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Changes in gene expression of the renin-angiotensin system in two-kidney, one clip hypertensive rats.

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Running title: Gene expression in 2K1C hypertensive rats

Summary

In investigation of the molecular mechanism of sustained hypertension in two-kidney, one clip (2K1C) hypertensive rats, possible changes in gene expressions of renin in the kidney and angiotensinogen in the liver were studied. In 2K1C rats 4 weeks after clipping, the plasma renin and angiotensin II levels were significantly higher than those sham-operated rats, but the plasma angiotensinogen levels were in similar in the two groups. At this time, expression of the renin gene in the ischemic kidney of 2K1C rats was 2.6 times that in sham operated rats (P<0.05), but expressions of the angiotensinogen gene were similar in the two groups. Sixteen weeks after clipping, the angiotensin II levels in 2K1C rats were plasma renin and not significantly higher than those in sham-operated controls, but expression of the renin gene in the kidney was still 2.2 times higher in 2K1C rats than in controls (P<0.05). The plasma angiotensinogen level was significantly higher in 2K1C rats than in controls (P<0.05), expression of the angiotensinogen gene in the liver was 2.9 and 2K1C rats than in controls (P<0.01). times higher in These results indicate that the roles of the renin-angiotensin system in maintenance of hypertension in 2K1C rats differ in the acute and chronic phases: in the acute phase, over expression of the renal renin gene coupled to increased renin secretion plays the main role in elevating the blood pressure. In the chronic phase, a counterregulatory mechanism may affect the post-transcriptional fate of renin. Consistent over-expression of the renin gene and mobilization of angiotensinogen gene, i.e., over-expression of the renin-angiotensin system, may contribute to maintenance of hypertension.

Introduction

The renin-angiotensin system plays a dominant role in regulation of water-electrolyte balance and blood pressure[1-3]. Particularly, overwhelming activation of this system has been considered to be a main cause of renovascular hypertension.

The two-kidney, one clip (2K1C) Goldblatt hypertension rat is a useful animal model for study of hypertension. However, studies using 2K1C rats have not given clear-cut results on the role of circulating plasma renin [4-15]: in early phase after clipping, the plasma renin level is elevated[6,7], whereas, in the chronic phase, it returns to nearly the basal level despite persistent high blood pressure[10,11]. For determination of the reason for the sustained high blood pressure in the chronic phase, various possible factors such as thromboxane and reconstruction of the vascular wall have been studied[16]. But the findings that inhibitors of the renin-angiotensin system, such as renin inhibitor[17], converting-enzyme inhibitor[18], and angiotensin II analogue[4,19], decrease the high blood pressure in the chronic phase suggest that the renin-angiotensin system still plays а significant role in 2K1C rats in this phase. Recently, we reported that increased activity of the tissue renin-angiotensin system, particularly, increased activity of the vascular converting-enzyme activity, contributes to maintenance of a high blood pressure in the chronic phase[4.5].

Recent advances in molecular biology now enable us to investigate changes in the components of the renin-angiotensin system in the kidneys and other tissues directly[1-3,25-32]. In this study, we examined the production of renin in the kidney and of angiotensinogen in the liver at the molecular level by quantifying their messenger RNAs (mRNA) in 2K1C rats in the early and chronic phases.

Materials and Methods

Six-week-old male Wistar rats weighing 200-225 g were used. They were divided into two groups: a 2K1C group with hypertension and a sham operated group. The rats were given regular rat chow (Clea Japan Ltd., Tokyo, Japan), with free access to tap water, and were housed under identical conditions.

Experimental Protocol

Two-kidney, one clip hypertension was induced as we reported previously[4]. Briefly, the rats were anesthetized with sodiumpentobarbital (50 mg/kg i.p.), and the left renal artery was partially occluded by a silver clip (inside diameter 0.2 mm), while the right renal artery was left intact. Control rats were submitted to a sham operation in which the clip was placed adjacent to the left renal artery.

The blood pressure were measured directly through a cannula inserted into the left femoral artery as reported previously[4], and animals with a mean blood pressure of over 150 mmHg at 4 and 16 weeks after clipping were included in the 2K1C group. The animals (5 per group) were randomly allocated to either the early group at 4 weeks, or the chronic group at 16 weeks after clipping. In both cases, a matched, sham-operated group was also studied. Rats were killed by exsanguination 4 or 16 weeks after clipping.

