Time Course of Stimulation of Renal Renin Messenger RNA by Furosemide

Min Chen, Jürgen Schnermann, Richard L. Malvin, Paul D. Killen, and Josie P. Briggs

Renin secretion responds rapidly to a variety of stimuli; however, reported changes in renal renin messenger RNA (mRNA) levels in vivo have been observed only after prolonged stimulation. Studies were designed to test whether rapid changes in renin mRNA levels can be produced in vivo. In the first series, Sprague-Dawley rats received furosemide (10 mg/kg) intraperitoneally and a low sodium diet (0.05% sodium); renin secretion was significantly stimulated at 8 or 16 hours after treatment, but renin mRNA levels did not change. In a second series, rats were pretreated with deoxycorticosterone acetate (200 mg/kg) and saline drinking water for 3 days and then killed 0, 2, 4, 8, or 48 hours after furosemide administration. The renin mRNA level was unchanged at 2 hours but was stimulated twofold at 4 and 8 hours and threefold at 48 hours. In additional animals, the response of renin mRNA 4 hours after furosemide was found not to be potentiated by the converting enzyme inhibitor quinapril (5 mg/kg). The results demonstrate that with acute stimulation, renin mRNA levels lag 2–4 hours behind the change in plasma renin levels. (Hypertension 1993;21:36–41)

Key Words • juxtaglomerular apparatus • kidney • gene expression • sodium • rats, inbred strains

A ctivation of the renin-angiotensin system is a critical response in maintenance of whole body volume homeostasis. In general, in most volume-depleted states, renin secretion is increased, and renal renin messenger RNA (mRNA) levels are augmented. Regulatory mechanisms established to influence the secretion of renin include the level of sympathetic nerve activity, the NaCl concentration at the macula densa, renal perfusion pressure, and the circulating levels of a number of hormones. Many of the stimuli that influence renin secretion have been shown to modify renin gene expression, but it is unclear whether the two responses share common regulatory mechanisms.

Renin secretory rate responds rapidly to a variety of stimuli, but the time course for changes in renal renin mRNA levels has not been studied in detail. Most in vivo studies of renin gene expression have assessed mRNA levels only after 24 hours or more of stimulation. There is substantial evidence accumulating in a variety of other systems that the mechanisms responsible for rapid or immediate changes in gene expression are different from the mechanisms that produce delayed changes. Definition of the time course of the response of mRNA levels to stimulation is therefore a useful step in assessing the possible control mechanisms. For example, there is evidence that cyclic AMP may be an important potential regulator of renin mRNA synthesis. Since cyclic AMP-mediated changes in gene transcription can occur very rapidly, a substantial lag in the response of renin mRNA would raise the possibility of operation of other mechanisms. No previous studies have undertaken a careful assessment of the time course of the response of renin mRNA to acute stimulation in vivo. We elected to examine the response to furosemide because furosemide is known to produce a rapid and predictable stimulation of renin secretion. The immediate secretory response to furosemide is believed to reflect, at least in part, inhibition of macula densa transport, and other factors such as decreases in renal perfusion pressure or increases in the renal sympathetic nerve activity may contribute to delayed effects. We chose to perform the experiments in deoxycorticosterone acetate (DOCA)-salt–loaded animals because DOCA-salt loading is known to suppress renin mRNA levels; thus, we speculated that an early stimulatory effect might be easier to detect in this setting. The results demonstrate that under these conditions, renin mRNA levels lag behind plasma renin levels but can change relatively rapidly, with an increase detectable in 4 hours.

Methods

Animals

Experiments were performed on male Sprague-Dawley rats weighing 250–300 g (Charles River Breeding Laboratories, Wilmington, Mass.). In all studies, animals were decapitated, blood was obtained for measurement of plasma renin levels, the right kidneys were removed for measurement of renal renin content, and the left kidneys were prepared for extraction of RNA.

