Differences in Pattern of Plasma Angiotensinogen in Native and Nephrectomized Rats

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SUMMARY Rat plasma contains two distinct forms of angiotensinogen (Ao-1 and Ao-2) that can be found in single animals in a distinct ratio. The ratio of Ao-1 to Ao-2 was determined by separation of Ao-1 and Ao-2 from 1 ml of plasma from individual rats on an SP-Sephadex C-50 column. Plasma from rats of three different strains, Wistar, Wistar-Kyoto (WKY), and spontaneously hypertensive rats (SHR), was investigated. In Wistar rats native plasma contained Ao-1 and Ao-2 in a ratio of 2.6:1. Twenty-four hours after nephrectomy, which increased the total Ao content 4.1-fold, this ratio was changed to 1.1:1. In native WKY and SHR the ratio of the two forms was similar to that in Wistar rats: 2.4:1 and 2.8:1, respectively. After nephrectomy the ratio of Ao-1 to Ao-2 was changed to 1.1:1 and 0.78:1 in WKY and SHR, respectively, while the total Ao content increased 4.9-fold and 8.2-fold in the two strains. Endogenous plasma renin inactivated the two forms of Ao, with a $K_m$ of $4.0 \pm 0.46$ and $3.7 \pm 0.43 \mu$M and a $V_{max}$ of 176 ± 15.5 and 155 ± 12.7 nM/hr, respectively. These results suggest that 1) Ao-1 and Ao-2 are synthesized in equimolar amounts, 2) the clearance of Ao-2 is faster than that of Ao-1 in control rats, and 3) under conditions of stimulated synthesis (i.e., after nephrectomy), the plasma content of Ao-2 increases faster than that of the more highly glycosylated form, Ao-1. (Hypertension 9: 339-344, 1987)

KEY WORDS • angiotensinogen • renin-angiotensin system • Wistar rats • Wistar-Kyoto rats • spontaneously hypertensive rats • nephrectomy • plasma renin • carbohydrate differences • kinetics

ANGIOTENSINOGEN (Ao), the precursor of the peptide hormone angiotensin, is synthesized and secreted by the liver. In rat plasma, this glycoprotein exists in two different forms (Ao-1 and Ao-2), which have recently been isolated. The two forms differ in their molecular weight by about 4000 Da, and this difference has been shown to reside in the carbohydrate moiety. In contrast to plasma, cerebrospinal fluid apparently contains only one molecular weight form of Ao, though an Ao messenger RNA (mRNA) from liver and brain has been reported. Based on minor differences found in the amino acid determination of Ao-1 and Ao-2, it has been suggested that Ao-2 may have one less carbohydrate binding site than Ao-1. This possibility seems unlikely, since only one polypeptide sequence deduced from the complementary DNA (cDNA) sequence analysis of rat Ao has been described as containing two potential glycosylation sites. Very recently, Campbell et al. obtained two differently glycosylated Ao forms by in vitro translation using liver mRNA, rabbit reticulocyte lysate, and pancreatic microsomes, thus supporting the idea that either site can be linked with an N-glycan.

The synthesis and secretion of Ao by the liver recently have been studied in vivo using isolated liver perfusion, liver tissue slices, isolated rat hepatocytes, and hepatoma cells in culture, as well as by in vitro translation of rat liver mRNA. In these studies, the influences of various effectors, such as glucocorticoids, estrogens, prostaglandins, angiotensin II, adrenalectomy, and nephrectomy on the synthesis and secretion of Ao, was studied without distinction between the two forms of Ao.

The aim of the present study was to show that the two differently glycosylated forms of Ao are present in individual animals. Nephrectomy, which is a potent stimulus of Ao synthesis, was used to investigate whether an independent change in the plasma levels of both forms occurs. To show that both forms are present in different rat strains, we used Wistar rats and Wistar-Kyoto rats.
Kyoto rats (WKY). In addition, the experiments were performed with spontaneously hypertensive rats (SHR), since the Ao plasma levels are often elevated in hypertension (for review, see Reference 22). We also studied the kinetics of each form independently at increasing levels with endogenous plasma renin to ascertain whether the differences in glycosylation of the two forms influence the generation of angiotensin.

Materials and Methods

With the rats under ether anesthesia, blood, to which EDTA (0.15%) was added, was obtained from the carotid artery of Wistar rats, WKY (all weighing 250–300 g), and SHR (weighing 200–220 g) before and 24 hours after nephrectomy. The plasma from each animal was collected separately and stored at —20°C until use. Each series was performed with six animals.

