Effect of Converting Enzyme Inhibition on Renin Synthesis and Secretion in Mice

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We have investigated the relative importance of renal renin stores and de novo synthesis during stimulation of renin secretion and the role of transcription and posttranscriptional factors in providing increased synthesis of renin. When enalapril was administered to previously untreated mice, plasma renin concentration increased 40-fold within 1.5 hours, and remained at a high level for the 8 days of the experiment. Renal renin decreased by 82% after 24 hours and thereafter increased to levels higher than controls. Calculations of renin turnover, based on data for the rate of metabolism of renin in plasma, indicated that most of the renin released in the first 24 hours could be accounted for by the decrease in renal renin stores, indicating that de novo synthesis played only a minor role. After 24 hours, however, when both plasma renin concentration and renal renin increased, the calculated rate of renin synthesis increased to nearly 40 times the rate in controls. When enalapril was administered to mice that had been depleted of plasma and renal renin by chronic sodium loading, plasma renin concentration increased markedly within 1.5 hours, but to only half the level achieved in the previously untreated mice. No decrease in renal renin occurred, suggesting that the renal renin remaining after chronic sodium loading was not available for release. Renal renin messenger RNA increased 4.5-fold after 6 hours, and after 8 days had increased to 5.0 times the level at day 0. The increase in calculated rate of renin synthesis was maximal between 5 and 8 days, when it was 54 times greater than at day 0. During enalapril treatment, there were marked increases in the granulation of the juxtaglomerular cells and in the amount of rough endoplasmic reticulum and Golgi apparatus they contained. These results suggest that posttranscriptional factors play a major role in determining the rate of renin synthesis. (Hypertension 1989;14:385–395)

The importance of the renin-angiotensin system in blood pressure and volume homeostasis is well established, and an extensive body of work exists concerning factors that regulate release of renin into the circulation. Less is known about the intracellular mechanisms involved during variations in the rate of renin release by juxtaglomerular cells. Two issues that remain unclear are: 1) the relative importance of stored renin and de novo synthesis when renin release is stimulated, and 2) whether the rate of renin synthesis is determined by messenger RNA (mRNA) levels or posttranscriptional factors. Changes in the amount of intracellular organelles of protein synthesis, such as ribosomes, endoplasmic reticulum, and Golgi, may also be important during stimulation of renin synthesis, and require further elucidation.

Rat kidneys contain sufficient renin to allow secretion at the normal rate for up to 100 days. This suggests that acute stimulation of secretion could occur without the need for any mechanism other than release of renin from granules. However, some evidence exists for the secretion of newly synthesized renin, and others have shown blunting of acute release in the presence of the protein-synthesis inhibitor cycloheximide. Others have failed to detect the predicted (if de novo synthesis is not involved) decrease in renal renin after large increases in secretion. This may merely reflect the fact that kidney renin stores are so large relative to plasma levels. In single-renin-gene mice, the ratio of kidney to plasma renin is much lower than in rats, and kidney stores are sufficient to maintain normal levels of secretion for only about 10 days. A fall in renal renin after acute stimulation of renin secretion should thus be easier to demonstrate in mice, which we have used in this study.

Previous work on the role of transcriptional control in renin synthesis is conflicting: nonproportional changes in plasma renin concentration (PRC), renal renin, and renin mRNA in response to stimu-
lation by sodium depletion and converting enzyme inhibitor (CEI) have been reported, as has the opposite finding of a close correspondence between renin mRNA and secretory rate in response to the same stimuli. However, there are insufficient data in these reports to calculate the rate of synthesis of renin. To do so would require that both PRC and renal renin were measured in mice during stimulation by CEI administration. These mice received no treatment before CEI administration. The second group of experiments was designed to evaluate the role of transcriptional and posttranscriptional events during the transition from suppression to stimulation of renin synthesis. These mice were treated with 9α-fludrocortisone and saline drinking water for 6 weeks before CEI administration, as we have shown previously that this regimen causes suppression of renin synthesis and secretion.In addition to measurements of PRC and renal renin, renin mRNA studies and electron microscopic studies were performed in this group. Mice in both groups were killed at intervals during CEI administration, and the data obtained used to calculate the rate of renin synthesis. For measurement of renin mRNA levels, a method of quantification was developed with due consideration given to adequate controls. The electron microscopic studies were undertaken to follow the progression of morphological changes throughout CEI administration.