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Protocol 1. In the first experimental series, 14 Sprague-Dawley rats were injected with furosemide (10 mg/kg i.p.). Standard rat chow was replaced with pellets of low sodium chow (0.05% sodium, ICN Biomedicals, Inc., Costa Mesa, Calif.). Rats were killed at 0, 8, and 16 hours after furosemide administration (n=4 for each period of treatment); two additional rats were killed at 4 hours for mRNA determination only.

Protocol 2. The second group of experiments was performed in rats pretreated with DOCA (Sigma Chemical Co., St. Louis, Mo.) and a high salt intake to suppress baseline renin mRNA levels. DOCA-implregnated silastic (200 mg/kg body weight) was implanted subcutaneously, and 0.9% NaCl was given as drinking water. All animals in this group received this pretreatment for 3 days.

A total of 26 rats were used in these studies that examined the time course of the response of renin mRNA to furosemide, with animals killed 0, 2, 4, 8, or 48 hours after furosemide administration (10 mg/kg i.p.) (n=5 or 6 for each treatment group). After administration of furosemide, standard rat chow was replaced with low sodium pellets (0.05% sodium) and tap water was given as drinking fluid.

To test whether inhibition of converting enzyme would potentiate the response to furosemide, eight animals pretreated as above received two doses of a converting enzyme inhibitor, quinapril (2.5 mg/kg i.p., Warner-Lambert/Parke Davis, Ann Arbor, Mich.) 12 and 24 hours before receiving furosemide (10 mg/kg i.p.) or vehicle. Rats were killed 4 hours after furosemide administration (n=4 for each treatment).

Determination of Renal Renin Content and Plasma Renin Levels

The kidneys were excised, weighed, and homogenized in 0.1% bovine serum albumin (BSA) in distilled water. The homogenate was centrifuged at 4°C, and the supernatant was stored at -20°C until assay. Renin was measured from both plasma and kidney samples with the antibody trapping technique using purified homologous antibody. The initial homogenate was centrifuged at 4°C, and the supernatant was stored at -20°C until assay. Renin was measured by both Northern blot and dot blot techniques performed individually on RNA from each experimental animal. For Northern blot analysis, total kidney RNA samples (12 μg) were denatured in 5% formaldehyde, subjected to electrophoresis through a 1% agarose, 6% formaldehyde gel, and transferred onto nylon filters (Zeta Bind, New England Nuclear, Boston, Mass.). For dot blot analysis, serial twofold dilutions of total RNA from 10 μg to 0.625 μg were prepared in 40 mM NaH₂PO₄ solution and dotted onto nylon filters using a 96-well vacuum manifold (Schleicher & Schuell Inc., Keene, N.H.). The RNA was fixed to the filter by baking in vacuo at 80°C for 2 hours. The filters were prehybridized for 1 hour at 65°C in hybridization buffer consisting of 1% BSA, 7% sodium dodecyl sulfate (SDS), 0.5 M NaH₂PO₄, and 1 mM EDTA and then hybridized with cDNA probes in the same buffer at 65°C overnight.

Quantification of Messenger RNA

 Autoradiographs were prepared at -80°C; preflashed film and intensifying screens were used. Exposure times within the linear range of film characteristics were established in preliminary experiments. Autoradiographs were scanned with a densitometer (UltraScan XL, LKB). No systematic variation was noted in the amount of β-actin or α1(IV) collagen mRNA after treatment with furosemide. For each Northern blot, absorbance of double bands of α1(IV) collagen mRNA were used for normalizing absorbance of renin mRNA band. Renin mRNA was quantified by measuring the ratio of absorbance of renin mRNA to α1(IV) collagen mRNA and then dividing that ratio by the ratio observed for the control on the same filter. On the dot blots, the slope in the linear range for both renin and β-actin was determined for each sample. The ratio of renin mRNA to that of β-actin mRNA was calculated and then compared with the control ratio on the same filters.