Assay of renin activity.

Before exsanguination, blood samples were obtained <u>via</u> an inserted cannula from animals that had recovered from ether anesthesia (5-6 hours after anesthesia). The samples were collected in cooled tubes containing ethylene diaminetetraacetic acid (EDTA-2Na: 1mg/ml whole blood) and centrifuged at 4° C, and plasma samples were stored at - 20° C for assay. The plasma renin concentration (PRC) was measured by the method of Goto et al.[20] in the presence of excess substrate of rat renin.

Assay of plasma angiotensinogen

Samples of 10 microlitre of plasma were diluted to 100 microlitre with 0.2M phosphate buffer, pH 7.0, and aliquots (25 microlitre) were incubated for 4 hours at 37^oC with 1 microlitre (1 Goldblatt unit) of human recombinant renin prepared by Murakami et al.[21] in 0.2 M phosphate buffer, pH 7.0, containing 125mM EDTA-2Na and 50mM phenylmethylsulfonylfluoride. During the incubation, angiotensinogen was completely converted to angiotensin I, which was measured by the method of Ikeda et al.[22].

Assay of plasma angiotensin II

Samples of 1 ml of freshly separated plasma were promptly concentrated in an Amprep C18 cartridge (Amersham, Aylesbury, U.K.) by

the method of Saito et al., [23] and their angiotensin II content was measured by the method of Shimamoto et al. [24] with a very sensitive and specific anti-angiotensin II antibody.

Purification of RNA

Rats were killed, and their kidney and liver were promptly removed, weighed, frozen in liquid nitrogen and stored at -80°C until RNA extraction. Total cellular RNA was extracted by the method of Chirgwin et al.[25]. Briefly, each kidney or liver was homogenized in 10 ml of a solution of 4M guanidine thiocyanate, 0.5% sodium N-lauryl 25mM sodium citrate (pH 7.0), 2M cesium chloride (CsCl), sarcosine. and 0.1M beta-mercaptoethanol. The RNA was precipitated by centrifugation through a 5.7M cushion of CsCl at 37,000 rpm. for 16-18 hours at 20°C in a RPS40T rotor (Hitachi Kouki Co. Ltd., Tokyo, Japan). The RNA was then solubilized in 0.2M sodium acetate, pH 4.8, diluted with water and quantified by measurement of its absorbance at 260 nm. The RNA was then stored at -80°C until use.

RNA analysis

Messenger RNA was quantified by Northern blot analysis. Aliquots of total RNA were lyophilized, and denatured by heating at 65°C for 15 min in glyoxal and dimethylsulfoxide (Nakarai Tesq. Co., Osaka, Japan). The denatured RNA was subjected to electrophoresis in 1.5% agarose gel in 10mM sodium phosphate buffer, pH 7.9 at 80 volts for 2-3 hours. The RNA was then capillary-blotted onto Hybond-N (Amersham, Aylesbury, UK) in 20 X saline-sodium-citrate (SSC: 1X SSC equals 0.15M

sodium chloride, 15mM sodium citrate, pH 7.0) as recommended by the manufacturer of membranes. The membranes were prehybridized at 42°C for at least 4 hours in hybridization buffer (5m1/100 cm²) consisting of 5X Denhardt's solution, 5X SSC, 50% formamide, 1% sodium dodecyl sulfate (SDS), 200 microgram/ml of denatured salmon sperm microgram/ml of yeast tRNA, and 10 microgram/ml each of DNA. 100 poly(A) and poly(C). The membranes were then hybridized at 42°C for 12-18 hours with fresh hybridization solution containing 1×10^{6} cpm/ml of ³²P-labeled full-length cDNA probes for rat renin or angiotensinogen (provided by Dr. Kevin Lynch, University of Virginia) and beta-actin (Toyobo Co. Ltd., Osaka, Japan). The cDNA probes were labeled by random oligonucleotide-primed synthesis in the presence of [³²P]dCTP (>1000 Ci/mmol; Amersham, Aylesbury, UK) to a specific activity of 10^8 - 10^9 cpm/ microgram. After hybridization, the membranes were washed sequentially in 2 X SSC, 0.1% SDS at room temperature (1 X 15 min), 1 X SSC, 0.1% SDS at 65°C (2 X 15 min), and 0.1 X SSC, 0.1% SDS at 65°C (2 X 15 min). Hybrids were detected by autoradiography at -80°C with hyperfilm-MP (Amersham) with an intensifying screen (E.I. du Pont de Nemous & Co., Wilmington, DE, USA).

