Active and Passive Immunization Against Angiotensin II in the Rat and Rabbit

EVIDENCE FOR A NORMAL REGULATION OF THE RENIN-ANGIOTENSIN SYSTEM

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ABSTRACT

Active or passive immunization has been used repeatedly as a tool in studies on the role of the renin-angiotensin system in the control of blood pressure and kidney function. The results are not consistent among each other, and they are also at variance with other studies using different approaches. To evaluate the possible causes of these discrepancies, the biological characteristics of angiotensin antibodies were studied in rats. Following the intravenous injection of angiotensin II antibodies (purified by affinity chromatography), the plasma concentration of the antibodies declined in a two-exponential curve with half times of 11 hours and 7 days, which probably reflect distribution in the extracellular space and elimination, respectively. Plasma angiotensin II levels rose from preinjection levels of 100 pg/ml to 12,000 pg/ml within 10 minutes and then declined concomitantly with the decline in antibody concentration. We calculated that only a small fraction of the circulating antibody was occupied by angiotensin II. Plasma renin concentrations were initially elevated both in controls and in antibody-injected rats, but they returned to the control level after 30 minutes and remained at that level throughout the rest of the experiment (10 days). This fact indicates that feedback mechanisms which control renin secretion, such as free plasma angiotensin II concentrations, are in the normal range. We therefore concluded that the renin-angiotensin system in antibody-injected rats was regulated at a normal level. Similar conditions seemed to exist in rabbits actively immunized against angiotensin II; these animals exhibited high concentrations of total immunoreactive angiotensin II (up to 200,000 pg/ml) and a small increase in plasma aldosterone and corticosterone concentrations.

One of the most frequently used principles in studies on the biological role of an endogenous humoral or local transmitter is to prevent its effects at the presumed site of action by either inhibiting its synthesis or preventing its access to the effector site. This principle has also been applied to studies on the role of the renin-angiotensin system in the control of arterial blood pressure, aldosterone secretion, and kidney function. The synthesis of angiotensin II, the principal effector of the system, can be blocked by inhibitors of renin (e.g., pepstatin) or converting enzyme (e.g., SQ 20,881). To prevent the interaction of angiotensin II with tissue receptors, antibodies against angiotensin II and, more recently, competitive antagonists of angiotensin II (e.g., 1-Sar-8-Ala-angiotensin II) have been used. A critical review on the suitability of the various agents has recently been published by Davis and his colleagues (1).

For the purpose of such studies, antibodies have the primary advantage of a prolonged persistence in the circulation compared with the short half-life of a few minutes or less of the low-molecular weight antagonists. However, studies with active immunization against angiotensin II (2-8) have failed to demonstrate any participation of the renin-angiotensin system in blood pressure control and the pathogenesis of renal hypertension, although in the latter condition such a participation has been convincingly demonstrated with other inhibitors (reviewed by Davis et al. [1]).

Passive immunization, i.e., injection of angiotensin II antiserum, appears to be a more promising tool (9-17). In the two-kidney model of renovascular hypertension (one renal artery clamped, the contralateral kidney left untouched), the injection of angiotensin antiserum reduces arterial blood pressure (10, 13-15). Reports on a blood pressure effect of antiserum in other forms and stages of renal hypertension and in normotensive animals are less consistent. Moreover, in most studies, the reduction in blood pressure following antiserum injection is only of short duration, i.e., a few minutes, despite continued blockade of the pressure effect of injected angiotensin (10, 12, 14, 15), a phenomenon that is usually taken as evidence for

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adequate angiotensin-blocking concentrations of antibody in the circulation.

However, some methodological aspects of these studies have not been taken into consideration, which may explain some of these inconsistencies and others to be discussed later. Crude undiluted antiserum, which has been used in most of these studies, not only contains heterologous proteins, renin, and renin substrate but also high concentrations of angiotensin II (up to 200 ng/ml). The affinity of the antibody for angiotensin II has not been determined in most of these studies. It may be assumed that low-avidity antibodies cannot effectively compete with angiotensin receptors for angiotensin II. The biological characteristics of the injected antibody such as distribution, biological half-life in the circulation, and loading with angiotensin II and the reaction of the renin-angiotensin system are not known, although these data are essential for the interpretation of results. To provide a more defined basis for such studies, we investigated some of these biological characteristics of purified angiotensin II antibodies.