Separation and Determination of Angiotensinogen 1 and 2

One milliliter of plasma was diluted 1:4 with saline and separated on an SP-Sephadex C-50 column (1.0 × 30 cm) on a linear ammonium acetate buffer gradient, 0.1 M (50 ml) to 0.5 M (50 ml), pH 5.0. The flow rate was 1 ml/hr. Then, 1-ml fractions containing Ao-1 and Ao-2 were pooled, dialyzed against 0.1 M ammonium bicarbonate buffer, pH 7.8, and lyophilized. Ao-1 and Ao-2 can be separated on this column (see Results).

Ao was determined in the fractions using an indirect angiotensin (ANG I) radioimmunoassay23 as well as a direct radioimmunoassay, which was performed according to the method of Bouhnik et al.24 Ten microliters of the effluent was diluted 1:200 with 0.1 M phosphate buffer, pH 7.5, containing 0.1% gelatin and 5 mM EDTA. Then, 50 μl of the diluted sample was added to 100 μl of a rabbit anti-Ao antibody solution (1:40,000 in the same buffer solution) and 250 μl of 125I-labeled Ao (3000 cpm) and incubated for 48 hours at 4°C. Bound and free angiotensinogen were separated using a goat antibody raised against rabbit immunoglobulin. This immunoglobulin was obtained by 50% ammonium sulfate precipitation of the antiserum. The 100 μl of 0.1 M phosphate buffer, pH 7.5, that was added to the incubation mixture contained 0.1% (wt/vol) of the lyophilized goat immunoglobulin. After a further 3-hour incubation at 20°C, 1.14 ml of 25% polyethylene glycol containing 1.5% Triton X-100 in 0.1 M phosphate buffer, pH 7.5, was added to the tubes, which were stirred intensively. The mixture was centrifuged at 5000 g for 30 minutes at 4°C, the supernatant was withdrawn, and the precipitate was counted. The standards ranged from 0.25 to 32 ng of Ao (i.e., 5–600 fmol). Each assay was the mean of a triplicate determination. Purified human Ao and sheep Ao were tested for cross-reactivity (the purification procedure will be described elsewhere). As shown in Figure 1, there was an almost linear decrease in B/B₀ between 10 and 150 fmol rat Ao and no binding of human and sheep Ao within the range tested. In addition, there was complete cross-reactivity of the antiserum with des-ANG I-Ao (data not shown). Therefore, the amount of des-ANG I-Ao can be calculated by subtracting the data for the direct radioimmunoassay from those of the indirect (ANG I) radioimmunoassay.

Pure Ao-1 was iodinated with iogen according to the method of Fraker and Speck.25 Iodinated Ao was separated from other conjugated products on a Sephadex G-25 sf column in 0.05 M phosphate buffer, pH 7.5, containing 0.25% gelatin (wt/vol). The incorporated radioactivity was 22 μCi/μg protein. The labeled protein was homogeneous when examined by sodium dodecyl sulfate disk electrophoresis and autoradiography.

Angiotensinogen Antibodies

Purified rat Ao-1, which was obtained according to a previously described method,4 was used as the antigen in rabbits. Five hundred micrograms of the protein was dissolved in 3 ml of saline and mixed with the same volume of complete Freund’s adjuvant. Then, 1 ml of the suspension was applied intracutaneously at 40 to 60 sites on the shaved skin of the rabbit’s back. This procedure was performed according to the method of Vaitukaitis et al.26 Two booster injections were given subcutaneously at 3-week intervals with the same quantity of antigen at about 20 sites; however,
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the antigen was dissolved in an incomplete Freund’s adjuvant–saline solution. One week after the second booster injection, the animals were bled. The specificity of the antiserum was tested by immunodiffusion according to the method of Ouchterlony, using the Ao radioimmunoassay and ANG I radioimmunoassay.

**Angiotensinogen-Free Plasma**

Ao was removed from plasma by affinity chromatography. Purified Ao antibodies were linked to aminopropylsilylexyl-CPG (Electro Nucleonics, Fairfield, NJ, USA) by glutaraldehyde, as already described. Then, 2 ml of plasma was applied to the affinity column (0.5 × 4 cm) and eluted with a flow rate of 2 ml/hr. The efficiency of removal of Ao from plasma exceeded 99%. The bound Ao was removed by elution with 3 M NaSCN.

**Turnover of Angiotensinogen by Plasma Renin**

To estimate Ao turnover by plasma renin, increasing amounts of both Ao-1 and Ao-2 were added to the Ao-free plasma containing endogenous renin. The amount of ANG I generated was determined after a 15- or 60-minute incubation with the ANG I radioimmunoassay.

**Statistical Analysis**

Results are expressed as means ± SD. The statistical significance of differences was calculated using Student’s t-test for paired data. A p value less than 0.05 was considered statistically significant.

**Results**

The plasma Ao levels in Wistar, WKY, and SHR before and after nephrectomy are shown in Table 1. The plasma Ao values, determined by the ANG I method (indirect) differed from those obtained by the direct Ao radioimmunoassay only within the range of the standard deviations. This suggests that plasma contains no or less than 10% inactive des-ANG I-Ao. No significant differences could be found between Wistar rats and SHR. In WKY, however, the plasma Ao values were about 40% higher than those in Wistar rats and about 20% higher than those in SHR.