Materials and Methods
Male Balb/C mice, 6–10 weeks old (24–30 g), were used in all experiments.

The first group of experiments studied the response to enalapril in previously untreated mice. Mice were given a 2 mg i.p. bolus of enalapril on day 0, and enalapril was also given in drinking water (100 mg/l) until the mice were killed. Water intake was monitored, and the average enalapril intake was 18 mg/kg/day. Mice were killed for assay of PRC and renal renin content on day 0 and 1.5 hours, 3 hours, 6 hours, 24 hours, 2 days, 3 days, and 8 days after commencement of enalapril (n=5 at 2 days, 3 days, and 8 days, and n=4 at all other times).

The second group of experiments studied the response to enalapril in renin-depleted mice. Mice were given 9α-fludrocortisone in 0.5% saline as drinking water for 6 weeks to suppress kidney and plasma renin levels. Average 9α-fludrocortisone intake was 300 μg/kg/day. The standard laboratory food was given. After 6 weeks, saline was replaced by tap water and diuretics were given for 2 days (furosemide 2 mg i.p. bolus and 100 mg/l in drinking water, and amiloride 12.5 mg/l in drinking water). 9α-Fludrocortisone was continued during this 2-day period. At the end of this period, 9α-fludrocortisone and diuretics were ceased, and this point is referred to as day 0 of the experiment. Some mice were killed after 6 weeks of treatment with 9α-fludrocortisone, both before and after the 2-day administration of diuretics, and were studied as described below. The remaining mice were then divided into two groups. Group 1 (no treatment) mice remained on standard chow and tap water from day 0 until termination. Mice were killed 24 hours, 2 days, and 8 days after day 0 and studied as described below.

Group 2 (enalapril) mice commenced enalapril therapy on day 0. The protocol of enalapril administration was exactly as described above for experiments on previously untreated mice. Mice were killed at day 0 and 1.5 hours, 3 hours, 6 hours, 24 hours, 2 days, 5 days, and 8 days after day 0 for measurement of PRC, renal renin, and renin mRNA (n=3 at 1.5 hours and 3 hours and n=6 at all other times). Some mice were also taken for perfusion fixation of kidneys and subsequent morphological studies.

Sample Collection and Renin Assay
Blood was obtained from conscious mice by orbital sinus puncture and collected in glass capillary tubes. Plasma was separated by centrifugation and kept on ice until used for renin assay. After blood collection, mice were immediately killed by cervical dislocation and both kidneys were removed. The left kidney was snap frozen and kept in liquid nitrogen until RNA extraction. The right kidney was homogenized in ice-cold buffered saline, and the homogenates were kept on ice until used for renin assay. Kidney homogenates and plasma samples were diluted in buffered saline and incubated in the presence of excess sheep renin substrate and 0.008% dimercaptoopropanol for 1 hour at 37°C. Renin concentration in samples was calculated after radioimmunoassay of angiotensin I (Ang I) generated in incubates, as described previously.

RNA Extraction
Three kidneys from the same experimental group were pooled for each extraction. The kidneys were homogenized for 60 seconds in 8 volumes of buffer containing 4 M guanidium thiocyanate, 5% 2-mercaptoethanol, and 0.5% N-lauroyl sarcosine. Cesium chloride (0.5 g/ml homogenate) was added, and the homogenate was layered over 1.5 ml of 5.7 M cesium chloride cushion in polyallomer centrifuge tubes. After centrifugation in an SW-41 rotor at 28,000 rpm for 18 hours, RNA pellets were dissolved in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA (TE) and precipitated in ethanol. For application to dot-blot assays and Northern analyses, the precipitated RNA was centrifuged in a desktop microfuge, the precipitates washed in 70% ethanol and dissolved in a volume of TE that was strictly propor-
tional to the weight of tissue from which the RNA had been extracted. Thus, equal volumes of the RNA solutions contained RNA from equal weights of tissue (rather than equal amounts of RNA), facilitating comparison of the resulting signals in terms of renin mRNA concentration or the amount of renin mRNA per gram of kidney tissue. The amount of RNA recovered was measured by absorbance at 260 nm to check for large differences that would suggest spillage or wastage.

