Immunoreactive Renin in Mouse Adrenal Gland Localization in the Inner Cortical Region

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SUMMARY The existence of renin in the adrenal gland of the mouse was determined by its enzymatic activity and by immunohistochemical techniques using monospecific antibodies to mouse submandibular gland renin. The adrenal gland of mouse was found to contain a very high level of renin significantly greater than other mouse tissues except for the kidney and submandibular gland. Also, the renin level in mouse adrenal was significantly higher than that in adrenals of other species. This renin activity was distinct from the nonspecific renin-like activity of acid proteases in that its activity was optimal at neutral pH and specifically inhibited by antirenin antibody. Adrenal renin increased upon nephrectomy indicating that it is not derived from the kidney. Immunohistochemical studies localized the renin-immunoreactive substance to cells in the inner region of the cortex. The intensity of staining was highest in the innermost region and decreased in cells in outer layers. (Hypertension 6: 275–280, 1984)

KEY WORDS • zona fasciculata • zona reticularis

IN 1938, Goormaghtigh and Handovsky1 recognized that the arterioles of the adrenal gland capsule contained epithelioid cells morphologically similar to the juxtaglomerular cells of the renal afferent arterioles. Later, renin-like activity was reported2–6 in the adrenal of various species of animals. However, the identity of the renin-like activity in tissues other than kidney was not clear. Earlier, such activity in tissues like brain was criticized as a nonspecific action of acid proteases.7–8 Recently, methods have been developed to distinguish specific renin from nonspecific renin-like activity of acid proteases by chromatographic separation and by its selective reactivity to antirenin antibodies in several tissues.9–14

Angiotensins play important roles in the release of aldosterone and catecholamine from the adrenal gland.15 Determination of its activity and precise localization within the adrenal gland will have important implications in clarifying the physiological role of adrenal renin. In the present studies, we have shown high levels of renin in mouse adrenal gland and have determined its localization in the inner region of the adrenal cortex by an immunohistochemical technique.

Materials and Methods

Animals

Adult male Swiss-Webster mice (35–40 g body weight) were maintained on regular chow and ad libitum water supply. Adrenal tissues were obtained under pentobarbital anesthesia: 1) before nephrectomy; 2) 24 hours after bilateral nephrectomy; and 3) 24 hours after bilateral nephrectomy plus submandibular sialoadenectomy. Animals were killed by exsanguination under pentobarbital anesthesia followed by perfusion with saline. Various other tissues were removed from animals in Group 3 and from female mice treated in the same way for the comparison of renin activity in these tissues. Adrenal glands were obtained from other species, as indicated in Table 1. All tissues were stored at −80° C until use.

Tissue Extraction and Assay Methods

Each tissue was homogenized at 4° C in 0.01 M pyrophosphate containing 0.1 M NaCl, pH 6.5, in a Brinkman Polytron homogenizer (Brinkman Company, Westbury, New York). The supernatant ob-
tained by centrifugation at 39,000 g for 40 minutes was used for subsequent studies. The protein concentration of the extract was measured by the Bradford dye-binding method using crystalline bovine serum albumin as a standard.

The following conditions were used for renin activity assay to minimize the contribution of nonspecific action of cathepsin D. Extracts (12.5 µl) were incubated with 100 µl of infractionated plasma of nephrectomized rats in 0.1 M N-Tris (hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) buffer, pH 7.0, containing 7.5 mM EDTA, 2.7 mM phenylmethanesulfonyl fluoride, and 5 mM diisopropylfluorophosphate for 1 hour at 37° C. The angiotensin I (AI) formed was determined by radioimmunoassay. Under the condition employed, recovery of AI added to the extract was 94%. Protease activity was determined using 14C-labeled hemoglobin as substrate by the method of Williams and Lin. The pH dependency of renin and protease activity was determined at between 3 and 8.7 pH using the following 0.2 M buffers: citrate-phosphate for 3.0 to 5.5 pH, morpholinoethanesulfonic acid for 6.0 pH, phosphate for 6.5 to 7.0 pH, Tris-HCl for 7.5 to 8.7 pH.

