Possible Role of the Vascular Renin-Angiotensin System in Hypertension and Vascular Hypertrophy

Ryuichi Morishita, Jitsuo Higaki, Mizuo Miyazaki, and Toshio Ogihara

To investigate the vascular renin-angiotensin system in two-kidney, one clip (2K1C) hypertension, we measured angiotensinogen messenger RNA (mRNA) in the aorta and aortic and plasma angiotensin II (Ang II) concentration in 2K1C rats during early (4 weeks) and chronic (16 weeks) phases. Four weeks after clipping, there was no significant change in aortic angiotensinogen mRNA in both groups. However, the levels of plasma and aortic Ang II in 2K1C rats were significantly elevated compared with levels in control rats (p<0.05). Sixteen weeks after clipping, aortic angiotensinogen mRNA in 2K1C rats did not differ compared with the level in control rats. The aortic Ang II level in 2K1C rats was significantly increased compared with that in control rats (p<0.05), whereas there was no significant difference in the plasma Ang II level between the groups during this chronic phase. During both phases, morphological studies in 2K1C rats showed arteriosclerotic changes, with a significant increase in the wall-to-lumen ratio (p<0.01). The present study is the first to demonstrate an increase in vascular Ang II levels and concomitant morphological arteriosclerotic changes during both the early and chronic phases in 2K1C rats. Together with the results of our previous study that demonstrated an elevation of vascular renin activity during the early phase and increased vascular angiotensin converting enzyme activity during the chronic phase, we conclude that the elevated vascular renin activity and vascular angiotensin converting enzyme activity during each phase may play a dominant role in the increase in vascular Ang II observed during both phases. (Hypertension 1992;19[suppl II]:II-62–II-67)

Recent reports that angiotensin converting enzyme inhibitors prevent neointimal proliferation after balloon injury and vascular amplifier development in spontaneously hypertensive rats have focused much attention on the vascular renin-angiotensin system. The same effect has also been observed in renal hypertensive rats, such as one-kidney, one clip (1K1C) and two-kidney, one clip (2K1C) hypertensive rats. In these models, the contribution of the vascular renin-angiotensin system to the hypertrophy of vascular smooth muscle cells (VSMCs) has been suggested. In the balloon injury model, Rakugi et al reported an increase in angiotensinogen messenger RNA (mRNA) in the neointimal tissue, using in situ hybridization. Previous reports suggest that angiotensin II (Ang II) induces mitogenic actions and stimulates DNA and protein synthesis, as well as being a hypertrophic and proliferative agent in VSMCs. However, it is not clear whether de novo vascular Ang II is associated with vascular hypertrophy in vivo or how vascular Ang II is synthesized. To evaluate the contribution of the vascular renin-angiotensin system to vascular hypertrophy and hypertension, we examined aortic Ang II content and angiotensinogen mRNA in 2K1C hypertensive rats, because neither of these factors related to the vascular renin-angiotensin system has yet been studied.

Methods

Experimental Protocol

Six-week-old male Wistar rats weighing 200–225 g were used. They were divided into a 2K1C group with hypertension and a sham-operated group. The rats were given a regular rat chow (Clea Japan Ltd., Tokyo), with free access to tap water, and the two groups were housed under identical conditions. We induced 2K1C hypertension using the method we have previously reported. The rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and the left renal artery was partially occluded by a silver clip (0.2-mm i.d.), 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.

We measured blood pressure directly through a cannula inserted into the left femoral artery as previously reported.7 Mean blood pressure was obtained 5–6 hours after rats had recovered from anesthesia and were conscious and in a steady state. Animals with mean blood pressure higher than 150 mm Hg at 4 and 16 weeks after clipping were included in the 2K1C group. Animals were randomly allocated to either the early group at 4 weeks or the chronic group at 16 weeks after clipping. For each group, a matched sham-operated group was also studied. Rats were killed by exsanguination 4 or 16 weeks after clipping.

Before exsanguination, 2-ml blood samples were obtained via an inserted cannula from animals that had completely recovered from ether anesthesia (5–6 hours after anesthesia) after blood pressure recording. The samples were collected in cooled tubes containing EDTA-2Na (1 mg/ml whole blood) and were centrifuged at 4°C. For the measurement of Ang II, plasma was stored with 2.5 mM phenylmethylsulfonyl fluoride at -70°C before assay. Samples of 1 ml freshly separated plasma were promptly concentrated in an Amprep C8 minicolumn (Amersham, Aylesbury, UK), as previously reported,8 and quantified as described below.