Quantification of Renin Messenger RNA
The DNA Synthesis Laboratory of the Howard Florey Institute, Melbourne, Australia, synthesized 30-mer oligonucleotide probes for mouse kidney renin and mouse actin. Chicken submaxillary gland renin complementary DNA (cDNA) was also provided by the Howard Florey Institute. The sequence of the renin oligonucleotide probe was 5'-TACCTCCCCCAGACACCCAGAAGTGGTG-3', which is complementary to the segment of the mouse Ren-1 gene encoding the Met 266 to Thr 275 amino acids of renin. The mouse submaxillary gland renin cDNA (pRn 1-4, 1,427 nucleotides long and containing the entire coding sequence of renin mRNA without the poly-A tail) was a gift from P. Corvol. Oligonucleotide probes were labeled with [32P]dadenosine triphosphate (ATP) and [32P]d cytidine triphosphate (CTP) by extending random primers with Klenow fragment (Bresatec, Adelaide, Australia). An aliquot of each RNA solution was denatured in formaldehyde at 62°C for 15 minutes and diluted in 15× standard saline citrate (SSC) (150 mM NaCl, 15 mM sodium citrate). Various dilutions of total RNA were dotted onto nitrocellulose filter papers, which were then vacuum baked at 80°C for 2 hours. Filters were then prehybridized for 2 hours at 42°C in 50% formamide hybridization buffer to which was added ATP to 100 μM. The mouse renin oligonucleotide probe was then added, and hybridization was allowed to occur overnight at the same temperature. Filters were then washed in 2x SSC plus 0.1% Triton-X at 50°C (four washes of 15 minutes each). Filters were autoradiographed for 12-48 hours with Kodak XAR-5 film. The strength of each signal on the autoradiogram was measured by the optical density recorded on a laser densitometer. For each tissue sample, three or four measurements of signal intensity at different dilutions of total RNA were obtained and analyzed by linear regression. The gradient of the linear regression equation obtained was used to compare renin mRNA content between different samples on a single filter.16 Autoradiographic intensity was found to be proportional to the amount of RNA applied to the filters, as evidenced by the high regression coefficients (r>0.99 for most samples, r>0.96 for all samples). To enable comparison between samples on different filters, a stock of RNA obtained from kidneys of a separate group of untreated mice was prepared for use as a standard, and aliquots of this were applied to each filter. Filters were subsequently washed in TE plus 0.1% sodium dodecyl sulphate at 80°C to remove the renin probe, then rehybridized with the mouse actin probe. After autoradiography, the same process was repeated for the tubulin probe, with the difference that the washing conditions were made more stringent (0.1x SSC, two washes of 30 minutes at room temperature, two washes of 30 minutes at 42°C). Regression analysis was performed for the actin and tubulin signals, and the average of these was taken as an index of mRNA recovery and used to correct the renin signals for any difference in mRNA yields between samples. Correction was required for only three of the 21 RNA extractions, and in one of these by only 20%.

To check the accuracy of the dot-blot assay, several samples were also subjected to Northern analysis and probed with the mouse submaxillary gland renin cDNA: total RNA was electrophoresed on a 1.0% agarose gel, transferred to nitrocellulose, then prehybridized, hybridized, and washed as described above for the tubulin cDNA probe. Densitometry was performed on the autoradiographs. Results for three samples are shown in Figure 1. Relative signal intensities, as measured by densitometry, were approximately equal for the dot-blot assay and Northern analysis. As a further control, several of the dot-blot assays were washed after hybridization with the renin oligo and reprobed with mouse submaxillary gland renin cDNA. The results were the same for the two probes (data not shown).

Primer Extension
A primer extension study was performed to compare the 5' untranslated regions of the renin transcripts formed under basal and CEI-stimulated conditions. A 30 nucleotide probe complementary to nucleotides 73 to 102 of the coding sequence of mouse kidney renin mRNA was used. One nanogram of this was 5' end-labeled with [32P]ATP and annealed to 50 μg total RNA from kidneys of control and enalapril-treated mice. Annealing took place at 60°C for 1 hour in a buffer containing 0.25 M KCl, 0.2 mM EDTA, and 2 mM Tris-HCl, pH 8.0. Primer extension was carried out by reverse transcriptase (Life Sciences, Florida) in the presence of actinomycin D and human placental RNAase inhibitor (Amersham). The reaction mixture was phenol/chloroform extracted, ethanol precipitated, and taken up in 5 μl 50% formamide dye. After heating, the mixture was applied to a 6% polyacrylamide sequencing gel. Molecular size markers were provided by digesting pBR322 with Hpa2 and end-filling with labeled dCTP.
FIGURE 1. Comparison of autoradiographic signals for three samples after Northern analysis and hybridization to mouse renin complementary DNA (cDNA) probe (top) and with dot-blot assays using 30-mer synthetic probe (bottom). Total RNA from kidneys of three mice treated with 9α-fludrocortisone (lane 1) and 8 days of enalapril administration (lane 2) was compared with a “standard” RNA derived from pooled kidneys (lane 3). Thirty micrograms RNA was applied to each lane of the Northern analysis. Amount of RNA, in micrograms, applied to dot blots is indicated at left. Intensity of each image was measured by laser densitometry. Ratio of products of area times density of the Northern signals in lanes 1, 2, and 3 was 20:205:100. After linear regression analysis, ratio of dot-blot signals in lanes 1, 2, and 3 was 27:229:100.