**Antiserum**

Antisera against pure mouse submandibular gland renin were raised in rabbits, as described previously. One of these antisera caused 50% inhibition of pure mouse submandibular gland renin (2.25 ng AI/hour) at 1:2000 dilution and complete inhibition at 1:100 dilution. It has inhibited renin in mouse kidney extract equally well. The proportion of renin-like activity that was inhibited by the antirenin antibody was determined by preincubating the extract with the antiserum (1:100) or with preimmune serum (1:100) for 24 hours at 4° C prior to the AI generation reaction described above. Antisera to hog, rat, and human renin were raised with pure hog, rat, and human renin preparations. These antibodies were used in the determination of specific enzyme activities of renin in tissue extract, as described previously. These antibodies were specific to renin and did not inhibit protease activity.

Renin activity was distinguished from protease activity also by chromatographic technique. Mouse adrenal extract obtained was applied to an immunoaffinity column (1 x 4 cm) containing Sepharose coupled to rabbit antimume submandibular gland renin IgG. This column had been equilibrated with 20 mM sodium pyrophosphate buffer, pH 7.0, containing 0.3 M NaCl. After the application of the sample, the column was washed thoroughly with the same buffer. Adsorbed materials were eluted with 0.15 M acetic acid containing 0.3 M NaCl. Each eluate fraction was neutralized immediately with 3M Tris HCl, pH 8.6.

**Immunohistochemical Study**

Pentobarbital-anesthetized adult male mice, which had undergone nephrectomy and submandibular sialoadenectomy 24 hours before the experiment, were perfused with warm (37° C) saline followed by Bouin’s solution. Adrenal glands were removed and postfixed in the same solution, dehydrated, embedded in paraffin; 5 µm sections were then cut.

Immunostaining by antimouse submandibular gland renin antibody was done using the avidin-biotin-complex method with diaminobenzidine tetrahydrochloride as peroxidase substrate. To test the specificity of the immunohistochemical reaction, two control sera were substituted for primary antisera: normal rabbit serum and antisubmandibular gland renin antisera absorbed with purified mouse submandibular gland renin.

Statistical analysis was performed using Student’s t test.

**Results**

Mouse adrenal renin activity was readily measurable (92.2 ± 11.1 ng AI/hour/mg protein) and more than 99% of the activity at pH 7.0 was inhibited by specific antibody against mouse submandibular gland renin. At 24 hours after bilateral nephrectomy, the adrenal renin activity was elevated to a significantly higher level (422 ± 98.1 ng AI/hour/mg protein, p < 0.001), and again the increased enzyme activity was almost completely inhibited by the antibody. Sialoadenectomy combined with nephrectomy did not affect the level of adrenal renin activity beyond the level of nephrectomized mice (571 ± 68.9 ng AI/hour/mg protein).

<table>
<thead>
<tr>
<th>Species</th>
<th>No.</th>
<th>Nephrectomy before sacrifice</th>
<th>Renin activity (ng AI/hr/mg protein)</th>
<th>Inhibition by antibody* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>11</td>
<td>+ t</td>
<td>571 ± 68.9%</td>
<td>98.2</td>
</tr>
<tr>
<td>Rat</td>
<td>5</td>
<td>+</td>
<td>85.9 ± 27.2</td>
<td>96.5</td>
</tr>
<tr>
<td>Dog</td>
<td>6</td>
<td>−</td>
<td>6.4 ± 0.6</td>
<td>−</td>
</tr>
<tr>
<td>Hog</td>
<td>8</td>
<td>+</td>
<td>2.4 ± 0.7</td>
<td>83.2</td>
</tr>
<tr>
<td>Human</td>
<td>2</td>
<td>−</td>
<td>1.9 ± 0.6</td>
<td>94.4</td>
</tr>
<tr>
<td>Rabbit</td>
<td>5</td>
<td>−</td>
<td>1.8 ± 0.5</td>
<td>−</td>
</tr>
<tr>
<td>Sheep</td>
<td>3</td>
<td>+</td>
<td>0.3 ± 0.1</td>
<td>−</td>
</tr>
</tbody>
</table>

Values are the means ± SEM of determinations.