Twenty-four hours after nephrectomy, plasma Ao levels were about four times higher in Wistar rats and about five times higher in WKY. The highest (eightfold) increase in plasma Ao levels was observed in SHR. Values obtained by direct Ao radioimmunoassay were approximately equivalent to those obtained by the ANG I radioimmunoassay.

As shown in Figure 2, the two Ao forms, Ao-1 and Ao-2, could be separated from each other by chromatography on an SP-Sephadex C-50 column. The recovery of both separated forms after chromatography was about 50% in Wistar rats and between 60 and 70% in WKY and SHR. This loss was due to nonspecific adsorption of active material by the gel. Both Ao forms were present in plasma of every rat at similar concentrations in comparable animals.

Ao-1, the more highly glycosylated form, represented the main angiotensin precursor in the plasma of Wistar rats, WKY, and SHR (Table 2). Its plasma levels were 2.6 times higher in Wistar rats, 2.4 times higher in WKY, and 2.8 times higher in SHR than those of the minor glycosylated form, Ao-2. After nephrectomy, which stimulated the Ao plasma levels fourfold to eightfold (see Table 1), the difference between Ao-1 and Ao-2 decreased. The plasma levels of Ao-1 and Ao-2 were approximately equivalent in nephrectomized Wistar rats and WKY.

As shown in Table 3, the ratio of the two Ao forms was equivalent in plasma of Wistar rats and WKY after subtracting these levels from those in native animals. In SHR postnephrectomy plasma levels of the minor glycosylated form, Ao-2, increased 60% above those of Ao-1; however, this increase was not statistically significant (p<0.1).

When the values of the two forms in native plasma and in plasma of nephrectomized rats were compared, a threefold to fourfold increase of Ao-1 and a sevenfold to eightfold increase of Ao-2 were noted in nephrectomized Wistar rats and nephrectomized WKY. In nephrectomized SHR, the increase in Ao-1 was about fivefold, while that in the minor glycosylated form, Ao-2, was more than 17-fold.

To investigate the enzymatic properties of endogenous plasma renin in the two differently glycosylated Ao molecules, Ao-free plasma was prepared by affinity chromatography on an anti-Ao-antibody column. Increasing amounts of Ao-1 and Ao-2 were added to this Ao-free plasma and incubated at 37°C for

<table>
<thead>
<tr>
<th>Group</th>
<th>ANG I (nmol/ml)</th>
<th>Ao (nmol/ml)</th>
<th>ANG I (nmol/ml)</th>
<th>Ao (nmol/ml)</th>
<th>ANG I (nmol/ml)</th>
<th>Ao (nmol/ml)</th>
</tr>
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<tbody>
<tr>
<td>Wistar (n = 6)</td>
<td>0.68 ± 0.06</td>
<td>0.64 ± 0.06</td>
<td>2.77 ± 0.35</td>
<td>2.50 ± 0.45</td>
<td>4.1:1</td>
<td>3.9:1</td>
</tr>
<tr>
<td>WKY (n = 6)</td>
<td>0.94 ± 0.02*</td>
<td>1.05 ± 0.20</td>
<td>4.58 ± 0.51*</td>
<td>4.35 ± 1.12</td>
<td>4.9:1</td>
<td>4.1:1</td>
</tr>
<tr>
<td>SHR (n = 6)</td>
<td>0.78 ± 0.10f</td>
<td>0.84 ± 0.02</td>
<td>6.37 ± 0.57f*</td>
<td>7.40 ± 2.60</td>
<td>8.2:1</td>
<td>8.8:1</td>
</tr>
</tbody>
</table>

Values are means ± SD. ANG I values obtained by indirect radioimmunoassay; Ao values obtained by direct radioimmunoassay. Nx = nephrectomy; Ao = angiotensinogen.

* p < 0.005, compared with respective value in Wistar rats.

† p < 0.05, †p < 0.005, compared with respective value in WKY.
various periods. As shown in Figure 3, there was an identical release of ANG I in plasma containing either Ao-1 or Ao-2 up to the Ao concentration found in native plasma (i.e., 0.8 nmol/ml). The kinetics of the two forms were studied with concentrations of Ao up to 23 nmol/ml. The $K_m$ values for Ao-1 and Ao-2, as calculated from the linear Lineweaver-Burk plot, were 4.0 ± 0.46 and 3.7 ± 0.43 μM, respectively. The $V_{max}$ value with Ao-1 and Ao-2 was found to be 176 ± 15.5 and 155 ± 12.7 nmol/hr, respectively ($n = 4$; Figure 4). These data show that plasma renin apparently does not prefer one form of Ao, which suggests that differences in the plasma levels of Ao-1 and Ao-2 may be due to variations in their synthesis or elimination but not to differences in the cleavage of each form by renin.