Calculation of Rate of Renin Synthesis

It is assumed that renin is not released into the circulation from any source other than kidney. We have used the single-renin–gene Balb/C strain of mice which, unlike 2-gene strains, expresses renin in the submaxillary gland only at a low level. Assuming that there is no intrarenal breakdown of renin, the rate of renin synthesis will be equal to the amount of renin secreted into the circulation, plus or minus changes in renal renin. Nakamura et al18 have studied renin metabolism in mice and found that renin was metabolized (removed or inactivated) in proportion to its concentration in plasma, with a half-life in plasma of approximately 13 minutes. The amount of renin secreted can thus be calculated if PRC, plasma volume (V), and the metabolic rate constant (K) for renin are known. Thus, if PRC is constant over the period considered, we may use the equation:

\[
\text{plasma renin turnover} = \text{PRC} \times \text{V} \times \text{K}
\]

V is assumed to equal 1.2 ml for all mice, and K=0.0337/min.18

Statistics

One-way analysis of variance was used to compare values obtained before and after each treatment. Subsequent analysis with Duncan’s Multiple Comparisons Test, with p values of 0.01 and 0.05, was used to compare values between specific time points within an experiment. Correlation and regression analysis was used to assess the significance of changes occurring over a number of consecutive time points in a given experimental group.

Morphology

All mice were anesthetized with methoxyflurane (Pitman Moore, Inc., Washington Crossing, New York) and maintained in 100% oxygen. The abdominal aorta and vena cava were exposed through a midline incision. Heparin (250 IU) was injected via the jugular vein. The lower abdominal aorta was cannulated with a 26G needle, and the vascular perfusion was commenced at 200 mm Hg using 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 and room temperature. The inferior vena cava just adjacent to the renal veins was severed. No buffer rinse was used, although a small amount of Hanks’ balanced salt solution (C.S.L., Melbourne, Australia) was used to fill the needle tip. After 5 minutes of vascular perfusion (approximately 100 ml fixative), both kidneys were dissected out, weighed, and then fixed for a further 2 hours. For electron microscopy, the kidney cortex was then cut into 1×1 mm strips, washed in 0.1 M phosphate buffer with 5% sucrose, postfixed in 1.5% osmium tetroxide for 1 hour, dehydrated through graded acetones and embedded in Araldite-Epon (Ladd, Burlington, Vermont). Thick plastic sections (1 μm) were cut on a Cambridge Huxley ultramicrotome and stained with methylene blue. Sectioning was continued until a granulated juxtaglomerular region was located. The block was then further trimmed, and thin sections (90–100 nm) were cut on diamond knives, stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop 102 microscope at 60 kv.

Results

Response to Enalapril in Previously Untreated Mice

There was a marked increase in PRC with enalapril (Figure 2): values obtained during enalapril treatment were shown by one-way analysis of variance to differ from values at day 0 (p<0.0001). PRC increased 40-fold after 1.5 hours and subsequent
Results are mean±SEM. Mice were killed at each of the time-points for measurement of plasma renin concentration (PRC) and renal renin content. AI, angiotensin I.

There was a 60% decrease in the average renin mRNA signal after 9a-fludrocortisone compared to controls (p<0.01) compared with controls. Changes in renin mRNA content were assessed by regression analysis of dot-blot assays. Comparison of signals was made by comparing the gradient of regression lines obtained from several dilutions of each sample. There was a 60% decrease in the average renin mRNA signal after 9a-fludrocortisone compared with controls (Table 1). The administration of diuretics was not statistically significant (p<0.05).

