Regulation of Angiotensin II in Rat Adrenal Gland

Ahsan Husain, Preenie DeSilva, Robert C. Speth, and F. Merlin Bumpus

Levels of angiotensin II immunoreactivity in the rat adrenal gland are over one hundredfold higher than those in plasma. It is unclear, however, whether the major source of adrenal angiotensin II immunoreactivity is intracellular synthesis by a local renin-angiotensin system, uptake by angiotensin II receptors, or both. Our studies show that angiotensin II immunoreactivity in the adrenal gland is predominantly attributable to angiotensin II (>75%). Angiotensin III (16%) and other angiotensin II fragments are also present. The majority of angiotensin II immunoreactivity (73%), renin activity (73%), and angiotensin II receptor binding activity (66%) in the adrenal gland is located in the capsular glomerulosa cell layers. Dehydration produced by 2% NaCl imbibition decreased these activities in the capsular-glomerulosa. In the fasciculata-medullary regions of the adrenal gland, dehydration decreased renin activity but not angiotensin II immunoreactivity or angiotensin II receptor binding activity. Combined data from control and dehydrated rats showed a close correlation of the capsular-glomerulosa angiotensin II Immunoreactivity with angiotensin II receptor binding activity ($r = 0.94$, $p < 0.001$) and a weaker, nonsignificant correlation with renin activity ($r = 0.66$, $p < 0.1$). In the fasciculata-medullary cell layers, no significant correlations were found between angiotensin II immunoreactivity and either renin or angiotensin II receptor binding activity. These data demonstrate that functionally distinct layers of the rat adrenal gland differentially regulate angiotensin II receptors and the renin-angiotensin system. These data also suggest that the majority of angiotensin II immunoreactivity in the adrenal capsular-glomerulosa is derived from receptor-mediated sequestration of extracellular angiotensin II by its receptors and is not due to intracellular synthesis of the peptide. (Circulation Research 1987;60:640-648)

Angiotensin II (Ang II) directly affects adrenal function by interacting with Ang II receptors on cortical glomerulosa and medullary chromaffin cells. These interactions cause an increase in the biosynthesis and release of aldosterone and catecholamines. Based on the discovery of renin activity in the adrenal gland, Ryan first proposed that locally synthesized Ang II may modulate adrenal function. Since that time, this concept has been supported by numerous studies documenting the presence of angiotensinogen and adrenal enzymes that are able to form and convert angiotensin I (Ang I) to the active octapeptide Ang II. These studies complement the overall concept that local Ang II generation is an important paracrine regulator of tissue function.

An important step toward understanding the role of Ang II in the adrenal gland is the identification of the biochemical mechanisms regulating the production of adrenal Ang II. Such a study is significant in part because conflicting evidence has been obtained regarding the regulation of Ang II and the enzymes that are important in its production in the adrenal gland. For instance, bilateral nephrectomy in rats markedly elevates adrenal glomerulosa renin activity, but Ang II levels in the adrenal glomerulosa are unaffected. In KCl-loaded rats, adrenal renin activity is increased and adrenal Ang II levels are decreased. Ang II levels in the adrenal gland may not be due to local synthesis alone. Selective uptake of circulating Ang II by Ang II receptors, which are present in the adrenal gland, may also be an important determinant of Ang II immunoreactivity (Ang II-ir) in the adrenal gland. Recent studies by Bianchi et al have shown that [123I]-Ang II is internalized by adrenal glomerulosa cells. This internalization process appears to be similar to those described for several other peptide hormones. Generally, this process, which involves specific cell surface hormone receptors, is a mechanism utilized by the cell to recycle receptors, and it yields an intermediate prelysosomal vesicle that contains high levels of the hormone. Endocytotic vesicles formed by receptor-mediated endocytosis have been shown to terminate in lysosomes and hence represent used hormone, destined for degradation, rather than a stored form of peptide hormone ready to be released on demand. Our study shows that adrenal Ang II levels are more closely related to adrenal Ang II receptor binding levels than to the activity of renin in this tissue.