**Methods**

**ANTIBODIES**

The purification by affinity chromatography and the characterization of antibodies against 5-Ile-angiotensin II have been described previously (18). The preparation used in this study had a titer of 1:200,000; its protein content was 2.3 mg/ml and its apparent affinity constant was $7 \times 10^4$ liters/mole. A binding capacity of 11.44 µg/ml (10.94 nmoles/ml) was calculated from double-reciprocal plots of radioimmunoassay standard curves. The cross-reaction with angiotensin I was 0.2%. The antibody preparation was free of renin, renin substrate, and angiotensin I and II.

**EXPERIMENTAL PROTOCOL**

Seven male Sprague-Dawley rats with a body weight of 275 ± 17 (sd) g received 0.2 ml of the antibody preparation in a total volume of 0.4 ml of saline injected into the saphenous vein under light ether anesthesia. Four control rats received 0.4 ml of saline. Blood samples (0.5 ml) were collected into tubes containing 25 µliters of 125 mM Na2EDTA and 25 mM o-phenanthrolin. To keep stimulation of renin secretion by closely spaced blood samplings as low as possible, the rats were divided into four groups (two antibody-injected rats and one control rat each). Blood was taken at 7 minutes, 90 minutes, and 10 hours from group 1, at 30 minutes, 90 minutes, and 10 hours from group 2, at 7 minutes and 4 hours from group 3, and at 30 minutes and 4 hours from group 4 (consisting of one antibody-injected rat and one control rat only). Subsequently, blood was obtained from all of the rats at 1, 2, 3, 4, 6, 8, and 10 days after antibody injection.

Until 6 hours following antibody injection, the rats were kept in the laboratory; subsequently, they were transferred to the animal unit, where they were housed at a constant temperature of 24°C with a relative humidity of 55% and lights on from 6 AM to 6 PM. The rats had free access to food (Ssniff-pellet diet) and tap water. Body weight and food and water consumption were recorded daily.

**PLASMA ANGIOTENSIN II ASSAY**

Following extraction of rat or rabbit plasma with a cation exchange resin according to the procedure described by Dusterdick and McElwee (19), the angiotensin eluted from the resin was estimated by radioimmunoassay. Details have been described previously (20). Complete dissociation of angiotensin II bound to antibody by the extraction procedure was ascertained by control experiments, in which angiotensin II, when it was added to antibody dilutions in buffer or antibody-containing plasma, was recovered by the extraction procedure to the same extent (85-90%) as it was from normal plasma. The radioactivity of [125I]-labeled angiotensin II added to antibody-containing plasma was completely removed from the plasma by the extraction.

**PLASMA ANTIBODY CONCENTRATION**

Serial dilutions of plasma were incubated at 0°C with 10 µg of monoiodinated [125I]-labeled angiotensin II in a total volume of 200 µliters of 0.1M Tris-acetate solution, pH 7.4, containing 0.2% human serum albumin. After 20 hours of equilibration, bound and free angiotensin were separated by plasma-coated charcoal (20). Following centrifugation, the charcoal pellet was counted in a well-type gamma scintillation counter. The titer is defined as the dilution of the antibody-containing preparation at which exactly 5 pg of radioiodinated angiotensin II is bound by the antibody. If this amount of antibody is taken as an arbitrary unit, the concentration in a given sample can be expressed as the reciprocal of the titer in arbitrary antibody units per 0.2 ml (the volume of equilibration). To eliminate possible interferences by angiotensin I or II, the plasma samples were extracted with a cation exchange resin prior to titer estimation, as described earlier.