**Rate of Renin Synthesis**

The data have been used to calculate the synthetic rate of renin using the equation described in the Materials and Methods section. For calculation of rate of synthesis at day 0, it is assumed that renal renin content before administration of enalapril was constant (i.e., not changing from day to day) and thus that the rate of renin synthesis was proportional to PRC. Thus, at day 0, the rate of synthesis is given by 380 ng Ang I/hr/ml x 1.2 ml x 0.0337/min, which equals 15.4 ng Ang I/hr/min, or 22 μg Ang I/hr/day. In the first 24 hours of enalapril administration, assuming an average PRC of 6,500 ng Ang I/ml/hr, the amount of renin released was 380 μg Ang I/hr. The amount of renin stored in the two kidneys decreased from 380 to 65 μg Ang I/hr during the same time, a depletion of 315 μg Ang I/hr/day. Thus, the calculated synthetic rate was 65 μg Ang I/hr renin per day in this 24-hour period. Between 24 and 48 hours, stored renin increased to 477 μg Ang I/hr. Assuming an average PRC of 5,650 ng Ang I/ml/hr, the synthetic rate was 741 μg Ang I/hr/day. Between 48 and 72 hours, both renal renin and PRC increased further, and the synthetic rate was 780 μg Ang I/hr/day. By day 8, PRC and renal renin had undergone further slight increases, and the calculated average synthetic rate between days 3 and 8 was 489 μg Ang I/hr/day. Thus, the calculated rate of renin synthesis increased up to 35-fold during enalapril treatment.

**Response to Enalapril in Renin-Depleted Mice**

Effect of saline plus 9a-fludrocortisone. The effects of chronic treatment with 9a-fludrocortisone and sodium loading on renin and renin mRNA levels in mice have been described in a separate publication. Mice that underwent this treatment for 6 weeks and were not killed at that time for renin assays were used in the present study. As shown in Table 1, the 6-week treatment period resulted in a 67% decrease in PRC (p<0.01) and a 62% decrease in renal renin (p<0.01) compared with controls. Changes in renin mRNA content were assessed by regression analysis of dot-blot assays. Comparison of signals was made by comparing the gradient of regression lines obtained from several dilutions of each sample. There was a 60% decrease in the average renin mRNA signal after 9a-fludrocortisone compared with controls (Table 1). The administration of diuretics was not statistically significant (p<0.05).
Table 2. Effect of Cessation of Sodium Loading

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>PRC (μg Ang I/hr/ml)</th>
<th>RRC (μg Ang I/hr/2kid)</th>
<th>mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>6</td>
<td>171±64</td>
<td>120±14</td>
<td>38 (38,38)</td>
</tr>
<tr>
<td>Day 1</td>
<td>3</td>
<td>72±10</td>
<td>185±22</td>
<td>...</td>
</tr>
<tr>
<td>Day 2</td>
<td>3</td>
<td>97±12</td>
<td>225±9</td>
<td>107</td>
</tr>
<tr>
<td>Day 8</td>
<td>3</td>
<td>95±18</td>
<td>301±14</td>
<td>80</td>
</tr>
</tbody>
</table>

Changes in plasma renin concentration (PRC), renal renin content (RRC), and renin messenger RNA (mRNA) when mice that had been treated with 9α-fludrocortisone and sodium loading for 6 weeks (with administration of diuretics on the 2 days before day 0 to reverse sodium loading) were returned to a normal sodium diet and 9α-fludrocortisone was stopped. Renin mRNA is expressed in standardized units relative to a reference preparation of RNA derived from pooled kidneys. Reference is 100 units and refers to renin mRNA concentration.

Effect of cessation of 9α-fludrocortisone. As a control for the effect of enalapril in this experiment, some of the mice that had been sodium loaded for 6 weeks and treated with diuretics for 2 days were returned to normal diet and tap water on day 0 and given no further treatment. PRC remained at low levels, whereas renal renin concentration increased progressively and by 8 days had returned to control (mice that did not undergo sodium loading) levels (Table 2). Renin mRNA increased sharply by 48 hours of cessation of 9α-fludrocortisone and thereafter decreased slightly approximating control levels by day 5.

