SUMMARY An apparent high molecular weight angiotensinogen (H-Aogen) can be separated from the usually predominant low molecular weight angiotensinogen (L-Aogen) by gel filtration of plasma. H-Aogen has been quantitated in plasma from normotensive menstruating women, estrogen treated women, normotensive pregnant women, women with pregnancy-induced hypertension (PIH), and women whose preexisting hypertension was exacerbated during pregnancy. When expressed as a percent of the total angiotensinogen, the H-Aogen levels were: menstruating women 4%, estrogen-treated women 10%, normotensive pregnant women 16%, women with PIH 25%, and pregnant women with exacerbated hypertension 28%. A significant difference (p < 0.01) was found between H-Aogen concentration in normotensive pregnant women and women with PIH (1.10 ± 0.12 and 1.73 ± 0.16 fig renin releasable angiotensin I/ml plasma respectively). In some hypertensive pregnant women, H-Aogen is the predominant form of angiotensinogen. Thus, H-Aogen should be recognized as a component of the renin-angiotensin system. (Hypertension 4: 729-734, 1982)

KEY WORDS · high molecular weight angiotensinogen · hypertensive pregnancy

ANGIOTENSINOGEN, a plasma protein, is the substrate for the enzyme renin. Although multiple forms of angiotensinogen have been detected by a variety of techniques, there has been only one report of a high molecular weight form of angiotensinogen (H-Aogen). Gordon and Sachin\(^1\) reported that an apparent H-Aogen could be separated from the predominant low molecular weight angiotensinogen (L-Aogen) by gel filtration. In plasma from normal menstruating women, the H-Aogen accounted for about 3% to 5% of the total angiotensinogen. The plasma from women taking oral contraceptives and from pregnant women were also examined by this technique. Although the different forms of angiotensinogen were not quantitated in these states, it was noted that the size of the peak of H-Aogen appeared to be larger in these states.

This report confirms and extends these findings. Plasma H-Aogen and L-Aogen have been quantitated in normotensive menstruating women, women receiving exogenous estrogen, normotensive pregnant women, women with pregnancy-induced hypertension (PIH), and women whose existing hypertension was exacerbated by pregnancy. Both the actual and relative amount of H-Aogen was increased in high estrogen states and the level in women with PIH was significantly elevated as compared to normotensive pregnant women.

Methods

Gel Filtration on Sephacryl S-200

A Sephacryl S-200 column (1.6 × 85 cm) was equilibrated with 0.05 M Tris-HCl-0.1 M NaCl-3 mM EDTA, pH 8. The sample, 1.0 ml containing 1 to 2 µg renin releasable angiotensin I (AI), was applied and the column eluted with the equilibrating buffer at a flow rate of 0.1 ml/min with 18-minute fractions being collected. When necessary, the plasma sample was diluted with the equilibrating buffer. This ensured that a relatively constant amount of angiotensinogen in a constant volume was applied to the column. The elution volume was determined by weighing tubes in groups of 10 before and after collection of fractions. The A\(_{280}\) nm of each fraction was determined, and an aliquot of each fraction with A\(_{280}\) nm > 0.005 was assayed for angiotensinogen. The angiotensinogen content of each tube under each peak was summed and expressed as percent of the total angiotensinogen recovered. The H-Aogen and L-Aogen content of the
original plasma was calculated by multiplying the percent of each times the angiotensinogen content of the plasma. The overall recovery of angiotensinogen for 22 samples assayed in this manner was 91% (range, 80% to 117%). Since the elution volume of H-Aogen was very constant, only fractions across the valley between H-Aogen and L-Aogen were assayed individually in some runs and the fractions preceding and following the valley were assayed as two pools. The overall recovery of angiotensinogen for 24 samples assayed in this manner was 94% (range 80% to 108%). The coefficient of variation (CV) for the same plasma run three times was 15%.