**PLASMA RENIN CONCENTRATION**

Renin concentration was estimated by incubating 50 µliters of the plasma sample with 200 µliters (400 pmoles) of a rat renin substrate preparation (21) containing 2 mM dimercaprotoylpropanol, 4 mM 8-hydroxyquinolin, and 10 mM Na2EDTA in 0.1M TES-NaOH buffer, pH 7.2, at 37°C. At 0, 60, and 120 minutes 50 µliters of the reaction mixture were removed, adjusted to pH 6.0 with 200 µliters of 0.1M ammonium acetate, and mixed with 0.1 ml of a suspension of a cation exchange resin (Biorad AG 50 W x 2, 100-200 mesh, H+ form). This mixture was transferred to a small column containing an additional 0.1 ml of the resin suspension. The column was washed with 5 ml of 0.1M ammonium acetate, pH 6.0, and angiotensin I was eluted with 2.0 ml of 10% methanolic ammonia. The eluate was dried at 30°C in a stream of filtered air, and the residue was dissolved in 400 µliters of 0.1M Tris-acetate solution, pH 7.4. Subsequently, angiotensin I was estimated by radioimmunoassay. Under these conditions, the recovery of angiotensin I from the column, when it was added in buffer solution or to plasma incubation mixtures, was 86 ± 5% (sd). Exact pH adjustment of the incubation mixture prior to column separation is essential for the dissociation of angiotensin.
I from the antibody, which, in spite of its low cross-reactivity with angiotensin I (0.2%), partially prevents the binding of angiotensin I to the exchange resin when the incubation mixture is passed through the column at more acid or more alkaline conditions. Under the conditions described, the antibody present in the plasma samples did not interfere with the generation of angiotensin I during incubation or with the recovery of angiotensin from the column. This fact was ascertained by incubating normal rat plasma with or without the addition of appropriate amounts of angiotensin II antibody and by adding the same amount of antibody to identical incubation mixtures after the incubation. There was no difference in angiotensin I generation between the three groups.

RABBIT EXPERIMENTS

Nine New Zealand white rabbits were immunized against 5-Ile-angiotensin II coupled to porcine gamma globulin by the carbodiimide method of Greenwood et al. (22). The coupling product was emulsified with complete Freund’s adjuvant and injected in 0.2-ml volumes (corresponding to 0.1 mg of angiotensin II) intramuscularly and subcutaneously fortnightly for 3-6 months. Sham-immunization was performed with the same emulsion without angiotensin II.

ESTIMATION OF ALDOSTERONE, CORTICOSTERONE, SODIUM, AND OSMOLALITY IN RABBIT PLASMA

Plasma aldosterone concentrations were estimated by radioimmunoassay using a modification of the method of Mayes et al. (23), and corticosterone was determined by the method of Vecsei (24). Serum sodium was estimated by flame photometry (Zeiss FA 2), and serum osmolality was determined by measurement of freezing point depression (Knauer osmometer).

Results

ANTIBODY KINETICS

The decrease in antibody concentration in the circulation following the intravenous injection of angiotensin II antibody is shown in Figure 1. A more rapid decline occurred during the first day followed by a slow but steady decrease in the subsequent days. This curve can be described as the sum of at least two exponential functions, which are obtained by extrapolation of the terminal slope and by plotting the differences from this slope to the experimental points, respectively. Due to the alternating blood sampling protocol (see Methods), the data (means ± se) were obtained from four antibody-injected rats at 7 minutes, 90 minutes, and 10 hours, from three antibody-injected rats at 30 minutes and 4 hours, and from two control rats each at 7, 30, and 90 minutes and 4 and 10 hours. Subsequently, each point represents data from all seven antibody-injected rats or four control rats, respectively. This protocol also applies to the data given in Figures 2 and 3.