Materials and Methods

Animal Models and Study Protocols

Wistar rats (Hilltop Laboratory Animals, Scottsdale, Penn.) weighing 250–290 g were housed in a constant temperature environment with a 12-hour light
cycle and fed a standard diet (Purina Rodent Chow Diet #5001, Ralston-Purina, St. Louis, Mo.). The rats were housed 4 per cage and allowed free access to food and fluid. Rats were dehydrated by substituting 2% NaCl for drinking water. Control rats received tap water. Fluid intake and body weights were measured daily. After 6 days of treatment, all rats were killed by decapitation, and the initial 2 ml of blood from the trunk was collected into chilled tubes containing sodium ethylenediaminetetraacetate (EDTA) as an anticoagulant. The plasma was separated from blood cells by centrifugation at 3,000 rpm at 4°C. The adrenals were rapidly removed, cleaned of fat on ice, and frozen on dry ice. The entire procedure was completed within 1 minute. In some studies, the cleaned adrenals were further separated into capsular (capsule-glomerulosa cell layers) and decapsular (fasciculata, reticularis, and medulla cell layers) tissue by cutting the tip of the adrenal and squeezing the gland between the thumb and index finger. The separate adrenal parts were rapidly frozen on dry ice. The plasma and tissue samples were stored at −90°C until processed for analysis. The left adrenal gland and its parts were processed for measurement of angiotensin peptides and the right adrenal gland for renin activity and Ang II receptor measurements.

Measurement of Adrenal and Plasma Angiotensins

Tissues were thawed at 4°C and extracted by a method similar to that used by Eng and Yalow. They were homogenized in 20 vol of 75% ethanol in 0.18 M HCl at 0°C using a Polytron (Brinkman Instruments, Westbury, N.Y.). At the beginning of the extraction, [125I]-Ang II (1,000 cpm, specific activity 2,200 Ci/mmol; New England Nuclear, Boston, Mass.) was added to all tissue and plasma samples for the estimation of recovery and degradation. The suspension was centrifuged at 30,000g for 20 minutes at 4°C. The apparent pH of the resultant supernatant was adjusted to 6.5 with 1 M NaOH, and the supernatant was evaporated to dryness in a speed-vac concentrator (Savant Instruments, Farmingdale, N.Y.). The residue was processed for radioimmunoassay (RIA) using the following procedure: Residues were resuspended in 0.1% aqueous trifluoroacetic acid (TFA) and applied to a C18 Sep-pak cartridge (Waters Associates, Milford, Mass.) and sequentially prewashed with 5 ml of the following solvents: methanol, tetrahydrofuran, hexane, methanol, methanol, and distilled water. The cartridge was washed with 10 ml 0.1% aqueous TFA and 10 ml 10% acetonitrile containing 0.1% TFA. Ang I-ir and Ang II-ir were then eluted with 30% acetonitrile containing 0.1% TFA. The latter fraction was dried and used for direct RIA or RIA-high pressure liquid chromatography analysis. Immunoreactive recovery of Ang II and radioactive recovery of [131I]-Ang II during the entire procedure was 75 ± 6% (n = 18) and 85 ± 5% (n = 18), respectively. Ang I-ir was determined by RIA using reagents obtained from New England Nuclear, and Ang II-ir was determined by RIA according to the method of Suzuki et al. 21

The following procedure was used for the high pressure liquid chromatography (HPLC) analysis of Ang II-ir. Ang II-ir was analyzed on a Nova-pak C18 column (Waters Associates), using a 12-minute gradient between 89% buffer A (25 mM sodium phosphate, pH 7.6, containing 5% acetonitrile) in buffer B (95% acetonitrile) and 68% buffer A in buffer B using curve 7 (Waters Associates gradient controller) at a flow rate of 1.5 ml/min. Six-second fractions were collected, and the Ang II-ir of each fraction was assessed by RIA. The column was calibrated using synthetic Ang II, [des-Asp1] Ang II (Ang III), [des-Asp1, Arg2] Ang II (hexapeptide-Ang II), and [des-Asp1, Arg2, Val3] Ang II (pentapeptide-Ang II). These synthetic standards were provided by Dr. M.C. Khosla (The Cleveland Clinic Foundation). The HPLC retention times of Ang II, Ang III, hexapeptide-Ang II, pentapeptide-Ang II, and [125I]-Ang II were 7.9, 10.2, 10.9, 9.4, and 10.3 minutes, respectively. Ang II antiserum used for the RIA showed 100% cross-reactivity with all of these C-terminal Ang II fragments but less than 0.05% cross-reactivity with Ang I and N-terminal Ang II fragments. Cross-reactivity of the antibody with the C-terminal Ang II tetrapeptide was also less than 0.05%. Three to eight processed adrenal glands or plasma samples were pooled and used for each HPLC analysis of endogenous Ang II-ir.