For accurate quantification of specific mRNAs, slot-blot analysis was carried out using a 72-well slot analysis apparatus (Sunplatic Co., Osaka, Japan). The RNA was denatured at 65°C in 50% formamide, 6% formaldehyde, 18mM Na_2HPO_4 and 2 mM NaH_2PO_4 , and applied to Hybond-N under vacuum. After blotting, the membranes were treated as described above. The exposed film was scanned with a densitometer (Gelman Instrument Co., Michigan, USA).

Statistical Analysis

Results are expressed as means <u>+</u> SEMs. The significances of differences between values for different groups were determined by Student's t-test.

Results

Table 1 summarizes the characteristics of 2K1C rats in the early (4 weeks after operation) and chronic (16 weeks after operation) phases after clipping and their respective controls (sham-operated rats). In both the early and chronic phases, 2K1C rats weighed significantly less than matched controls (P<0.01). Mean blood pressures were significantly higher in the two experimental groups than in the corresponding controls (P<0.01), but there were no differences in the heart rates of 2K1C and control rats. In the early phase, the weight of the clipped kidney was significantly less than that of the contralateral kidney (P<0.01), but later, this difference was lost, probably because of nephrosclerosis of the contralateral kidney as a result of sustained high blood pressure.

The changes in plasma renin, angiotensinogen, and angiotensin II are shown in Table 2. In the early phase, the plasma renin concentration was significantly higher in 2K1C rats with hypertension than in shamoperated controls (P<0.01). However, in the chronic phase the level of PRC in 2K1C rats decreased significantly (P<0.05) to a level that was not significantly higher than that of sham-operated rats. The plasma level of angiotensinogen in 2K1C rats was not significantly different from that in sham-operated rats in the early phase, but became significantly higher than that of the controls in the chronic phase (P<0.05).

The plasma angiotensin II level in 2K1C rats was significantly higher than that of the control rats in the early phase (P<0.01). In the chronic phase plasma angiotensin II concentrations are still more than double the control values. However, the difference is not significant because of the wide standard error.

Figures 1 and 2 show typical examples of northern blot analyses of renin mRNA in the kidneys of 2K1C hypertensive rats in the early and chronic phases and those of their controls. On northern blot analysis, renal renin mRNA was detected as a single band, a value which was consistent with previous reports [26,27].

Figure 3 shows results on the ratio of renin mRNA to beta-actin mRNA. In the early phase, the ratio in the ischemic kidney was 3.6-times that in the contralateral kidney (P<0.05), and 2.6-times that in the kidney of control rats (P<0.05). In 2K1C rats in the chronic phase, ratio in the ischemic kidney was 2.8-times that in the the contralateral kidney (P<0.05), and 2.2-times that in the control rats (P<0.05). The expression of renin gene in the contralateral, non clipped kidney of 2K1C rats tended to be suppressed in the early phase and significantly decreased in the chronic phase (P<0.01). no significant change in the value of beta-actin mRNA in There was either the early or chronic phase (4W; control 114.0+14.8 vs. nonclipped 108.0+7.7 vs. clipped 127.0+15.6 arbitrary densitometric units, 16W; control 83.8+11.1 vs. non-clipped 126.0+31.3 vs. clipped 107.5+12.5 arbitrary densitometric units).