Statistical Analysis

Results are expressed as mean±SEM. The relation between the densitometry readings from the dot blots and the amount of RNA loaded was computed by linear
TABLE 1. Plasma Renin, Renal Renin Content, and Renin Messenger RNA After Treatment of Furosemide and Sodium Restriction in Rats Not Given Deoxycorticosterone Acetate–Salt Pretreatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma renin (mGU/ml)</th>
<th>Renal renin content (mGU/mg kidney)</th>
<th>Renin mRNA levels (ratio to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.29±0.10</td>
<td>0.89±0.14</td>
<td>1.0</td>
</tr>
<tr>
<td>8 hours</td>
<td>1.42±0.28*</td>
<td>0.66±0.03</td>
<td>1.18±0.19</td>
</tr>
<tr>
<td>16 hours</td>
<td>0.96±0.25†</td>
<td>0.76±0.06</td>
<td>1.62±0.54</td>
</tr>
</tbody>
</table>

n, Number of rats; GU, Goldblatt units; mRNA, messenger RNA. Values are mean±SEM. *Normalized with β-actin. †p<0.01. §p<0.05.

regression analysis. All comparisons were made by analysis of variance (SYSTAT) followed by comparison of each experimental group with its control group using the Bonferroni multiple comparison correction.

Results

Protocol 1

Table 1 summarizes the changes in plasma renin, renal renin content, and renin mRNA levels in the rats given furosemide and low salt without any pretreatment. Renin mRNA values are presented as the ratio of renin mRNA to β-actin mRNA calculated from dot blot analysis compared with the control animals. Plasma renin was stimulated 8 and 16 hours after furosemide; renal renin content tended to fall slightly at 8 hours, and renin mRNA levels were modestly increased at 16 hours, but neither of these changes was significant when compared with control rats. There was also no detectable stimulation of renin mRNA levels at 4 hours after furosemide administration (n=2, data not shown).

Protocol 2

In rats pretreated with DOCA-salt for 3 days, plasma renin levels were significantly suppressed compared with rats without pretreatment (0.29±0.10 versus 0.06±0.01, p<0.01). In agreement with previous observations, this period of treatment was too short to result in significant suppression of renal renin content. After furosemide administration, plasma renin increased 2 hours after the diuretic was given and remained at a high level at 4, 8, and 48 hours. Renal renin content showed no significant change (see Table 2).

Figure 1 illustrates a Northern blot of kidney cortex RNA from individual animals in this series, probed for both renin, which identifies a 1.4 kb mRNA band, and α(IV) collagen, which has a characteristic doublet of approximately 6.0 kb size. Since no significant change in band intensity for α(IV) collagen mRNA was noted after furosemide, renin mRNA levels were compared with collagen mRNA levels. On this Northern blot, which is representative of the whole experimental series, clear-cut stimulation of renin mRNA was apparent only at 48 hours. However, in comparison with the band intensity for α(IV) collagen, values at 4 and 8 hours showed a tendency to increase.

More precise quantification was obtained by use of dot blot methods. Dot blots were performed on successive twofold dilutions of RNA from each animal, which provided determinations of mRNA abundance at five levels from 10 to 0.625 μg total RNA. Figure 2 shows a representative dot blot probed for renin or for β-actin, and Figure 3 shows average values of density for renin and β-actin mRNA. The average regression lines of β-actin mRNA and renin mRNA from the control rats and treated rats were determined from the data shown in Figure 3. There was no difference in slopes of β-actin mRNA between the control rats and treated rats with furosemide and low salt at any time point. The slope for the renin mRNA level was not increased 2 hours after furosemide administration. However, at 4, 8, and 48 hours, slopes significantly steeper than those from the control rats were observed (p<0.02, 4 and 8 hours; p<0.005, 48 hours). The ratio of renin mRNA at each time point to control values from the same filters, normalized with β-actin mRNA, was also determined from dot blot analysis and is shown in Table 2. Figure 4 summarizes the time course for the increase in plasma renin and renin mRNA after treatment with furosemide and low salt. Furosemide stimulated plasma renin at the first time point measured (2 hours). Renin mRNA levels

FIGURE 1. Representative Northern blot of kidney cortex RNA from rats pretreated with deoxycorticosterone acetate–salt, given furosemide (10 mg/kg i.p.), and killed 0, 2, 4, 8, or 48 hours after diuretic administration. In each lane 12 μg total RNA from kidney cortex was run. Filter was hybridized with rat renin complementary DNA (cDNA) (single arrow) and rat α(IV) collagen cDNA (double arrows). Figures below the graph indicate a comparison of the density ratios of renin messenger RNA (mRNA) to α(IV) collagen mRNA in the treated rats with that of the control rats.