Determination of Plasma Renin Activity and Aldosterone

Plasma renin activity was measured by RIA of Ang I generated by the sample. Plasma aldosterone was measured by direct RIA (Diagnostic Products, Los Angeles, Calif.).

Measurement of Adrenal Ang II Receptor and Renin Activity

Adrenal tissue was thawed and homogenized with a Polytron in 1 ml of ice-cold 20 mM sodium phosphate buffer, pH 7.1. The homogenate was centrifuged at 48,000g for 20 minutes at 4°C. The supernatant was used for the measurement of renin activity, and the pellets were resuspended in 1 ml of receptor assay buffer containing (in mM) NaCl 150, MgCl2 10, ethyleneglycol-β-amino,N,N,N',N'-tetraacetic acid (EGTA) 10, dithiothreitol 1, and sodium phosphate 50, pH 7.1. The pellet suspension was centrifuged and resuspended in receptor assay buffer. Ang II receptor binding was determined as described by Speth and Husain, except that [125I] [Sar1, Ile8] Ang II was used as the radioligand. Adrenal membrane suspensions (0.15 ml) and 0.1 ml of receptor assay buffer, containing [125I] [Sar1, Ile8] Ang II, 0.2% bovine serum albumin, with or without Ang II (1 µM), were incubated for 2 hours at 22°C. For the analysis of binding affinity (Kd) and maximal binding capacity (Bmax) of the membranes for [125I] [Sar1, Ile8] Ang II, 6 concentrations of the radioligand between 60 and 1,000 pM were used. Following the incubations, free and tissue-bound [125I] [Sar1, Ile8] Ang II was separated on GF/B filters (Whatman, Clifton, N.J.), which had been pretreated
with 0.1% bovine serum albumin over a vacuum manifold. The tubes and filters were rinsed 4 times with 3 ml of ice-cold 50-mM sodium-potassium phosphate buffer, pH 7.4. Radioactivity retained on the filters was assayed by gamma scintillation counting at an efficiency of 65–75%. Nonspecific [125I]-[Sar^1, Ile^8] Ang II binding (in the presence of 1 μM Ang II) was subtracted from total [125I]-[Sar^1, Ile^8] Ang II binding (in the absence of Ang II) to derive specific binding to the putative Ang II receptors. K_d and B_max were calculated from Scatchard plots as previously described. 24

The relative contribution of receptor affinity and binding site density to the total amount of ligand bound to the receptor is dependent on the concentration of the ligand used. The lower the concentration of ligand used, the greater the effect of receptor affinity on the amount of ligand bound. To estimate the amount of circulating Ang II that may be sequestered by the receptor in vivo, we chose a single radioligand concentration of 30 pM in regional studies on the adrenal gland to determine Ang II receptor binding activity since this was the approximate level of circulating Ang II found in normal and dehydrated rats.

Adrenal renin activity was measured in duplicate by the following procedure: Renin activity was measured by generation of Ang I from homologous rat plasma renin substrate at a pH of 7.4. In this assay, a 50-μl sample was incubated with 450 μl of 250 mg/ml rat renin substrate in 0.1 M sodium phosphate buffer, pH 7.4, containing 10 mM EDTA, 5 mM N-ethylmaleimide, and 0.5 mM phenylmethylsulfonyl fluoride. The renin substrate at a pH of 7.4. In this assay, a 50-μl sample was incubated with 450 μl of 250 mg/ml rat renin substrate at a pH of 7.4, containing 10 mM EDTA, 5 mM N-ethylmaleimide, and 0.5 mM phenylmethylsulfonyl fluoride. The Ang I generated at 37° C and 4° C was measured, and the difference was used to calculate renin activity.

Statistical Methods

Statistical analysis was performed using unpaired Student’s t test. Probability values of less than 0.05 were considered to be statistically significant. All values reported are mean ± SEM. Correlation coefficients were calculated by the method of least squares.

Results

Effects of 2% NaCl Imbibition on Plasma Levels of Renin Activity, Ang II-ir, Ang I-ir, and Aldosterone and Body Weights and Fluid Intake

Substitution of 2% NaCl for tap water produced a progressive reduction in body weight over a 6-day treatment period (Figure 1A). Increased fluid intake was evident in rats dehydrated with the NaCl-loading protocol (Figure 1B). As shown in Table 1, there were significant decreases in the plasma levels of renin activity (53%), Ang I-ir (65%), and aldosterone (85%) in dehydrated rats compared to control rats (p < 0.005 in each case). However, plasma Ang II-ir levels were not significantly different between the two groups.