High Performance Liquid Chromatography (HPLC)
The system used consisted of Waters 6000A pump, Waters model 710A sample processor, a guard column packed with Waters I-125 packing, either one or two Waters I-250 protein columns (0.78 x 30 cm), LKB Uvicord detector with 280 nm filter, Waters data module and Waters system controller. The columns were equilibrated with 0.05 M Tris-sulfate-0.15 M Na₂SO₄, pH 7.5 containing 2 units heparin/ml. The sample, 0.1 ml, was injected and the columns eluted with equilibrating buffer at a flow rate of 0.6 ml/min with 18 second fractions being collected. The elution volume was determined by weighing tubes in groups of ten before and after collection of fractions. Each fraction was assayed for angiotensinogen and H-Aogen and L-Aogen levels calculated as described above. The overall recovery of angiotensinogen for 11 samples assayed was 87% (range 74% to 103%).

Subjects
Blood was obtained from normotensive menstruating women, estrogen-treated women, women whose preexisting hypertension was exacerbated by pregnancy, and women with PIH; the plasma was stored at −20°C until assayed. Estrogen-treated women include those taking an oral contraceptive with a significant estrogen component and women being treated with Premarin. The operational definition of PIH was two blood pressure measurements of 140/90 mm Hg or greater occurring after the 24th week of gestation in a previously normotensive pregnant woman.

Angiotensinogen Assay
The incubation buffer, 0.6 M sodium phosphate, 36 mM EDTA, pH 6.0 containing gelatin (4 mg/ml) was brought to a boil and cooled to room temperature. The gelatin was used to minimize nonspecific adsorption of generated AI. The buffer was boiled to inactivate any protease activity that might be present. A mixture containing 0.25 ml buffer angiotensinogen (0.8 to 10 ng AI), 20 μl of human renin, and H₂O to bring volume to 1.0 ml, was incubated at 37°C for 3 hours. Aliquots were assayed for AI by the radioimmunoassay (RIA) procedure of Haber et al. except that lysozyme (Sigma grade 1) 1 mg/ml was used as carrier protein. The buffer, 1 liter, containing the lysozyme, was treated with 1 ml phenylmethylsulfonyl fluoride (PMSF), 50 mg/ml ethanol, and then filtered through a 0.45 μm membrane. An aliquot of an incubation blank containing renin but no angiotensinogen was added to each RIA tube comprising the standard curve. A control plasma was assayed in each run. The intraassay CV was 5.8% (n = 24) and the interassay CV was 12% (n = 81). The mean recovery of AI (8 or 4 ng) added to the assay of the control plasma at the start of the incubation was 92% (range 86% to 101%, n = 4).

Human renin was prepared by procedure A of Haas et al. The renin, 2 ml, was treated with 10 μl PMSF, 50 mg/ml ethanol. It was ascertained that each batch of renin would release all of the AI in 5 μl of normal human plasma within 1.5 hours under the above assay conditions.

Plasma Renin Activity (PRA) Assay
A mixture containing 0.25 ml, 0.60 M sodium phosphate-36 mM EDTA, pH 5.4, 0.24 ml H₂O, 0.5 ml plasma, and 10 μl PMSF, 50 mg/ml ethanol, was prepared in an ice bath. The pH of the mixture was 6.0. One-half of the mixture was incubated at 37°C for 1 hour and then placed in an ice bath. The other half of the mixture was kept in the ice bath. Aliquots of both mixtures were assayed for AI by RIA as described above. The value for the 0°C sample was subtracted from that of the 37°C sample in calculation of the PRA. The intraassay CV was 6.1% (n = 10) and the interassay CV was 8.8% (n = 24).

Rat Pressor Assay
This assay was done in a nephrectomized pentolinium-treated rat according to the procedure of Boucher et al. using an AI standard.