*Inhibition of renin activity by homologous antirenin antibody.

+Submaxillary sialoadenectomy was also performed.

p < 0.001 vs other species.

§Adrenal glands were removed as part of a radical operation for renal cancer.
A predominant portion of the activity at pH 7.0 in these tissues was inhibited by antisubmandibular gland renin antibody (Table 2). Except for the kidney and submandibular gland, the adrenal gland showed the highest activity among these tissues, followed by the spleen, which was previously reported to have high renin level. No significant difference in the renin activity of the adrenal gland was observed between the male and female mice.

The pH profiles of adrenal renin activity in extracts preincubated with or without the antibodies are shown in Figure 1. Renin activity sensitive to antibodies had a neutral pH optimum. This was clearly different from the nonspecific (antibody-insensitive) renin-like activity of proteases, which was optimal at pH 4.0. Moreover, as shown in Figure 2, renin and protease activities in mouse adrenal extract were clearly separated by the immunoaffinity chromatography.

Renin-specific immunohistochemical staining was found in cells located in the innermost region of the adrenal cortex, which is the inner layer of the zona reticularis-fasciculata (Figure 3). The intensity of the specific staining varied among the cells. Staining was most intense in the innermost region of the cortex and decreased gradually toward the outer region. The nuclei remained unstained. No staining was observed in the cells of zona glomerulosa, outer portion of zona fasciculata, and adrenal medulla.

Control staining with normal rabbit serum or anti-submandibular gland renin antisera absorbed with purified mouse submaxillary renin did not show positive staining in any cells in the adrenal gland.

### TABLE 2. Renin Activity in Various Tissues and Plasma of Nephrectomized and Sialoadenectomized Mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Renin activity (ng AII/hr/mg protein)</td>
<td>Inhibition by antibody* (%)</td>
</tr>
<tr>
<td>Adrenal</td>
<td>571 ± 68 9t</td>
<td>496 ± 74.5t</td>
</tr>
<tr>
<td>Spleen</td>
<td>326 ± 45.1</td>
<td>80.4 ± 11.5</td>
</tr>
<tr>
<td>Aorta</td>
<td>175 ± 27.6</td>
<td>68.5 ± 8.7</td>
</tr>
<tr>
<td>Testis</td>
<td>138 ± 20.0</td>
<td>87.8 ± 4.7</td>
</tr>
<tr>
<td>Liver</td>
<td>67.6 ± 6.3</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>Upper GI tract†</td>
<td>50.5 ± 16.2</td>
<td>Not detected</td>
</tr>
<tr>
<td>Lung</td>
<td>48.2 ± 14.2</td>
<td>—</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>41.5 ± 10.4</td>
<td>—</td>
</tr>
<tr>
<td>Lower GI tract§</td>
<td>27.9 ± 3.9</td>
<td>—</td>
</tr>
<tr>
<td>Heart</td>
<td>17.3 ± 4.1</td>
<td>—</td>
</tr>
<tr>
<td>Brain</td>
<td>7.6 ± 1.6</td>
<td>—</td>
</tr>
<tr>
<td>Anterior pituitary</td>
<td>2.2 ± 0.2</td>
<td>—</td>
</tr>
<tr>
<td>Posterior pituitary</td>
<td>Not detected</td>
<td>—</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.7 ± 0.2</td>
<td>—</td>
</tr>
<tr>
<td>Kidney</td>
<td>188 ± 72.0 × 10³</td>
<td>—</td>
</tr>
<tr>
<td>Submaxillary</td>
<td>1790 ± 185 × 10³</td>
<td>—</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM of five animals.