Ang II-ir, Ang I-ir, Renin Activity, and Ang II Receptor in the Whole Adrenal Gland of Normal and Dehydrated Rats

Figure 2A shows the resolution by reversed phase HPLC of the synthetic standards: Ang II, Ang III, hexapeptide-Ang II, and the pentapeptide-Ang II. Figures 2B and 2C show the respective separation of endogenous Ang II-ir in the adrenal gland and plasma of control rats. A summary of the HPLC separation of endogenous Ang II-ir (Table 2) shows that the predominant form of Ang II-ir in the adrenal gland and plasma of both control and dehydrated rats was chromatographically identical to Ang II. In the adrenal gland, Ang III was the second most prevalent form of Ang II-ir, while in the plasma the second most prevalent form of Ang II-ir was the pentapeptide-Ang II. Addition of [125I]-Ang II to the plasma or tissue at the time of extraction showed a single symmetric peak of radioactivity occurring at the retention time of [125I]-Ang II on subsequent HPLC analysis. The HPLC recovery of [125I]-Ang II from this system exceeded 90%, and no other radioactivity peak was observed, indicating that the observed immunoreactive Ang II fragments in plasma and adrenal extracts were not an artifact of the extraction procedure.

Ang II-ir levels in the rat adrenal gland ranged between 2.2 and 3.4 ng/g tissue wet weight; the adrenal glands from control rats weighed 23 ± 2 mg (n = 9) and from dehydrated rats 26 ± 3 mg (n = 9). The adrenal concentration of Ang II-ir (assuming 1 ml of plasma is equal to 1 g) was approximately 100 times higher than that of the plasma (Table 1) in the control group. There was essentially no correlation between adrenal content and plasma concentration of Ang II-ir.

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Effect of drinking 2% NaCl (broken line) in place of tap water (solid line) for 6 days on the body weight (Panel A) and cumulative fluid intake (Panel B) of rats. 2% NaCl imbibition produced a significant reduction in body weight and stimulated thirst. Each group contained 15 rats. † = p < 0.05; ‡ = p < 0.01.
Ang Il-ir were resolved on a Nova-Pak C₁₈ column by HPLC as [des-Asp¹, Arg²] Ang II (Hexa); [des-Asp¹, Arg², Val³] Ang II (Penta).

Panel A: Standard Ang II and its C-terminal fragments — Ang II; [des-Asp¹] Ang II (Ang III); [des-Asp¹, Arg²] Ang II (Hexa); [des-Asp¹, Arg², Val³] Ang II (Penta). Panel B adrenal gland Ang II-ir and Panel C plasma Ang II-ir were resolved on a Nova-Pak C₁₈ column by HPLC and Ang II-ir in the fractions measured by Ang II RIA.

FIGURE 2. Determination of the nature of Ang II-ir in rat adrenal gland and plasma. Panel A: Standard Ang II and its C-terminal fragments — Ang II; [des-Asp¹] Ang II (Ang III); [des-Asp¹, Arg²] Ang II (Hexa); [des-Asp¹, Arg², Val³] Ang II (Penta). Panel B adrenal gland Ang II-ir and Panel C plasma Ang II-ir were resolved on a Nova-Pak C₁₈ column by HPLC and Ang II-ir in the fractions measured by Ang II RIA.

Table 1. Renin Activity (RA), Angiotensins, Aldosterone, and Ang II Receptor (Rec) In Control and Dehydrated Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue</th>
<th>Ang II-ir (pg/gland or ml plasma)</th>
<th>Ang I-ir (pg/gland or ml plasma)</th>
<th>RA (ng Ang I/ht gland or ml plasma)</th>
<th>Aldosterone (pg/ml)</th>
<th>Ang II Rec quantity (fmol/gland)</th>
<th>Ang II Rec affinity (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Adrenal</td>
<td>66 ± 3.1(9)</td>
<td>26.0 ± 4.0(9)</td>
<td>3.80 ± 0.50(7)</td>
<td>nd</td>
<td>184 ± 20(7)</td>
<td>185 ± 8(7)</td>
</tr>
<tr>
<td>Dehydration</td>
<td>Adrenal</td>
<td>22 ± 5.4(9)*</td>
<td>24.8 ± 2.9(8)</td>
<td>1.14 ± 0.11(8)*</td>
<td>nd</td>
<td>159 ± 27(8)</td>
<td>235 ± 20(8)†</td>
</tr>
<tr>
<td>Control</td>
<td>Plasma</td>
<td>31.6 ± 8.5(9)</td>
<td>164 ± 28(9)</td>
<td>12.7 ± 1.19(9)</td>
<td>260 ± 33(7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydration</td>
<td>Plasma</td>
<td>32.8 ± 6.1(8)</td>
<td>57 ± 14(9)</td>
<td>6.0 ± 1.26(9)‡</td>
<td>45 ± 10(5)*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Number in parenthesis refers to the number of observations. Significance of difference from control as determined by Student's t test: *p<0.001, †p<0.05, ‡p<0.005; nd = not determined.