Effect of enalapril. Enalapril was commenced on day 0. Subsequent values for PRC, shown in Figure 3, were significantly different from the day 0 values (p<0.0001, one-way analysis of variance). There was a 36-fold increase after 1.5 hours (p<0.01), and a 44-fold increase after 3 hours (p<0.01), compared with day 0 (Figure 3). After 3 hours, PRC decreased somewhat; the values obtained at 6 hours, 24 hours, and 48 hours did not differ significantly from each other, but were less than the values obtained at 1.5 hours and 3 hours (p<0.05). PRC increased significantly between 48 hours and 8 days (p<0.05). The values of PRC attained in this experiment were not as high as those in previously untreated mice (Figure 2); at 1.5 hours, for example, PRC was 50% less than in the previous experiment.

In contrast to the rapid changes in PRC, renal renin content did not change significantly during the first 24 hours of enalapril administration. Regression analysis did not show a significant trend of renal renin with respect to time during this period (r=−0.26, 0.1<p<0.5). Subsequently, however, renal renin increased, and by day 8 was nine times higher than the day 0 level, and four times higher than control levels (p<0.05).

Renin mRNA levels increased 2.7-fold within 1.5 hours of commencing enalapril and by 6 hours had increased 4.5-fold (Figure 4). There was little further change, and at day 8 the level was fivefold greater than before enalapril. Thus, the maximum average level of renin mRNA obtained was only 2.5-fold greater than the level in control (untreated) mice. The highest single measurement, obtained on
day 8, was 3.0 times greater than the average control level and 8.5 times greater than the lowest single measurement, obtained after 6 weeks of 9α-fludrocortisone treatment (Figure 1).

Rate of Renin Synthesis

The data have been used to allow a calculation of the rate of renin synthesis during intervals of the experiment (Figure 5). For the calculation of synthetic rate in the 9α-fludrocortisone-treated group before administration of enalapril, it is assumed that at the time of assay, 6 weeks after the beginning of the experiment, renal renin content was constant (i.e., not changing from day to day), and thus that renin synthesis was proportional to PRC. Thus, at day 0, the rate is given as 171 ng Ang I/hr/mlx1.2 mlx0.0337/min, which equals 6.9 ng Ang I/hr/min, or 9.9 μg Ang I/hr/day. When enalapril was given, the calculated synthetic rate increased sharply, and continued to increase over the 8-day period. After 24 hours of enalapril, PRC had increased but renal renin remained unchanged from day 0, and the calculated synthetic rate was 17-fold higher than before enalapril. As noted above, the mRNA level increased about 4.5-fold within 6 hours of enalapril and increased little thereafter. Calculated synthesis, however, continued to increase over the duration of enalapril administration. Between days 5 and 8, there was a large increase in renal renin while PRC also increased; the calculated synthetic rate during this period was twofold greater than between days 2 and 5 whereas mRNA hardly changed. Comparing the values at day 8 with those at day 0, there is a 54-fold increase in calculated synthetic rate and a fivefold increase in renin mRNA level.

Primer Extension

The mouse Ren-1 gene contains two TATA boxes located 61 and 97 nucleotides upstream from the initiation codon.17 Only the TATA box nearest the 3' end has been shown to be functional in mouse kidney.17,19 A primer extension experiment was performed on RNA from both untreated and enalapril-treated mice to examine the possibility that different mRNA transcripts are present in the two situations. The results are shown in Figure 6 and indicate that only the downstream (3') TATA box is functional, regardless of stimulation by CEI administration. The beginning of the transcripts map to a position approximately 35 nucleotides upstream from the initiating (methionine) codon.

Morphology

After treatment with 9α-fludrocortisone, juxtaglomerular cells contained dense irregularly shaped granules, often characterized by vacuoles within the granule matrix (Figure 7). Lysosomes were abundant and membrane whorls and granular material were often seen outside the cell. The Golgi was shrunken and granular endoplasmic reticulum (GER) was sparse. Enalapril caused a significant increase in the synthetic activity of the cells. By 6 hours, stacks of dilated GER were present. Enlarged Golgi profiles with many more cisternae were also present in these areas, containing both homogenous, crystalline, and vesicular matrices. Small and large granules were present within the cytoplasm, as well as some lysosomes. After 2 days of enalapril treatment,
the cells contained medium-sized rounded granules. The most abundant organelles within the cytoplasm were dilated GER and Golgi profiles. After 8 days, large lakes of dilated GER were often found between stacks of less dilated GER. Four to five Golgi profiles were often found in one region of the cell with granular material detectable in small buds of endoplasmic reticulum. Vesicles, protogranules, and mature granules were also present. It was not uncommon to find this synthetic activity within cells containing many myofilaments, indicating the "turning on" of synthetic activity in contractile cells.