Measurement of Angiotensin I and II

Heparinized saline (20 IU/ml) was infused from the apex of the heart to wash out the blood. The aorta was promptly removed without excess fat, frozen in liquid nitrogen, and stored at -70°C until use. On the day of extraction, the aorta was thawed at 4°C, weighed, and homogenized by a polytron in 0.1N hydrochloric acid. Each specimen was centrifuged at 20,000g for 30 minutes at 4°C. For the extraction of Ang II, the supernatant was applied to an octyl minicolumn (Amprep C8) that was prewashed with 4 ml methanol and 4 ml of 0.1% trifluoroacetic acid, by the method of Saito et al.9 After the column was washed with 10 ml of 0.1% trifluoroacetic acid, Ang II was eluted with 2 ml ethanol/water/trifluoroacetic acid (80:19.9:0.1, vol/vol). The eluate was dried by a centrifugal concentrator in a vacuum (CC-181, Tomy, Tokyo). The resultant residue was resuspended in 100 μl of 0.1% trifluoroacetic acid. High-performance liquid chromatographic characterization was performed as previously described.9 In the appropriate fraction, samples were collected and dried in a vacuum centrifuge and redissolved in 0.1 M Tris acetate, pH 7.4, containing 2.6 mM EDTA-2Na, 1 mM phenylmethylsulfonyl fluoride, and 0.1% bovine serum albumin. Immunoreactive Ang II was measured by radioimmunoassay using antibody (kindly provided by K. Shimamoto, Sapporo Medical College). The sensitivity of this assay was 0.1 pg per tube. The cross-reactivity was 100% for angiotensin III and less than 0.1% for angiotensin I (Ang I).10 Immunoreactive Ang I was also measured by radioimmunoassay using antibody purchased from Amersham.

RNA Analyses

For each extraction, the aortic medial smooth muscle layer without excess fat from three animals was pooled. Total cellular RNA was extracted by the method of Chirgwin et al.11 RNA was precipitated by centrifugation through a 5.7 M cushion of cesium chloride at 35,000g for 16 hours at 25°C to pellet the RNA. The RNA was then solubilized in 0.2 M sodium acetate, distilled with water, and quantitated by absorbance at 260 nm. RNA was stored at -70°C until use. mRNA was quantified by Northern blot analyses, as previously reported.12 Aliquots of total RNA were lyophilized and denatured by heating at 65°C for 15 minutes in glyoxal and dimethyl sulfoxide. The denatured RNA was subjected to electrophoresis in a 1.5% agarose gel in 10 mM sodium phosphate buffer (pH 7.0) at 80 V for 2–3 hours. It was then capillary blotted onto Hybond-N membranes (Amersham) in 20× SSC (1× SSC is 0.15 M sodium chloride, 15 mM sodium citrate) as recommended by the membrane manufacturer. The membranes were prehybridized at 42°C for 3–4 hours in a buffer containing 5× Denhardt’s solution, 5× SSC, 50% formamide, 1% sodium dodecyl sulfate, 200 μg/ml salmon sperm DNA, 100 μg/ml yeast transfer RNA (tRNA), 10 μg/ml poly(A), and 10 μg/ml poly(C). The blots were then hybridized in the same buffer at 42°C overnight with phosphorus-32–labeled full-length complementary (cDNA) for rat angiotensinogen (kindly provided by K. Lynch, University of Virginia). Probes were labeled by random oligonucleotide-primed synthesis in the presence of [32P]dCTP. After hybridization, the blots were washed twice with 2× SSC containing 0.1% sodium dodecyl sulfate for 15 minutes each time at room temperature, and three times with 0.2× SSC containing 0.1% sodium dodecyl sulfate at 65°C for 30 minutes each time. Autoradiography was performed using Hyperfilm-MP (Amersham) with intensifying screens (Du Pont Co., Wilmington, Del.) at -70°C for 48–72 hours. The film was then scanned with a densitometer (Shimazu Instruments Inc., Kyoto, Japan). All Northern blots were repeated three times for each experiment.

Aortic Measurements

The abdominal aorta of each rat was dilated and perfusion fixed at the mean atrial pressure and removed intact and placed in a vial of 4% paraformaldehyde for later analysis. A segment between the branches of the renal and superior mesenteric arteries was taken and sectioned in a cryostat at -26°C. The sections were stained with hematoxylin and eosin and viewed with a ×4 objective and magnified ×137 with a Sony video monitor. The medial-intimal area was measured by a video analysis system with a digitizer. If the internal elastic lamina and media
showed any evidence of crenation or compression, the vessel was not used. At least three sections were analyzed from each vessel. A pilot study was performed to determine whether storage of aortas in 4% paraformaldehyde alters medial-intimal area and demonstrated no significant change in luminal diameter or medial-intimal area.