PLASMA CONCENTRATIONS OF ANGIOTENSIN II

As shown in Figure 2, a dramatic increase in plasma angiotensin concentration from control levels of about 100 pg/ml to 12,000 pg/ml occurred within 30 minutes following antibody injection. During the following 24 hours, the angiotensin concentration fell to 2,000 pg/ml; it then decreased slowly in the subsequent days. The high value at 10 hours as compared with that at 4 hours is probably due to the experimental protocol, since these two points were obtained from separate groups. In addition, the rats were transferred from the laboratory to the animal units between the fourth and tenth hours. Control rats also exhibited a small initial rise in plasma angiotensin II concentrations, which returned to stable levels after 30 minutes.

PLASMA RENIN CONCENTRATIONS

Plasma concentrations of renin, as shown in Figure 3, were initially elevated in both the antibody-injected and the control group, with no significant difference between the two groups. This short-lasting elevation probably reflects stimula-
tion of renin secretion by the experimental procedure (preparation of the saphenous vein and two anesthesia periods within 30 minutes) and explains the initial rise in the angiotensin II concentration in the control rats. The low angiotensin II concentration at 4 hours is also reflected in the lower renin concentration at this time. During the rest of the first day and the subsequent days of the experiment, there was no difference in renin concentration between antibody-injected rats and controls.

Hematocrit values were determined at days 2, 7, and 10 of the experiment; there was no difference between the two groups. There were also no differences in body weight gain and food and water intake (Fig. 4) between the two groups.

**ACTIVE IMMUNIZATION**

All immunized rabbits had developed antibodies after 3–6 months; their titers ranged from 1:5,600 to 1:640,000. Plasma angiotensin II concentration was markedly elevated in immunized rabbits compared with that in controls, ranging from 440 to 207,000 pg/ml plasma (Table 1). The three rabbits with the highest angiotensin II concentrations had the highest antibody concentrations, and the lowest antibody titer was associated with the lowest angiotensin II concentration. Plasma angiotensin II concentration in sham-immunized rabbits ranged from 50 to 190 pg/ml. The mean plasma aldosterone concentration in six immunized rabbits was 29.4 ± 3 (SE) ng/100 ml, which is elevated above the normal mean value for sham-immunized rabbits, 18.4 ± 2.9 ng/100 ml (Table 2). Changes in plasma corticosterone levels paralleled those in aldosterone. Serum sodium and serum osmolality were similar in both groups.

**Discussion**

Following the injection of angiotensin antibodies, the plasma concentration of the antibodies declined in a curve that can be described as the sum of at least two exponential functions. This change is in good agreement with the kinetics of gamma-G-immunoglobulins in general (25). Half-lives reported for the slow component, which reflects catabolism of the globulin, vary between 5.3 and 7.2 days in the rat. The fast component (or components) represents distribution in the extracellular space, with half-times ranging from a few hours to 1 day (for tabulation of data see ref. 25). It can therefore be assumed that angiotensin antibodies injected intravenously distribute in the extracellular volume with a half-time of 11 hours and are catabolized with a half-life of 7.0 days.
TABLE 2

Plasma Concentrations of Angiotensin II, Corticosterone, Aldosterone, and Sodium and Plasma Osmolality in Rabbits Actively Immunized against Angiotensin II and in Sham-Immunized Rabbits

<table>
<thead>
<tr>
<th></th>
<th>Immunized rabbits</th>
<th>Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensin II (pg/ml)</td>
<td>57751 ± 22685</td>
<td>82.6 ± 20.8</td>
<td>0.001</td>
</tr>
<tr>
<td>Corticosterone (ng/100 ml)</td>
<td>4.1 ± 0.5</td>
<td>2.4 ± 0.4</td>
<td>0.001</td>
</tr>
<tr>
<td>Aldosterone (ng/100 ml)</td>
<td>29.4 ± 3</td>
<td>18.4 ± 3</td>
<td>0.001</td>
</tr>
<tr>
<td>Sodium (mEq/liter)</td>
<td>143 ± 0.6</td>
<td>142.1 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Osmolality (mosmoles/kg)</td>
<td>308.7 ± 5.7</td>
<td>298.8 ± 4.9</td>
<td></td>
</tr>
</tbody>
</table>