FIGURE 2. Plasma Renin, Renal Renin Content, and Renin Messenger RNA After Furosemide in Animals Given Deoxycorticosterone Acetate–Salt Pretreatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma renin (mGU/ml)</th>
<th>Renal renin content (mGU/mg kidney)</th>
<th>Renin mRNA levels (ratio to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.06±0.01</td>
<td>0.80±0.24</td>
<td>1.0</td>
</tr>
<tr>
<td>2 hours</td>
<td>0.21±0.08†</td>
<td>0.75±0.19</td>
<td>1.09±0.30</td>
</tr>
<tr>
<td>4 hours</td>
<td>0.25±0.08†</td>
<td>0.78±0.08</td>
<td>2.26±0.81†</td>
</tr>
<tr>
<td>8 hours</td>
<td>0.33±0.10†</td>
<td>0.83±0.14</td>
<td>1.90±0.24†</td>
</tr>
<tr>
<td>48 hours</td>
<td>0.38±0.10†</td>
<td>0.72±0.31</td>
<td>3.24±0.85†</td>
</tr>
</tbody>
</table>

n, Number of rats; GU, Goldblatt units; mRNA, messenger RNA. Values are mean±SEM. *Normalized with β-actin. †p<0.01. §p<0.05.
FIGURE 2. Representative dot blot of RNA extracted from kidney cortex from rats pretreated with deoxycorticosterone acetate–salt, given furosemide (10 mg/kg i.p.), and killed at 0, 2, 4, 8, or 48 hours. Serial twofold dilutions of total RNA from 10 to 0.625 µg were loaded on the filter. Panel A: Dot blot was probed for renin. Panel B: Same dot blot was probed for ß-actin; results from 5 µg total RNA are shown.

lagged behind, but were significantly stimulated as early as 4 hours.

Since furosemide is a potent renin secretagogue, additional experiments were performed to test whether the stimulation of renin mRNA provoked by furosemide might be blunted as a consequence of the increase in angiotensin levels resulting from the increased renin secretion. Furosemide was administered as in the previous study, but in addition to pretreatment with DOCA-salt, animals received the converting enzyme inhibitor quinapril. A representative Northern and dot blot are shown in Figure 5. Rats were killed 4 hours after administration of furosemide or vehicle. In rats pretreated with DOCA-salt and then given quinapril, renin mRNA levels were higher than those in rats given only DOCA-salt (ratio quinapril-treated rats compared with control rats, 3.90±1.16, p<0.05 compared with 1.0, n=5), but there was no evidence for an additional stimulatory response 4 hours after administration of furosemide (see Figure 5).

FIGURE 3. Line graph shows densitometric analysis of all dot blots probed for renin messenger RNA (mRNA) and ß-actin mRNA from rats pretreated with deoxycorticosterone acetate–salt and given furosemide (10 mg/kg i.p.). Densitometric units for ß-actin mRNA (panel A) and renin mRNA (panel B) are shown plotted against kidney cortex total RNA. Data are mean±SEM. Regression lines, calculated by the method of least squares, yielded >0.99 for all groups. Slopes for ß-actin did not differ from control at any time point. Slope of renin mRNA was unchanged at 4 hours, but the slopes were significantly increased at 2, 4, 8, and 48 hours (p<0.02 at 4 and 8 hours; p<0.005 at 48 hours).