Statistical Analysis

Results are expressed as mean±SEM. The significance of differences between values for different groups was determined by the unpaired Student’s t test.

Results

During the early phase, plasma Ang II concentration in 2KIC rats showed a significant increase compared with that in control rats (p<0.05), as shown in Figure 1. However, during the chronic phase, no significant difference was observed. During the early phase, aortic Ang II concentration in 2KIC rats was significantly higher than that in control rats (p<0.05). During the chronic phase, aortic Ang II levels increased in 2KIC rats (p<0.05), in spite of normalization of plasma Ang II levels. During the chronic phase, 2KIC rats showed a tendency toward increased Ang II levels compared with levels in the early phase, but the change was not statistically significant. There was no significant change in vascular Ang I levels during either the early or chronic phase in both groups.

Figure 2 shows the levels of aortic angiotensinogen mRNA. There was no significant difference in aortic angiotensinogen mRNA levels during the early and chronic phases between the two groups. β-Actin mRNA showed no significant change in these studies (data not shown). Figure 3 shows typical examples of light micrographs of representative cross sections of two abdominal aortas evaluated in these studies. During both the early and chronic phases, wall thickness and medial-intimal area in 2KIC rats were significantly greater than in control rats, as summarized in Table 1. Similarly, the wall-to-lumen ratio in 2KIC rats was significantly greater than that in control rats during both phases.

FIGURE 1. Top panel: Bar graph shows changes in plasma angiotensin II concentration in control (open columns) and two-kidney, one clip (2KIC) rats (closed columns). *p<0.05 vs. sham rats. Bottom panel: Bar graph shows aortic angiotensin I and II concentrations in control (Sham) and 2KIC rats. Vertically shaded columns show angiotensin II and horizontally shaded columns show angiotensin I concentrations. Values represent mean±SEM of five to eight animals.
Early Chronic

Discussion

Previous epidemiological studies have clearly demonstrated that hypertension is a major risk factor for the development of atherosclerosis.13 In renal hypertensive rats, such as 2K1C and 1K1C hypertensive rats, several investigators have reported a significant increase in arteriolar wall-to-lumen ratio and increased vascular tone in smaller arterioles.314 In our study, the aortic wall-to-lumen ratio in 2K1C rats was increased even during the early phase, and during the chronic phase, a further increase was observed. However, the mechanisms responsible for this phenomenon are poorly understood. Owens15 reported that captopril and hydralazine had similar blood pressure-lowering effects at the doses used but that captopril was more effective in preventing increases in aortic muscle cell content and VSMC polyploidy. Captopril was also shown to significantly reduce smooth muscle cell size and medial smooth muscle content in Wistar-Kyoto rats. The same studies were performed in 1K1C3 and 2K1C4 hypertensive rats. In 1K1C rats, regression of vascular hypertrophy was observed after captopril treatment at a dose that did not lower blood pressure effectively. These observations suggest that factors other than blood pressure per se are involved in the VSMC hypertrophic response and that Ang II may play a role in the regulation of VSMC mass both in hypertensive and normotensive rat strains. Our findings that angiotensin converting enzyme inhibitors and a renin inhibitor suppress locally generated Ang II support the hypothesis that the efficacy of angiotensin converting enzyme inhibitors may be due to inhibition of local Ang II production. However, to our knowledge, there have been no detailed reports concerning the level of Ang II in vascular tissue. In the present study, we clearly demonstrated elevation of Ang II concentration in the aorta of 2K1C rats during both the early and chronic phases, using a newly established sensitive radioimmunoassay coupled with high-performance liquid chromatography.9 On the other hand, this study showed unchanged Ang I during both the early and chronic phases. It is not clear how vascular Ang II is produced from angiotensinogen or Ang I. As Hilgers et al16 reported that infused Ang I increased Ang II release from vascular tissues using rat hind limb perfusion, circulating Ang I may, in part, contribute to the production of vascular Ang II. Or, the release of Ang I, Ang II, or both from vascular tissues may affect vascular angiotensin levels. However, further studies are needed to clarify the mechanism of the vascular renin-angiotensin system.