in control and dehydrated rats (Figure 3). In the control group, the adrenal concentration of Ang I-ir (1.15 ± 0.17 ng/g, n = 9) was 7 times higher than that of plasma Ang I-ir (0.164 ± 0.028 ng/ml, n = 9). In the dehydration group, the Ang II-ir content of the adrenal gland was 67% less than the control group (p<0.01), but the adrenal gland content of Ang I-ir did not differ from the control group (Table 1).

The renin activity level in the adrenal gland of control rats (165 ± 21 ng/hr/g tissue wet weight) was 13 times higher than that in plasma (Table 1). Adrenal gland renin content was decreased to 30% of control in dehydrated rats (Table 1). Based on data from control rat adrenals only, no significant correlation was found between Ang II-ir and renin activity (r = −0.38, n = 7). With the combined control and dehydrated rat data, a systematic error in residuals (i.e., the difference between the experimental and calculated values of the dependent variable) was noted when a fit of the data to a linear equation was attempted (calculated curve not shown). This finding indicates that the correlation between adrenal renin activity and Ang II-ir was not linear (Figure 4). The validity of this statistical approach has been discussed by Ellis and Duggleby. However, the data fit a nonlinear equation, indicating a significant nonlinear relation between these variables (r = 0.86, p<0.0001).

Dehydration produced a 27% decrease in adrenal Ang II receptor affinity (p<0.05), but it did not significantly affect the quantity of adrenal Ang II receptors (Table 1). No linear correlations were found between adrenal content of Ang II-ir and either the quantity (r = −0.04) or the affinity (r = 0.33) of Ang II receptors in control and dehydrated rats. When Ang II receptor binding activity, derived from the lowest ligand concentration used in the Scatchard analysis rather than receptor quantity or affinity, was plotted against adrenal Ang II-ir, we found a significant correlation between these variables, using either the control rat data only (r = 0.81, p<0.05, n = 7, y = 1.1x + 22.5) or the combined control and dehydrated rat data (r = 0.59, p<0.05, n = 15, y = 1.9x − 22.4).

Regional Distribution of Ang II-ir, Renin Activity, and Ang II Receptor Binding Activity

The distributions of Ang II-ir, renin activity, and Ang II receptor binding activity in the adrenal capsular-glomerulosa and decapsular fasciculata-medullary cell layers were determined in control and dehydrated rats. The capsular-glomerulosa contained 72.6 ± 1.9% of the adrenal Ang II-ir (n = 5), 73.4 ± 5.7% of the renin activity (n = 5), and 65.6 ± 5.3% of the Ang II receptor binding activity (n = 5), but this region

Table 2. Nature of Ang II-ir in Adrenal Gland and Plasma of Control and Dehydrated Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue</th>
<th>Percentage of total Ang II-ir detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ang II</td>
</tr>
<tr>
<td>Control</td>
<td>Adrenal</td>
<td>76</td>
</tr>
<tr>
<td>Dehydration</td>
<td>Adrenal</td>
<td>81</td>
</tr>
<tr>
<td>Control</td>
<td>Plasma</td>
<td>65</td>
</tr>
<tr>
<td>Dehydration</td>
<td>Plasma</td>
<td>76</td>
</tr>
</tbody>
</table>

*Ang III = [des-Asp¹] Ang II; †hexapeptide = [des-Asp¹, Arg²] Ang II; ‡pentapeptide = [des-Asp¹, Arg², Val³] Ang II.
FIGURE 3. Relation between plasma Ang II-ir levels and the adrenal gland Ang II-ir content of control (○) and dehydrated (●) rats. No correlation was found between these two pools of Ang II-ir.

represented less than 25% of the wet tissue weight (n = 9).