**Discussion**

The existence of two different angiotensinogen molecules in rats was observed by Sen et al. using HgCl$_2$ stimulation. The two Ao forms were separated and characterized after purification from pooled plasma of nephrectomized rats. Available evidence suggests that the differences in molecular weight and isoelectric point between Ao-1 and Ao-2 are due to different gly-
of Ao is balanced by synthesis, consumption, and by several factors. In native animals, the plasma level of Ao-2 in native and nephrectomized rats may be caused by the two forms of Ao do not differ in their affinity for chromatography and isoelectric focusing. Although some overlap of the band pattern in isoelectric focusing of the two forms can be seen (Figure 2A), neuraminidase treatment and sodium dodecyl sulfate disk electrophoresis confirm that they do not belong to identical molecules (unpublished observation, 1986).

When we compared the plasma Ao content in native Wistar rats, WKY, and SHR, we found small but statistically significant differences in the three strains. The data obtained by both Ao assay systems used — ANG I and Ao radioimmunoassay — were almost equivalent. However, after nephrectomy, which is a well-known stimulus of plasma Ao content, the plasma Ao concentrations in Wistar rats, WKY, and SHR differed greatly. The lowest plasma levels as well as the lowest increase after nephrectomy were found in Wistar rats. The highest Ao content of native plasma was found in WKY, while the highest increase after nephrectomy was measured in SHR.

Separation of the two forms of Ao by SP-Sephadex C-50 showed a 2.4 to 2.8 times higher content of the more highly glycosylated form, Ao-1, than of Ao-2. After nephrectomy, which increased the total plasma Ao levels fourfold to ninefold in the three strains, this difference was decreased and the plasma levels of Ao-1 and Ao-2 were approximately equal in Wistar rats and WKY. This variation in the ratio of Ao-1 to Ao-2 in native and nephrectomized rats may be caused by several factors. In native animals, the plasma level of Ao is balanced by synthesis, consumption, and elimination of Ao, and Ao-2 may be eliminated faster than Ao-1 by the kidney, possibly because of its lower molecular weight and glycosylation rate. After nephrectomy, the consumption of Ao decreases very rapidly because of the loss of renin production and the rapid elimination of renin. This decrease leads to some increase in plasma Ao levels, and the increase in Ao levels apparently is due predominantly to an increased synthesis. This view is supported by a recent publication that reported increased formation of Ao in reticulocyte lysate primed with liver mRNA from nephrectomized rats. The identical plasma level of either form of Ao in nephrectomized Wistar rats and nephrectomized WKY suggests that they are synthesized, secreted, and eliminated in an equimolar ratio following nephrectomy. In addition, the more than 17-fold increase in the plasma level of Ao-2 in SHR suggests that Ao-2 increases more quickly under stimulated conditions than Ao-1.

To exclude a difference in consumption (i.e., the possibility that the two Ao forms are differently cleaved by renin), enzyme kinetic data were performed with Ao-1 and Ao-2. These studies showed that the two forms of Ao do not differ in their affinity for endogenous plasma renin, as the apparent $K_m$ values for the two forms were almost identical: 4.0 and 3.7 $\mu$M for Ao-1 and Ao-2, respectively. These $K_m$ values are in agreement with those published for total rat Ao (2.8 $\mu$M) and for the separated forms (5.0 $\mu$M for the Ao-1 equivalent form and 5.6 $\mu$M for the Ao-2 equivalent form). Interestingly, in the presence of endogenous renin, both Ao-1 and Ao-2 were cleaved to less than 1% of total plasma Ao per hour in vitro at 37°C. However, we do not know to what extent Ao-1 and Ao-2 are consumed in vivo. Lewicki et al. reported that the half-life of rabbit Ao was about 8 hours, which suggests that the turnover of Ao is not due to circulating renin alone. Recently, the possibility was raised that vascular smooth muscle cells may synthesize renin. Although it has been suggested that this local synthesis makes little contribution to the vascular renin.
inlike activity, since the aortic reninlike activity falls to very low levels after nephrectomy,32 this tissue renin should increase the consumption of Ao in vivo in native rats. However, it is unlikely that this tissue renin has enzymatic properties that differ from those of the circulating enzyme and, thus, may contribute to the different levels of Ao-1 and Ao-2 in native plasma. In addition to renin, other enzymes, such as trypsin,34 kallikrein,33 and cathepsin G,36 may contribute to the generation of ANG I and angiotensin II. However, neither the rate of angiotensin generation in vivo by these enzymes nor their enzymatic properties in respect to Ao-1 and Ao-2 is known. Thus, presently available evidence indicates that the different levels of the two Ao forms are not due to different enzymatic consumption.

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