+
Y	-	-	-	-	±	-	-	-	+	+	+	-	+	-	-	±	+	+
PLC-δ1*	-	-	-	+	-	+	-	-	+	-	+	-	-	+	+	-	+	+

Chromosome: +, present in 50% cells; ±, present in 20-50% cells;
(-), present in < 20% cells; -, present in 0%
* Amplification of rat specific sequence: +, amplified; -, unamplified

(b) Numbers of hybrids, which showed discordance between segregation of rat chromosomes and amplification of a rat specific PLC-δ1 sequence in rat × mouse somatic cell hybrid clones

Gene	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	X	Y
PLC-δ1	11	8	10	9	13	8	3	0	6	9	10	8	10	9	8	8	9	7	8	9	8	8

to find whether this isozyme itself functions as a hypotensive factor or an unknown gene nearby PLC-δ1 locus is responsible for lower blood pressure on chromosome 8. However, this PLC-δ1 locus is evidently a new candidate associated with lower blood pressure in original SHR other than BP-SP2 locus on chromosome X.

We hypothesize that PLC-δ1 mutation occurred while SHR were produced from WKY. Recalling that the original process adopted in the selection of the SHR strain from WKY (23), where a significant elevation in the blood pressure of the strain was observed only after the passage of three generations, we can conclude that this major change occurred, therefore, during a very short period of time in contrast to the production of other hypertensive rat strains. During this short time period, several genes associated with blood pressure underwent changes, some causing major blood pressure elevation while others acting to suppress these otherwise lethal effects. Therefore, we inferred that PLC-δ1

gene itself or nearby linked gene on chromosome 8 changed to lower blood pressure during this period. Obviously further study is necessary to explain the actual function of the PLC-δ1 gene in the development of hypertension in SHR and possibly in human essential hypertension.

On the other hand we failed to find cosegregation between genotype of NGFRR, which is a microsatellite marker located close to BP-SP1, and blood pressure using our F_2 progeny. This means that the BP/SP-1 could be a gene responsible for salt sensitive blood pressure elevation, consistent with the report by Jeunemaitre *et al.* who found no linkage between the human angiotensin converting enzyme gene and essential hypertension without sodium loading in humans (24). We propose here that BP-SP1 locus does not directly contribute to blood pressure elevation in SHR, or that this locus in SHRSP has the effect merely for further augmentation of the blood pressure in SHR.

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