All values are means ± SE.

quantitative considerations, an arbitrary antibody unit was used (see Methods). Accordingly, each rat received 200,000 antibody units. The plasma concentration after 7 minutes was about 4,000 units/0.2 ml (Fig. 1); thus, the volume of distribution was 10 ml, which is in fair proximity of the expected plasma volume (11.7 ml) in these rats (26). The total distribution volume in the body following equilibration can be derived from the intercept of the slow terminal slope of the decay curve with the ordinate. It was calculated to be about 26 ml, which means that the antibody did not distribute evenly in the total extracellular space (which in these rats was approximately 65 ml [27]) and, furthermore, that under equilibrium conditions 60% of the antibody present in the body was distributed in the interstitial space and only 40% was present in the circulation.

The only evidence for the presence of angiotensin antibodies in the interstitial space has been provided by Eide et al. (8), who found similar concentrations in renal lymph and plasma. This observation was made, however, in actively immunized rabbits.

Since angiotensin antibodies injected into the circulation are expected to bind circulating angiotensin, thereby preventing it from being metabolized, it is not surprising to find elevated angiotensin concentrations (Fig. 2). The quantitative pattern, however, is remarkable.

Only 7 minutes after antibody injection, the angiotensin II concentration had increased a hundredfold over control values; there was an additional small increase until 30 minutes, and thereafter angiotensin II concentrations declined rapidly during the first day and much more slowly during the subsequent days. It is obvious that the angiotensin II concentration curve closely resembles the antibody decay curve. Part of the immunoreactive material probably represents fragments of angiotensin II. Since the known fragments have a similar or smaller affinity to the antibody than does angiotensin II (20), it may be assumed that the relative contribution of fragments to the total immunoreactive material referred to as angiotensin II is not larger than it is in normal plasma.

Despite high angiotensin II concentrations, the antibody was far from being saturated. The antibody concentration at 7 minutes was about 21,500 arbitrary units/ml (for definition see Methods) with a total binding capacity of 245,000 pg of angiotensin II. Thus, only 4.1% of the binding sites of the antibody were occupied, provided that the value for the binding capacity, which had been measured in vitro, is also valid under in vivo conditions. The same calculation for the subsequent experimental periods from 30 minutes through 10 days showed that the total angiotensin II concentrations corresponded to 4.7, 3.2, 1.5, 3.3, 1.7, 2.6, 2.9, 2.2, 3.3, 3.1, and 3.0% of the total binding capacity of the respective antibody concentrations. Thus, there was a remarkable constancy of the fractional antibody occupation throughout the whole experiment. It seems reasonable to conclude that the constancy reflects a steady state of angiotensin turnover, which is reached within a few minutes following the injection of antibody.

This conclusion is supported by the renin concentration values shown in Figure 3. Except for a short initial rise in renin concentration, which was probably due to the experimental stress at the beginning of the experiment since it also occurred in the controls, renin concentrations were in the normal range throughout the entire experiment. These results are not only consistent with the concept of a steady state, but, moreover, they suggest that this steady state is close to, if not identical with, that in untreated rats. This suggestion is based on the well-documented negative feedback control of renin release by angiotensin. Elevations of blood angiotensin II concentration within the physiological range inhibit the secretion of renin (28, 29), whereas a competitive antagonist
of angiotensin II (1-Sar-8-Ala-angiotensin II) produces a severalfold increase in plasma renin concentration (30, 31). Therefore, any deviation from normal free concentrations of angiotensin II would tend to stimulate or depress renin secretion, which is apparently not the case in the antibody-injected rats. It may thus be concluded that normal renin concentrations in these rats reflected normal free angiotensin II concentrations, despite enormous concentrations of total angiotensin II.