In the dehydrated rat group, the adrenal capsular-glomerulosa content of Ang II-ir, renin activity, and Ang II receptor binding activity decreased by 91% (p < 0.025), 81% (p < 0.001), and 50% (p < 0.05), respectively, compared to the controls group (Figure 5A). There was a close correlation (r = 0.94, p < 0.001) between Ang II-ir and Ang II receptor binding activity (Figure 5B) and a weaker correlation (r = 0.66, p < 0.1) between Ang II-ir and renin activity (Figure 5C) in the capsular-glomerulosa cell layers of control and dehydrated rats.

The adrenal fasciculata and medullary cell layer renin activity of the dehydration group was decreased by 59% (p < 0.05), but Ang II-ir (56% decrease) and Ang II receptor binding activity (1% increase) were not significantly different from the control group (Figure 6A). No significant correlations between Ang II-ir and either Ang II receptor binding activity (Figure 6B) or renin activity (Figure 6C) were observed in the decapsulated adrenal gland.

Discussion

These experiments deal with five major issues that are relevant to a local renin-angiotensin system and to angiotensin-mediated events occurring within the adrenal gland. Three of these issues can be definitively resolved by this study, and two others can be at least partially resolved.

1. Are the levels of renin activity, Ang I, and Ang II in the adrenal gland present only in the blood contained within the gland? No. The adrenal renin activity, Ang I, and Ang II-ir levels are between sevenfold and one hundredfold higher than their respective concentrations in the blood. Moreover, there was no significant correlation between adrenal Ang II-ir and circulating Ang II-ir levels. Furthermore, dehydration induced by 2% NaCl imbibition caused differential
Changes in plasma and adrenal concentrations of the renin-angiotensin system components. The level of Ang II-ir ranges between 2.2 and 3.4 ng/g wet tissue weight in the whole adrenal. The level of Ang II-ir in the rat adrenal capsular-gomerulosa cell layers is approximately 8 ng/g wet tissue wt and represents the highest reported tissue concentration of this peptide in the body. The levels of Ang II-ir found in the adrenal gland in this study were 3 times higher than those reported by Nakamaru et al. This difference may reflect the methods utilized for tissue processing following sacrifice. In this study, the glands were removed from rats and flash-frozen within 1 minute of decapitation, whereas Nakamaru et al anesthetized the animals and perfused the adrenals prior to removal of the glands.

2. Does the Ang II-ir in the adrenal gland represent Ang II? Yes. The presence of Ang II and its known immunoreactive fragments was resolved by HPLC

(Figure 2), and the majority of the Ang II-ir derived from the adrenal was shown to migrate identically to the biologically active peptides Ang II (76%) and Ang III (16%) (Table 2). Both Ang II and Ang III show similar potency in stimulating aldosterone secretion from the adrenal cortex. Less than 8% of the total Ang II-ir was due to the less biologically active C-terminal Ang II fragments, the hexapeptide, and the pentapeptide. It is unlikely that this measurement reflects degradation of Ang II during the extraction procedures since there was no evidence to support degradation in recovery experiments with exogenously added radiolabelled Ang II.

3. Is there a regional localization of the components of the renin-angiotensin system within the tissue? Yes. Approximately two-thirds of the total adrenal content of renin activity and Ang II-ir was localized in the capsular-gomerulosa layers, which account for less than 25% of the mass of the adrenal gland.

4. Does local intracellular synthesis of Ang II by renin account for all of the Ang II present in the tissue? Probably not. On the one hand, total renin activity in the adrenal gland of the control animals was sufficient to produce 58 times the adrenal concentration of Ang II per hour. There is also a correlation, albeit nonlinear, between whole adrenal gland renin activity and adrenal Ang II-ir levels. On the other hand, examination of the data from adrenal capsular-gomerulosa layers and adrenal capsular-medullary layers separately revealed a linear but low correlation between adrenal renin activity and adrenal Ang II-ir. In the former analysis, a major flaw was the fact that heterogeneous renin and Ang II containing cell layers were taken together. In the latter analysis, the limited number of observations imposed a constraint on the conclusions.