On Northern blot analysis, hepatic angiotensinogen mRNA was detected as a single species of 1.7 kb (Fig. 4 and 5), a value which was consistent with previous estimates [28,29]. Table 2 shows that in the early phase there was no significant difference between the ratios of angiotensinogen mRNA to beta-actin mRNA in 2K1C hypertensive rats and (control rats 100% vs. 2K1C rats 140+51%), but that, in the controls chronic phase, the ratio in 2K1C rats was 2.9-times that in control rats (P<0.01, control rats 100% vs 2K1C rats 291+98%). Either in the early or chronic phases, the value of beta-actin mRNA did not change between two groups (4W; control rats 264.3+45.9 vs. 2K1C rats 275.0+80.4, 16W; control rats 276.8+36.9 vs. 2K1C rats 285.5+64.3 arbitrary densitometric units).

Discussion

The contribution of the renin-angiotensin system to the development and maintenance of 2K1C hypertension has been debated[4-15]. In some studies increases in plasma and kidney renin were observed in both the early[12,30-32] and chronic phases[14], but, in others including our own[4,5] the plasma and kidney renin levels were found to decrease to nearly normal levels in the chronic phase [10,11,15]. These discrepant results may due to differences in the methods used for blood sampling, anesthesia of the animals, and the technical difficulties in measurement of the kidney renin content. In the present study, we obtained blood samples through a cannula implanted into an artery of conscious, unrestricted rats, and reconfirmed that in 2K1C rats, the plasma renin level increased in the early phase, but decreased in the chronic phase despite a persistently high blood pressure. The increase of plasma renin in the early phase was associated with an increased plasma angiotensin II level, consistent with the classical concept that plasma renin is a rate-limiting enzyme of the circulating reninangiotensin system.

Recent progress in molecular biological techniques enabled us to study the effects of renal ischemia on the gene expressions of components of the renin-angiotensin system[32,33]. In 2K1C rats in the early phase, expression of the renin gene in the kidney was increased, suggesting enhanced renin synthesis. However, in this phase the expression of the angiotensinogen gene in the liver and the plasma angiotensinogen concentration did not change. These findings suggest that in the early phase, increased expression of the renin gene induced by renal ischemia may play a major role in elevation of the blood pressure of 2K1C rats. In contrast, in 2K1C rats in the chronic phase, the expression of angiotensinogen gene was increased, and furthermore the renin gene was still increased, as previously reported by Samani et.al.[32]. However, we found that decrease in the plasma renin level did not coincide with that of renin mRNA in the kidney, consistent with a previous report by Nakamura et.al. [26]. Probably, unidentified post-transcriptional inhibitory factors, such as decreased activation of prorenin to renin, or enhanced clearance of plasma renin by the kidney and the liver, decreased the plasma renin activity.

On the other hand, increased expression of the angiotensinogen gene and an elevated plasma angiotensinogen level were intriguing features

at a molecular level in 2K1C rats in the chronic phase. The discrepancy between the almost 3 fold increase in liver angiotensinogen mRNA levels and the much less dramatic rise in the plasma angiotensinogen levels may be due to the posttranscriptional factors. Previously, angiotensinogen production and gene expression stimulated by many factors, such as angiotensin II[34], were glucocorticoids[35], or others[36,37]. On the other hand, Dzau et. al.[38] reported that angiotensinogen production and release were inhibited by the presence of renin per se. Their reports mentioned that the production of angiotensinogen in the liver was controlled by the balance between renin and angiotensin II[38]: In 2K1C rats in the early phase, expression of the angiotensinogen gene was reduced to a normal level by marked increase in the plasma renin level, but stimulated by angiotensin II, or, over expression of angiotensinogen gene was slow. However, in the chronic phase, expression of the angiotensinogen gene and the plasma angiotensinogen level increased, probably due to shift of the balance between renin and angiotensin II, or possible to some other unknown mechanisms.

Our results show the direct measurement of change in the plasma angiotensin II level in 2K1C rats. The results clearly showed that both the plasma angiotensin II level and the plasma renin concentration were increased in the early phase, as previously reported by Wallace et.al.[39]. However, in our study the ratio of plasma angiotensin II to renin was clearly higher in 2K1C rats in the chronic phase, suggesting that increase in the plasma angiotensinogen level may contribute to generation of angiotensin II. Furthermore, we found previously that in 2K1C rats, the activity of the converting enzyme in the vascular wall increases in the chronic phase, whereas plasma converting enzyme activity does not increase[4]. The source of plasma angiotensin II is still unknown, but the increases in plasma angiotensinogen level and tissue converting enzyme activity may act cooperatively to increase the plasma angiotensin II level. Probably, in 2K1C rats in the chronic phase, the increased plasma angiotensin II level may explain the persistent hypertension, as Morton et.al.[40] reported that there was a significant correlation between plasma angiotensin II and blood pressure.