Discussion

We have obtained data for PRC and renal renin content at various intervals during continuous CEI administration in mice and used this data to calculate the rate of renin synthesis, using the equation described in Materials and Methods. The equation uses average values of PRC and renal renin content, and thus incorporates and amplifies the variation inherent in these measurements. Further, the equation is based on assumptions about renin metabolism in plasma and kidney that are unlikely to be strictly correct. In particular, although it is well accepted that circulating active renin is produced solely by the kidney, this may not be true for strains of mice that produce large amounts of active renin in the salivary glands. The choice of Balb/C mice was made for this reason, as salivary renin is very low in this strain. It would be preferable to validate the use of this strain by showing disappearance of circulating active renin after nephrectomy. However, this experiment was not performed. Thus, the calculated results are presented here merely to enhance qualitative judgments about changes in renin synthesis for comparison between different situations and are not claimed to provide accurate figures for synthetic rate per se. The results of these calculations have been used to assist our analysis of two groups of experiments investigating stimulation of renin synthesis and secretion.

The first group of experiments, performed in previously untreated mice, examined the relative importance of renin stores and de novo synthesis for renin secretion during stimulation by CEI administration. There was a large increase in renin secretion within 1.5 hours of commencement of enalapril. During the first 24 hours, renal renin content decreased progressively, as shown by the significant negative correlation between renal renin content and duration of enalapril administration in this period. After 24 hours, the kidneys had been depleted of 82% of their initial content, and our calculations suggest that this could account for most of the renin that appeared in plasma during the
first 24 hours. A decrease in PRC from its initially very high level occurred between 1.5 hours and 24 hours, coinciding with the decrease in renal renin, and the linear correlation between PRC and renal renin during this period was statistically significant. Between 24 hours and 8 days, as renal renin increased, PRC also increased, but this was not a statistically significant linear correlation. Thus, there appeared to be an important dependence of PRC on renal renin stores during the first 24 hours of stimulation by CEI, whereas de novo synthesis made only a small contribution to PRC. After 24 hours, there was increased synthesis of renin, as implied by simultaneous increases in renal renin and PRC, but no significant correlation between renal renin and PRC.

The relation between renin synthesis rate and renin mRNA levels was investigated in the second group of experiments. Changes in mouse kidney renin mRNA were measured using a quantitative dot-blot hybridization assay with a synthetic 30-mer mouse kidney renin DNA probe. The motivation to use dot-blot assays (rather than Northern analysis) and an oligo-DNA probe (rather than a full-length cDNA probe) was that they facilitated rapid processing of the large number of samples used in these experiments. The use of dot-blot assays and of oligo-DNA probes has previously been criticized for the tendency to record large background signals.\textsuperscript{12,20} We have found that this problem is avoided by the use of suitably stringent hybridization and washing conditions. We have previously reported that background hybridization of the 30-mer renin probe in dot-blot assays, as tested by hybridization to liver RNA, produced a signal that was not detectable by the laser densitometer.\textsuperscript{11} In the present study, several samples were measured on both a dot-blot assay (with the oligo probe) and a Northern analysis (with a mouse submaxillary gland cDNA probe), and the relative autoradiographic intensities were similar in each case, validating the use of dot-blot assays throughout the study.

Renin mRNA concentration could not be expressed in absolute terms, due to the lack of a renin mRNA standard. However, relative concentrations were expressed by comparison of all results with a reference RNA stock prepared from kidneys of mice not used in the experiment. The reference renin mRNA concentration was taken to be 100 units/g tissue. Control mice used in the experiment had an average mRNA concentration of 78 units/g. The lowest level obtained after 6 weeks of sodium loading was 27 units/g, and the highest level, after 8 days of CEI administration, was 229 units/g. The ratio of average renin mRNA levels in the day 0 and
enalapril day 8 groups was 1:5. The ratio of PRCs in these groups was 1:32, and the ratio of renal renin content was 1:9. Clearly, PRC increased disproportionately to renin mRNA after CEI administration.