Results
The chromatograms obtained from gel filtration on Sephacryl S-200 of plasma from a normotensive menstruating woman, a normotensive pregnant woman, and a hypertensive pregnant woman are shown in figures 1–3. In each instance the angiotensinogen was clearly separated into two fractions. The fraction that eluted first was subsequently referred to as "H-Aogen," while the latter fraction, which was usually the predominant form, was referred to as "L-Aogen." Since the H-Aogen peak was well resolved from the L-Aogen peak, quantitation of the total amount of H-Aogen and L-Aogen was achieved. Separation of H-Aogen and L-Aogen was also carried out on Waters I-250 HPLC columns. To verify that the two methods were equivalent, 11 samples were fractionated both ways. The H-Aogen concentration of these samples ranged from 0.079 ng AI/ml plasma. The plot of the H-Aogen values obtained by HPLC vs the H-Aogen values obtained by gel filtration on Sephacryl S-200 yielded a straight line with a slope of 0.922, an intercept of 0.087, and a correlation coefficient of 0.976. Since equivalent separation was achieved on two different types of gels, the separation achieved appears to be based on differences in the hydrodynamic radius of the molecules.
The results of quantitation of H-Aogen in 57 women in either normal estrogen or high estrogen states are given in Table 1. As has been amply documented in the past, the values demonstrated that total angiotensinogen increased in high estrogen states with a more than fourfold increase demonstrated in the pregnant state. The level of H-Aogen was low in normal menstruating women but increased in high estrogen states. The relative increase in H-Aogen was much higher than that for L-Aogen. Thus, in the normotensive pregnant women there was a 16-fold increase in H-Aogen and less than a fourfold increase in L-Aogen. The most striking finding of this study was a statistically significant difference between the H-Aogen levels in the PIH group and the normotensive pregnant group (p < 0.01). A statistically significant difference was also obtained when the highest H-Aogen value in the PIH group was excluded (p < 0.01). The individual values of H-Aogen for the three pregnant groups are shown in Figure 4. Although there was considerable overlap, it will be noted that there were six values out of 19 in the PIH group that were distinctly above the highest value for

<table>
<thead>
<tr>
<th>Female group</th>
<th>No.</th>
<th>Angiotensinogen (μg Al/ml)</th>
<th>Percent high mol wt angiotensinogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>High mol wt</td>
</tr>
<tr>
<td>Normotensive menstruating</td>
<td>7</td>
<td>1.59 ± 0.13</td>
<td>0.068 ± 0.011</td>
</tr>
<tr>
<td>Normotensive pregnant</td>
<td>13</td>
<td>7.07 ± 0.44</td>
<td>1.10 ± 0.12</td>
</tr>
<tr>
<td>Pregnancy-induced hypertension</td>
<td>19</td>
<td>6.95 ± 0.39</td>
<td>1.73 ± 0.16</td>
</tr>
<tr>
<td>Hypertension exacerbated by pregnancy</td>
<td>8</td>
<td>6.47 ± 0.52</td>
<td>1.78 ± 0.35</td>
</tr>
<tr>
<td>Estrogen-treated</td>
<td>10</td>
<td>5.19 ± 0.26</td>
<td>0.50 ± 0.047</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Al = angiotensin I.
normotensive pregnant women. In the group whose hypertension was exacerbated by pregnancy, one value was grossly elevated while the rest were within the range found for normotensive pregnant women.

The above values for both H-Aogen and L-Aogen were expressed in terms of μg AI/ml of plasma. Thus, when these values were expressed as percentages, a representation was obtained of the relative number of each type of renin substrate molecules present. The distribution of percent H-Aogen in each group studied is shown in figure 5. The values for the high estrogen states, estrogen-treated women, and all of the pregnant women were clearly elevated above the value for normal menstruating women. The majority of the values in the PIH group (12/19) and half of the values in the pregnancy-exacerbated hypertensive group (4/8) were above the highest value in the normotensive pregnant group. In one woman in the pregnancy-exacerbated hypertension group, 61% of the renin releasable AI was present in the H-Aogen fraction. Thus, in the majority of the hypertensive pregnant women there was a marked alteration in the relative proportion of the type of renin substrate present.