Ang II, the final product of the renin-angiotensin pathway, influences vascular growth by a variety of mechanisms. Paquet et al6 reported that Ang II stimulates protein and DNA synthesis and that it exerts a mitogenic action on spontaneously hypertensive rat cells, with induction of c-fos and c-myc. Ang II also induces proliferation of aortic myocytes.6 Ang II is reported to be a potent hypertrophic agent but to have no detectable mitogenic activity in cultured rat VSMCs.17 Although it is not clear whether Ang II induces hyperplasia or not, many reports have shown that Ang II does induce vascular hypertrophy. Thus, Ang II has a direct effect on VSMC growth. Ang II is also reported to increase protein synthesis and subsequently cause cellular hypertrophy by stimulation of platelet-derived growth factor production.18 These results suggest that the action of Ang II may be mediated by production and/or release of neuropeptides or polypeptide growth factors. It is still unknown how these factors interact and regulate vascular growth. Thus, increased vascular Ang II in

<table>
<thead>
<tr>
<th>Vessel wall dimension</th>
<th>2K1C Early</th>
<th>Sham</th>
<th>2K1C Chronic</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wall thickness (μm)</td>
<td>48.0±4.4*</td>
<td>32.7±1.5</td>
<td>53.5±1.9†</td>
<td>33.9±1.1</td>
</tr>
<tr>
<td>Medial-intimal area (×10^4 μm^2)</td>
<td>49.7±5.8*</td>
<td>36.9±4.2</td>
<td>65.1±5.5††</td>
<td>44.6±3.2</td>
</tr>
<tr>
<td>Wall/lumen ratio</td>
<td>0.42±0.01†</td>
<td>0.35±0.02</td>
<td>0.53±0.01††</td>
<td>0.35±0.01</td>
</tr>
</tbody>
</table>

Values are mean±SEM for five to six animals. 2K1C, two-kidney, one clip rats.

*p<0.05, †p<0.01 vs. sham rats.

†p<0.05, ††p<0.01 vs. early phase.
renal hypertensive rats may influence vascular growth and induce vascular hypertrophy by the angiotensin-induced actions as cited above.

Our studies revealed that even during the early phase in 2K1C rats, wall thickness and wall-to-lumen ratios increased compared with values in control rats. During the chronic phase, vascular hypertrophy in 2K1C rats was still observed and tended to increase compared with the degree of hypertrophy observed during the early phase, consistent with previous reports. It is very likely that these structural changes may be related to the increased vascular Ang II directly or indirectly through Ang II-induced actions. The findings that angiotensin converting enzyme inhibitors, but not hydralazine, decrease vascular hypertrophy in 1K1C, 2K1C, and spontaneously hypertensive rats support the hypothesis that vascular Ang II may influence vascular hypertrophy. However, the mechanism by which Ang II induces vascular hypertrophy in vivo still is not clear. Further studies are needed to determine the mechanism of vascular growth.

Recent progress in the field of vascular biology has demonstrated the existence of the autocrine-paracrine system in vessels. Vascular angiotensinogen mRNA has been reported to be present in VSMCs. In the present study, we demonstrated the presence of angiotensinogen mRNA in the vessel wall in 2K1C rats, and the level of vascular angiotensinogen mRNA did not differ between 2K1C and control rats during the early and chronic phases. In 1K1C rats, the same results have been previously reported, that angiotensinogen mRNA expression in the vessel wall was decreased or unchanged in chronic 1K1C hypertension, using in situ hybridization. These findings may suggest an abundance of vascular angiotensinogen. However, increased vascular angiotensin converting enzyme activity has been reported in 1K1C rats. Similarly, we reported elevated vascular renin activity during the early phase and elevated vascular angiotensin converting enzyme activity during the chronic phase in 2K1C rats (10.5 ±1.3 milliunits/mg protein in sham-operated rats versus 19.7±2.7 milliunits/mg protein in 2K1C rats; p<0.05) and an
increased rate of Ang II production in the blood vessels. These results, including those of our group, suggest that angiotensinogen expression cannot account for the activation of the vascular renin-angiotensin system. Therefore, the increased Ang II production rate described previously and increased Ang II concentration in blood vessels reported in this study are not due to enhanced angiotensinogen synthesis but are rather due to increased vascular renin during the early phase or increased angiotensin converting enzyme activity during the chronic phase.

In conclusion, our results demonstrated an increased vascular Ang II level during both the early and chronic phases in 2K1C rats, which may contribute to the development and maintenance of hypertension and subsequent arteriosclerosis in 2K1C rats through Ang II-induced hypertrophy or proliferation of VSMCs. This study also suggested that elevated vascular renin activity during the early phase and increased vascular angiotensin converting enzyme activity during the chronic phase may be major determinants of the increased vascular Ang II level. It is possible that vascular angiotensinogen may not contribute to vascular hypertrophy in 2K1C hypertension.

References


Key Words: angiotensin II • renovascular hypertension • angiotensinogen • renin-angiotensin system • hypertrophy
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