From the presented study, we conclude that the molecular mechanism for the development and maintenance of high blood pressure in 2K1C rats differs in the early and chronic phases: In the early phase, rapidly increased expression of the renin gene in the kidney may play a central role in the development and maintenance of high blood pressure. On the other hand, in the chronic phase, consistent overexpressions of the genes for all components in the renin-angiotensin system may contribute to maintenance of high blood pressure, in spite of unidentified post-transcriptional inhibition of kidney renin.

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Table 1.

Characteristics of 2K1C hypertensive rats in the early and chronic phases and respective control rats.

Groups	Body weight (g)	Mean BP (mmHg)	lleart rate (beats∕∎in)	Kidney weight (g)
4 weeks 2K1C	299 <u>+</u> 21 [*]	194 <u>+</u> 6 [#]	438 <u>+</u> 15	R 1.39 <u>+</u> 0.08
Control	389 <u>+</u> 9	117 <u>+</u> 3	442 + 18	L 0.81 <u>+</u> 0.06 1.06 <u>+</u> 0.03
16 weeks 2K1C	335 <u>+</u> 21 [*]	202 <u>+</u> 9 [*]	438 <u>+</u> 15	R 1.23 \pm 0.12
Control	400 <u>+</u> 11	126 <u>+</u> 5	450 <u>+</u> 8	1.02 ± 0.03 1.05 ± 0.03

Values are means <u>+</u> SEMs for groups of 5 animals. R; right (intact kidney), L; left (clipped side) **P<0.01, <u>vs.</u> control rats.

Table 2.

Levels of plasma renin, angiotensinogen, and angiotensin II in 2K1C rats in the early and chronic phases and control rats.

Groups	PRC (ngAI/ml/hr)	Angiotensinogen (ngAI/ml)	Angiotensin II (pg/ml)
4 weeks			
2K1C	$30.0 + 3.2^{**}$	735 + 94	$15.8 + 4.0^*$
Control	9.3 $\frac{-}{1}$ 1.1	829 + 58	5.5 + 2.1
16 weeks			
2K1C	16.2 + 4.2	971 + 88 [*]	14.7 + 7.5
Control	8.9 ± 1.3	695 + 50	6.8 ± 1.3

Values are means + SEMs for groups of 5 animals. **P<0.01, *P<0.05 <u>vs.</u> control rats. Figure 1. Representative Northern blots of RNA (40 microgram) hybridized with a rat renin probe from kidneys of 2K1C hypertensive rats 4 weeks after-clipping and control rats.

Lane: 1. control rat

- 2. clipped kidney of a 2K1C rat
- 3. non-clipped kidney of a 2K1C rat
- Figure 2. Representative Northern blots of RNA (40 microgram) hybridized with a rat renin probe from kidneys of 2K1C hypertensive rats 16 weeks after-clipping and control rats.

Lane: 1. control rat

- 2. clipped kidney of a 2K1C rat
- 3. non-clipped kidney of a 2K1C rat
- Figure 3. Percent change in the ratio of renin to beta-action mRNA in the early (4 weeks) and chronic (16 weeks) phases of 2K1C rats and control rats. Values are means + SEMs for 4 animals.

*p<0.05 vs. control rats

**p<0.01 vs. control rats

⁺p<0.05 <u>vs.</u> contralateral kidney

- Figure 4. Representative Northern blots of RNA (25 microgram) hybridized with a rat angiotensinogen probe from the livers of 2K1C hypertensive rats 4 weeks after-clipping and control rats.
- Figure 5. Representative Northern blots of RNA (25 microgram) hybridized with a rat angiotensinogen probe from the livers of 2K1C hypertensive rats 16 weeks after-clipping and control rats.







Fig. 3





Fig. 5