The question of local intracellular Ang II synthesis by adrenal glomerulosa cell renin can best be resolved by the studies of Strittmatter et al, who showed the absence of angiotensin-converting enzyme (ACE) within or on the cell surface of rat glomerulosa cells. Since ACE is required for the conversion of Ang I to Ang II, intracellular formation of Ang II in the glomerulosa cell by a renin-dependent pathway is unlikely. Intracellular formation of Ang II by the adrenal medullary cells utilizing a renin-dependent pathway is also unlikely since ACE in the adrenal medulla appears to be localized on the plasma membrane. The correlation that exists between adrenal gland Ang II-ir and renin activity may suggest that local synthesis of Ang II initiated by renin does contribute to Ang II levels. A tentative mechanism for local Ang II production in the adrenal gland is presented below.

Since adrenal renin activity is high and there appears to be an abundance of angiotensinogen in the adrenal, based on the demonstration of high levels of angiotensinogen mRNA in the rat adrenal, production of Ang I in the adrenal is likely. However, formation of Ang I by a renin-dependent pathway probably does not occur intracellularly for the following reasons: 1) Subcellular fractionation of the rat adrenal zona glomerulosa demonstrates the presence of Ang I in the soluble fractions.
representing the extracellular fluid and the cytosolic compartments, and 2) Ang I was not associated with adrenal renin-containing granules (authors' unpublished results). Ang I-ir levels in the adrenal gland are about 7 times greater than those found in plasma and may represent locally produced peptide within extracellular spaces of the adrenal. Since there is no ACE within glomerulosa cells, conversion of locally synthesized Ang I to Ang II probably occurs in adrenal blood vessels via vascular or circulating ACE. The rate and direction of blood flow in the adrenal is such that the highest levels of Ang II probably occur in cortical sinuses. These sinuses carry blood perfused through the various cortical layers to the adrenal medullary epinephrine-enriched cells. Remaining Ang I could also be converted to Ang II in the medulla by ACE, which is present on the sarcolemma of chromaffin cells. Thus, release of adrenal glomerulosa renin due to specific stimuli would produce Ang II, which, in addition to circulating Ang II, may activate adrenal medullary Ang II receptors.

5. Can receptor-mediated endocytosis of Ang II account for the presence of Ang II within the tissue? The answer to this question is likely to be yes. There is a strong correlation (r = 0.94) between Ang II receptor binding activity and Ang II-ir levels in the adrenal capsular-glomerulosa layers in the combined control and dehydrated groups of rats. Internalization of Ang II, mediated by adrenal Ang II receptors, may be dependent on a combination of receptor quantity, receptor affinity for ligand, and extracellular concentration of the peptide. To take these three factors into account, we estimated the total binding activity of adrenal membranes at a subsaturating level of the ligand; this measurement simultaneously accounts for the effects of receptor affinity and density in ligand binding. The concentration of ligand chosen for this measurement was 30 pM because this is the approximate level of circulating Ang II found in normal and dehydrated rats. When Ang II receptor binding to the whole adrenal, determined at a low radioligand concentration, was compared to whole adrenal Ang II-ir, there was also a significant correlation. Moreover, there appear to be sufficient levels of Ang II receptors to bind the quantities of Ang II present within the adrenal gland. If 1 pg of Ang II-ir represents approximately 1 femtomole of Ang II, Table 1 indicates that there are about 3 times as many Ang II receptors as Ang II-ir in the whole adrenal of control rats. This ratio is even higher in the adrenals of the dehydrated group of rats. Although the concentration of Ang II-ir appears to be approximately sixfold higher than the Ang II receptor binding reported for the capsular-glomerulosa layers of control rats in Figure 5, these binding assays were carried out with a subsaturating concentration of radioligand. If a Kd of 184 is assumed for the adrenal capsular-glomerulosa Ang II receptors (derived from the value determined for the whole adrenal gland), the 30 pM concentration would bind to only 14% of the total receptors, and thus, the binding values would reflect only about one-seventh the total Ang II receptor density in the capsular-glomerulosa layers. Therefore, the extrapolated value for the level of Ang II receptors in the adrenal glomerulosa is also higher than the level of Ang II-ir in that portion of the adrenal.

Although a high correlation between Ang II receptor binding and Ang II-ir levels is consistent with a receptor-mediated endocytosis mechanism generating the Ang II-ir levels in the adrenal gland, it does not constitute a proof that such a mechanism occurs in vivo. However, the recent study by Bianchi et al., showing binding and internalization of [125I]-Ang II by adrenal glomerulosa cells, and the well-described demonstration of receptor-mediated endocytosis for other peptide hormones indicate that measurement of Ang II receptor binding levels may be a good indicator of potential receptor-mediated sequestration of Ang II by the adrenal gland.