*Inhibition of renin activity by homologous antirenin antibody.
†p < 0.005, vs other tissues and plasma.
‡The esophagus, stomach, and duodenum were included.
§The small and large intestines were included.

**Discussion**

The results described here indicate that mouse adrenal gland contains a high level of renin-like enzyme activity compared with the adrenal gland of other species and other tissues of the mouse. This enzyme is immunologically related to renin and is distinct from acid proteases such as cathepsin D.

The localization of renin-like activity or renin in the adrenal gland has been reported on the basis of biochemical determination of renin enzyme activity in dissected portions of the gland. The activity has been observed predominantly in the cortex of rat and human adrenals.
FIGURE 2. Chromatographic separation of renin activity (—) and protease activity (—) in mouse adrenal on antirenin immunoglobulin-Sepharose column.

FIGURE 3. Paraffin sections of mouse adrenal gland stained immunohistochemically using anti-mouse submaxillary renin antiserum. Dark staining was seen in cells of the inner layer of the cortex, but not in outer layers of the cortex or in the medulla. Intensity of specific staining gradually decreased toward outer regions of the cortex. A. ×100. B. ×400. c = cortex; m = medulla.
human adrenal glands\textsuperscript{14} and in the medulla of bovine\textsuperscript{4} and hog adrenal glands (unpublished data of this laboratory). In the present study, separation of the cortical tissue from the medulla was not attempted because of the small size of the mouse adrenal gland. However, immunohistochemical study demonstrated localization of immunoreactive renin in the inner cortical region, which may be considered as the zona reticularis and the inner portion of the zona fasciculata of the mouse. In the mouse, the zona reticularis is not always observable. Thus, the renin stain is mostly in the zona fasciculata. This observation, however, does not exclude the possibility that renin is present in other regions, in view of the limited sensitivity of the histochemical technique.

The increase in the adrenal renin activity after bilateral nephrectomy is in agreement with the observation in the rat,\textsuperscript{13} supporting endogenous production rather than entrapment of plasma renin. Of interest is the observation that adrenal corticosterone production also increased after bilateral nephrectomy.\textsuperscript{25} However, the mechanism of increase of corticosterone and adrenal renin may have been different. The former may be more likely due to an increase of ACTH in response to the stress of nephrectomy. On the other hand, ACTH treatment or hypophysectomy did not affect the adrenal renin level (our unpublished observation), indicating that adrenal renin may be independent of pituitary. Other biological changes in the blood accompanying the uremic state, which includes electrolyte abnormality, acid-base imbalance, azotemia, and so forth, should also be considered.

Recent reports indicate the presence of All in the rat adrenal gland even after bilateral nephrectomy.\textsuperscript{26, 27} The concentration of All is particularly high in the capsular region as compared to the inner cortical regions. The All receptors were shown by autoradiography\textsuperscript{28} to exist at high concentrations in the zona glomerulosa and medulla. Although it may still be premature to attempt integrating these findings with the present observation of adrenal renin localized in the inner cortical region and to formulate a unified view on the possible relationship of renin localization in the inner cortical region with All in the capsular region, several alternative possibilities may be proposed for the role of adrenal renin. Adrenal renin may exert its effect by generating AI through an intracellular mechanism in a manner similar to that in cases of cultured neuroblastoma cells\textsuperscript{29} and cultured juxtaglomerular cells.\textsuperscript{30} It is likely that the AI and All thus formed may be released in a paracrine fashion to act on adjacent cells. A similar paracrine mechanism may be possible for renin itself. In this mechanism it may be secreted to generate angiotensins locally as a paracrine agent. However, our preliminary measurements of renin in the rat adrenal vein do not indicate release of adrenal renin in a large enough quantity to influence the renin level in adrenal vein plasma.

Whether intra-adrenal renin participates in the regulation of steroidogenesis and catecholamine synthesis and release is yet to be elucidated by further studies.

**Acknowledgments**

The authors are indebted to Dr. James C. McKenzie for his stimulating discussion, and to Edward Price, Jr., for his able technical assistance.

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