Some of the characteristics of the three pregnant groups studied are given in table 2. The two hypertensive groups exhibited definite hypertension with the mean diastolic pressure being more than 25 mm Hg above the mean for the normotensive group. The samples that were studied were obtained late in the third trimester of pregnancy. There was no significant difference in age between the PIH group and the normotensive pregnant group while the pregnancy exacerbated hypertension group was slightly older. There were no significant differences in the PRA values. Since these were not collected under conditions where posture or sodium intake was noted, however, definitive conclusions cannot be drawn from the PRA results. In the PIH group, 10 were primigravidas, nine exhibited proteinuria, and 14 were edematous. There was no obvious correlation between any of these parameters and abnormal elevation in either the absolute or relative quantity of H-Aogen.

The results of analysis of serial samples from two normotensive pregnant women are shown in figure 6. H-Aogen levels rose early in pregnancy and then remained relatively constant until delivery. L-Aogen levels tended to increase throughout pregnancy. When values for H-Aogen were expressed as percent total angiotensinogen, maximum values were reached at the same point and then these decreased slightly during the rest of the pregnancy. Serial samples in the third trimester from one woman with pregnancy exacerbated hypertension have been analyzed. As can be seen in figure 7, H-Aogen rose dramatically throughout this period with the values almost doubling from the 33rd week of pregnancy to the 40th week of pregnancy. During this period of time the percent H-Aogen also increased steadily from an initial value of 34% to a final value of 61%. Obviously, data on serial samples from more hypertensive pregnant patients need to be analyzed in order to establish whether this phenom-
TABLE 2. Physical Characteristics of the Different Pregnant Groups

<table>
<thead>
<tr>
<th>Female group</th>
<th>Blood pressure (mm Hg)</th>
<th>Weeks gestation</th>
<th>Age (yrs)</th>
<th>PRA (ng Al/ml/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normotensive pregnant</td>
<td>Systolic 118 ± 1.6</td>
<td>37.8 ± 0.5</td>
<td>26.6 ± 1.7</td>
<td>9.2 ± 1.4</td>
</tr>
<tr>
<td>Pregnancy-induced hypertension</td>
<td>Systolic 148 ± 7.0</td>
<td>38.4 ± 0.7</td>
<td>25.7 ± 1.3</td>
<td>7.6 ± 1.1</td>
</tr>
<tr>
<td>Pregnancy exacerbated hypertension</td>
<td>Diastolic 71 ± 1.4</td>
<td>38.4 ± 0.7</td>
<td>25.7 ± 1.3</td>
<td>7.6 ± 1.1</td>
</tr>
<tr>
<td>Pregnancy exacerbated hypertension</td>
<td>Diastolic 97 ± 9.6</td>
<td>38.4 ± 0.7</td>
<td>25.7 ± 1.3</td>
<td>7.6 ± 1.1</td>
</tr>
<tr>
<td>Pregnancy exacerbated hypertension</td>
<td>Diastolic 103 ± 2.9</td>
<td>38.4 ± 0.7</td>
<td>25.7 ± 1.3</td>
<td>7.6 ± 1.1</td>
</tr>
<tr>
<td>Pregnancy exacerbated hypertension</td>
<td>Diastolic 81 ± 1.2</td>
<td>38.4 ± 0.7</td>
<td>25.7 ± 1.3</td>
<td>7.6 ± 1.1</td>
</tr>
<tr>
<td>Pregnancy exacerbated hypertension</td>
<td>Diastolic 122 ± 2.8</td>
<td>38.4 ± 0.7</td>
<td>25.7 ± 1.3</td>
<td>7.6 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SEM. PRA = plasma renin activity.

Figure 6. Plot of high molecular weight angiotensinogen (H-Aogen) and low molecular weight angiotensinogen (L-Aogen) vs weeks gestation for two normotensive women.

Figure 7. Plot of high molecular weight angiotensinogen (H-Aogen) and low molecular weight angiotensinogen (L-Aogen) vs weeks gestation for a woman whose hypertension was exacerbated during pregnancy.