To resolve the question of proportionality between rate of synthesis and mRNA levels, calculations of renin synthetic rate were made, as described in Materials and Methods. The ratio of calculated synthetic rates at day 0 and day 8 of enalapril was 1.54. Figure 6 shows that, although the renin mRNA level virtually peaked within 6 hours of commencing enalapril, calculated synthetic rate continued to increase over 8 days. Clearly, factors other than mRNA levels are crucial in determination of the degree of stimulation of renin synthesis caused by CEI administration. A primer extension experiment has ruled out the possibility that renin mRNA transcripts with different 5′ leader sequences are formed during stimulation by CEI administration. The increase in amount of organelles of protein synthesis in response to CEI administration was apparent within 6 hours, but continued to develop further over subsequent days. Taken together the results indicate that, although an increase in renin mRNA is an important component of the synthetic response to CEI administration, a large (approximately 10-fold) increase in translational efficiency of the renin transcripts must also occur. The increase in amount of ribosomes and endoplasmic reticulum may, at least in part, account for this enhanced efficiency. The increase in Golgi may be required to allow processing of the translational products to proceed at maximal rate.

Despite an increase in plasma renin when enalapril was given to mice that had undergone prior treatment with 9α-fludrocortisone and sodium loading, there was no decrease in renal renin content. However, when enalapril was given to previously untreated mice, renal renin fell dramatically over the first 24 hours, and the calculations suggest that release of stored renin could account for most of the renin released during this period. The reason for the failure of renal renin to fall in the previously sodium-loaded mice is unclear; one possibility is that some of the renal renin was stored in a form that was not detected by our assay. However, we have consistently failed to demonstrate inactive renin in mouse kidney when the kidneys are handled in the present manner, using either acid-activation or trypsin-activation (unpublished results). An alternative explanation may be that the renal renin remaining after chronic sodium loading was unavailable for release, an analogous situation to the unavailability for release of renin in the unclamped kidney in two-kidney Goldblatt hypertension after removal of the clamped kidney.21 Thus, the large increase in PRC that occurred within 1.5 hours of CEI administration may have resulted from de novo synthesis. However, it is difficult to understand how synthesis could have achieved such a high level so early in the response to enalapril, whereas in previously untreated mice an increased synthesis was not clearly apparent until day 2. A possible explanation is that the administration of diuretics during the last 2 days of 9α-fludrocortisone treatment may have "primed" the synthetic apparatus of the juxtaglomerular cells, facilitating a more rapid expression of the stimulatory effect of CEI administration. The greatly increased numbers of ribosomes and endoplasmic reticulum present within 6 hours of enalapril administration would be consistent with this explanation. Unfortunately, equivalent morphological studies in the study of previously untreated mice, which could have confirmed or refuted this explanation, were not performed.

It has been shown that the effect of CEI administration on renin release is mediated via removal of the negative feedback of angiotensin II (Ang II).22 The inhibition of renin release by Ang II was first demonstrated by Vander and Geelhoed,22 who postulated a direct effect of Ang II on the renin secretory mechanism. In the present study, however, we have shown that the response of the juxtaglomerular cells to CEI administration involves a complex set of reactions that occur over different time scales. The earliest response (except when kidneys had undergone prior depletion of renin) was release of renin from existing renin stores. This effect was virtually maximal within 1.5 hours, and previous work from our laboratory has shown that a near-maximal effect occurs within minutes.24 This rapidity of onset would be consistent with a direct effect of removal of Ang II, which has a very short half-life in plasma. Other responses to CEI administration, however, were more gradual; the level of renin mRNA also increased within 1.5 hours, but did not reach a near-maximum value until about 6 hours, whereas the increase in cellular organelles of protein synthesis, evident within 6 hours, continued to develop over several days. The calculated rate of renin synthesis increased on the first day, but increased more markedly over subsequent days, and the amount of renin stored in the kidneys, which decreased or remained constant on the first day, afterwards increased progressively up to day 8.

The complexity of the cellular response to stimulation of renin by CEI administration is evident. In addition to release of stored renin and increased transcription of the renin gene, there is an obvious requirement for increased transcription of ribosomal RNA genes and probably a large number of genes encoding proteins involved in the synthesis and functioning of cellular organelles. We have previously shown, in the rat, that replacement of Ang II abolishes the increase in renal renin, as well as PRC, that occurs with CEI administration.23 This suggests that the entire range of responses may be attributable to removal of Ang II. However, these need not be direct effects. For example, removal of Ang II may cause release of renin from granules that in turn initiates an intracellular signal, which if prolonged, stimulates the synthetic response. Studies of the relevant gene pro-
motors and their DNA-binding factors will be necessary to elucidate these mechanisms.

References


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