This conclusion raises the question of what normal free concentrations of angiotensin are. Total angiotensin II concentrations, ranging from 52 to 142 pg/ml (20), do not necessarily represent free angiotensin II. There are reports on angiotensin II binding proteins in rat and human plasma (32-35). It is conceivable that these proteins have sufficient capacity and affinity for angiotensin II to reduce free concentrations of angiotensin II to very low levels. This speculative view is supported by the present study. From the apparent association constant and the binding capacity of the antibody, it can be calculated that free angiotensin II concentrations in antibody-injected rats are below 1 pg/ml. For reasons outlined earlier, it may be assumed that similar concentrations of free angiotensin II exist in normal rats, i.e., probably only a small fraction of the total plasma angiotensin II is biologically active. It is tempting to speculate further that the binding factors may vary in concentration and affinity, thus permitting changes in free angiotensin II concentrations without variations in total angiotensin II concentration.

In actively immunized rabbits, a situation similar to that in antibody-injected rats seems to exist. Renin concentrations reported by Oates and Stokes (36) are elevated only early in the course of immunization but are in the normal range after 3 months of immunization, despite continuous booster injections and an increase in the antibody titers. Normal plasma renin levels in immunized rabbits have also been reported by Johnston et al. (3). The very high total angiotensin II concentrations represent only 1-2% of the total binding capacity of the antibody. Free concentrations of angiotensin II are expected to be in the range of 0.5 to 5 pg/ml in immunized rabbits, if the calculation made for rat plasma is applied to rabbit plasma. Walker et al. (37) have reported an average of 4 pg/ml of free angiotensin II in angiotensin II-immunized rabbits. Thus, even in actively immunized rabbits, a steady state with probably normal free concentrations exists. It is conceivable that the renin-angiotensin system is regulated at a normal level in these rabbits and can respond to stimuli as well as it does in the nonimmunized rabbit. This situation would explain the lack of protection against the development of renal hypertension in such animals (2-7). A slight increase in plasma aldosterone and corticosterone in immunized rabbits (Table 2) is in accordance with elevated rather than decreased angiotensin II levels.

In several studies on the pathogenesis of renal hypertension, the injection of angiotensin II antiserum into animals with experimental renal hypertension failed to reduce the elevated blood pressure or a decrease was observed only for a short period of a few minutes (10, 12-15). These results have been interpreted as evidence against the participation of the renin-angiotensin system in the development of hypertension in the animal model studied. A different explanation has been offered by Thurston and Swales (16), who have proposed that angiotensin is formed and acts at vascular sites not accessible to the large antibody molecule. This explanation is based on the observation that in the same animal model (two-kidney renovascular hypertension) the angiotensin II antagonist, 1-Sar-8-Ala-angiotensin II, effectively reduces the elevated blood pressure.

In view of the results obtained in the present study, we favor an alternative interpretation. If crude antiserum is infused or injected, the angiotensin II present in the serum is already in equilibrium with the antibody. The injection may therefore have no effect at all on the levels of endogenous free angiotensin. But even if angiotensin levels are influenced, this disturbance will be of very short duration; a steady state is probably restored within a few minutes, i.e., the animal is regulating its renin-angiotensin system at a normal level. Consequently, no long-term blood pressure decrease is to be expected even under conditions in which the renin-angiotensin system significantly contributes to the maintenance of elevated blood pressure. However, under conditions in which angiotensin concentrations are changing rapidly, in particular, when angiotensin is injected, the antibody in the circulation will function as a “buffer.” The large free binding capacity will neutralize the high concentration of angiotensin, and the antibody will subsequently release “excess” angiotensin more slowly at sites with lower free concentrations, where it is rapidly destroyed by angiotensinases. This mechanism explains the observation that in antibody-injected animals no blood pressure response can be observed on injection of angiotensin (10, 12, 14, 15). The degree of unresponsiveness will be critically dependent on the concentration of the
injection solution, the speed of the injection, the affinity of the antibody, and the circulating conditions. Thus, lack of a blood pressure response cannot be taken as evidence for complete blockade of all responses to angiotensin. This fact is illustrated by the observation that in rabbits immunized against angiotensin II the injection of angiotensin II results in increased aldosterone secretion without any blood pressure response (38).

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**Correction**

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