Discussion

These studies show that angiotensinogen can be fractionated into two components by gel filtration, that a mechanism exists in the human body for elevating the absolute quantity and the relative percent of the apparent high molecular weight component in high estrogen states, and that in some women who become hypertensive during pregnancy, or whose hypertension is exacerbated by pregnancy, this apparent high molecular weight component is elevated above the level seen in normotensive pregnant women. Indeed, in some
women the high molecular weight form may become the predominant form of angiotensinogen. Three women have been studied in whom H-Aogen comprised from 48% to 61% of the total angiotensinogen. Thus, it appears that H-Aogen should be considered a significant component of the renin-angiotensin system.

Whether H-Aogen could be an artifact produced during the in vitro handling of the sample should be considered. The most obvious artifact would be that H-Aogen is an aggregate or polymer of L-Aogen. Most of the evidence available at present does not support this contention. The H-Aogen in the plasma from pregnant women has been shown to elute at the same volume upon repetitive gel filtration at pH 8. No evidence of any dissociation was noted.5 Equivalent results were obtained when the gel filtration was performed at pH 4.7.5 It has been noted that storage of plasma samples at −20°C for over 1 year did not change the amount of H-Aogen present. Also, the fact that H-Aogen can rise after a physiological stimulus, pregnancy, and fall again after parturition, and that this rise and fall in H-Aogen was not necessarily correlated with the rise and fall in L-Aogen, suggests that H-Aogen in plasma from pregnant women is not an aggregate of L-Aogen.

Although the H-Aogen fraction has been treated as a single entity in this work, it should be kept in mind that more than one high molecular weight form of angiotensinogen is present in the H-Aogen fraction. Previously, Gordon and Sachin1 noted that the peak elution volume of H-Aogen fraction in menstruating women was less than the peak elution volume of H-Aogen present in pregnant women. This finding has been confirmed.3 Thus, it would appear that there are at least two high molecular weight forms of angiotensinogen. Only the form with the highest apparent molecular weight is detectable in menstruating women. The other form with an apparent lower molecular weight is the predominant form in the pregnant state. Since these two forms are not resolved on the gel filtration systems presently used, it is not possible to determine whether the form seen in menstruating women is elevated in the pregnant state.

The true molecular weight of either form of H-Aogen is unknown. Gordon and Sachin1 obtained an estimate of these molecular weights by gel filtration of the native protein under nondenaturing conditions. A molecular weight of 350,000 for the predominant form of H-Aogen in plasma from pregnant women and a molecular weight of 450,000 to 500,000 for H-Aogen present in plasma from menstruating women were estimated. These values should not be considered as true values. The method employed yields valid estimates only if the shape of all the proteins studied, standards and unknowns, are the same and there is no evidence that this criterion was met.

Tewksbury and Dart4 have been able to determine the molecular weight of a subunit from one of the H-Aogens in plasma from pregnant women by gel filtration in 6 M guanidine hydrochloride because even after reduction, alkylation, and exposure to dissociation in 6 M guanidine hydrochloride, renin will release 10% of the initial AI after dialysis against water. The molecular weight of the polypeptide chain from H-Aogen which contained AI and that of purified L-Aogen determined by this method were found to be identical. Since the yield of AI was very low, it is not known from which of the H-Aogens the detected polypeptide was derived. Accurate estimates of the molecular weights of the H-Aogens and the number of polypeptide chains present in each H-Aogen can be obtained only after purified material is available for further study.

The mechanisms responsible for producing elevated blood pressure in pregnancy have not been defined, although many theories have been proposed. The finding of significant alterations in H-Aogen in hypertensive pregnant women provides a new avenue for investigation. Defining the control and function of H-Aogen may provide some insight into one of the causes of hypertension in pregnancy.

Acknowledgments

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High molecular weight angiotensinogen levels in hypertensive pregnant women.

D A Tewksbury and R A Dart

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