In the adrenal fasciculata plus medullary cell layers, no significant correlation was found between Ang II-ir and either Ang II receptor binding or renin activity. This poor correlation suggests a more complex relation between factors regulating Ang II in these cell layers than that found in the capsular-glomerulosa cells. Renin and Ang II receptors have been shown to be present in the adrenal medullary chromaffin cells. Our studies on the brain renin-angiotensin system (RAS) demonstrate the presence of renin activity in rat brain nerve terminals and show a microregional association between renin and catecholamines in the central nervous system. The possibility of an RAS in the neurons projecting to the adrenal medulla has not been examined, but our studies on the central nervous system RAS suggest that the peripheral nervous system may also utilize Ang II in cell-to-cell communication or to modulate neurotransmitter outflow. Thus, it is possible that the combined RAS of the chromaffin cells and the autonomic nerves in the adrenal medulla may have contributed to the difficulty in the analysis of factors regulating adrenal medullary Ang II-ir.

The traditional concept of the peripheral renin-angiotensin system involves extracellular synthesis of the active hormone Ang II in the blood. With the discovery of intracellular stores of renin and Ang II in tissues other than the kidney came the idea that there might be intracellular synthesis of Ang II. In support of this hypothesis, several studies have determined that all of the components necessary for the production of Ang II exist within several tissues and could be identified within cultured cell lines. Closer examination of the localization within tissues of the components of the renin-angiotensin cascade has led to a reevaluation of the concept of intracellular Ang II synthesis. In particular, there does not appear to be subcellular colocalization of the components of the system within the same cell. Thus, while the concept of local Ang II synthesis within tissues remains viable, this process does not appear to occur intracellularly.

Studies of the localization of ACE in the adrenal gland indicate that this enzyme does not occur in the adrenal glomerulosa cell but is confined to blood vessels and external membranes of the adrenal medullary...
cells. This finding suggests that synthesis of Ang II in the adrenal also occurs extracellularly, assuming that there is no enzyme that is capable of directly converting angiotensinogen to Ang II within adrenal cells.

Since the evidence in support of an intracellular synthesis of Ang II is questionable, the most likely source of the intracellular stores of Ang II-ir in adrenal cells is via an uptake mechanism such as receptor-mediated endocytosis. As noted above, there is a strong correlation between adrenal Ang II receptor binding and adrenal Ang II levels, and the amount of Ang II receptors appears to be sufficient to explain the observed levels of Ang II-ir in the adrenal. Therefore, we propose that adrenal stores of Ang II-ir represent used Ang II that has been bound to Ang II receptors and taken up into endocytotic vesicles and is destined for degradation by lysosomes. A basic tenet of this hypothesis is that the adrenal stores of Ang II are not indicative of releasable adrenal stores of Ang II to be used as a hormone to cause a response. Thus, determination of adrenal Ang II levels is not an indicator of the functionality of the adrenal renin-angiotensin system, but rather it is an indicator of the amount of stimulation of the adrenal by Ang II. These observations may also be applicable to other tissues where local Ang II synthesis has been hypothesized on the basis of the intracellular localization of Ang II.

Although these experiments are consistent with the hypothesis that receptor-mediated endocytosis is the source of tissue stores of Ang II, the possibility that Ang II in the adrenal is produced intracellularly by a nonrenin-dependent mechanism (e.g., via an enzyme that directly converts angiotensinogen to Ang II) cannot be ruled out. The presence of such enzymes has been described in other tissues, but this mechanism has not yet been investigated in the adrenal gland. At this juncture, we can only state that intracellular Ang II synthesis via a renin-mediated enzyme in the adrenal cannot explain Ang II-ir levels in this tissue. However, receptor-mediated endocytosis is probably the source of the intracellular stores of Ang II in the adrenal gland.

Acknowledgment

Our thanks to JoAnne Holl for her skillful preparation of this manuscript.

References

4. Lewis GP, Reit E: The action of angiotensin and bradykinin on the superior cervical ganglion of the cat. J Physiol (Lond) 1965;179:538–553


**KEY WORDS**: adrenal gland • angiotensin II • renin-angiotensin system • angiotensin II receptors • adrenal glomerulosa