FIGURE 4. Line graph shows time course of the changes in plasma renin and renal renin messenger RNA (mRNA) levels in rats pretreated with deoxycorticosterone acetate–salt and then given furosemide (10 mg/kg i.p.) and a low salt diet. Relative renin mRNA values were determined from dot blot analysis, normalized with ß-actin. Data are mean±SEM. PRA, plasma renin activity; GU, Goldblatt units. *p<0.05, **p<0.01.

Discussion

Time Course of the Response of Renin Messenger RNA

In the present experiments, we studied the time course of changes in renin mRNA levels after furosemide administration. In rats pretreated with exogenous mineralocorticoid plus a high salt intake, furosemide caused a significant increase in renin mRNA levels that could be detected as early as 4 hours after diuretic administration. The pattern followed the time course of the change in plasma renin levels, although stimulation of renin mRNA levels lagged at least 2 hours behind the stimulation of plasma levels (Figure 4). The results indicated that renin mRNA levels can show prompt responses in vivo. Nonetheless it is of note that the response appears not to be immediate, in that no stimulation was detectable at 2 hours, although secretion was clearly stimulated at this earlier time point. Responses of gene transcription can be produced by steroid hormones in intact animals within 15 minutes of...
injection of the hormone,\textsuperscript{10} and cyclic AMP-mediated changes in gene transcription can also occur within minutes.\textsuperscript{11} A lag in responses in gene expression of a few hours is characteristic of secondary responses\textsuperscript{10,11} and may indicate the participation of other genes or changes in mRNA stability in the response of renin to furosemide.

Stimulation of renin gene expression by furosemide was not detectable in 16 hours in animals that were not pretreated with DOCA and high salt, although renin secretion was markedly increased by the diuretic in this group of rats. These results are consistent with previous experiments showing that renin mRNA levels do not necessarily rise whenever secretion is stimulated. For example, Nakamura and his colleagues\textsuperscript{6} found nonproportional changes in plasma renin concentration, renal renin content, and renin mRNA levels in response to sodium depletion and captopril treatment for 15 days. Pratt and coworkers\textsuperscript{27} observed no change in renin mRNA levels with mild salt depletion even though PRA increased significantly. Barrett and coworkers\textsuperscript{4} have observed a disproportionate stimulation of renin secretion relative to renin mRNA levels with converting enzyme inhibition. These observations taken together indicate that renin secretion and renin mRNA synthesis do not invariably show proportionate responses. However, it is probably not valid to infer that the mechanisms for regulation of renin secretion and renin mRNA are distinct. The failure to detect changes in renin mRNA levels in our control series and in previous studies may reflect the methodological difficulty in assessing small absolute changes in mRNA levels. The techniques used to determine renin mRNA levels in our studies and in previous works\textsuperscript{4,9} assess relative changes between control and stimulated values, and the sensitivity depends on the n-fold stimulation, not on the absolute magnitude of the change. A small change in renin mRNA level superimposed on a high baseline may not be detectable, whereas the same change may be more easily recognized if mRNA baseline levels are low.

Stimulation of renin secretion without a change in renin mRNA might result from a feedback effect of angiotensin II on renin gene expression. There is substantial evidence that angiotensin II exerts an inhibitory control on renin mRNA synthesis.\textsuperscript{28} We speculated that interrupting this negative feedback pathway by converting enzyme inhibitor administration might potentiate an effect of furosemide on renin mRNA levels. In rats pretreated with DOCA-salt and quinapril, renin mRNA levels were stimulated when compared with rats given DOCA-salt alone, consistent with previous observations that converting enzyme inhibitor administration increases renin mRNA levels.\textsuperscript{4,7,9} In these animals, however, furosemide did not produce a detectable stimulation of renin gene expression at 4 hours. Thus, angiotensin II inhibition would appear not important in limiting the increase in renin mRNA levels produced by furosemide, at least at the 4-hour time point.

**Role of the Macula Densa**

A body of experimental work in intact animals performed 10–15 years ago\textsuperscript{14,29} led to the tentative conclusion that the acute stimulation of renin secretion by furosemide is at least partially a consequence of inhibition of macula densa NaCl transport. This conclusion has recently received direct support from studies using isolated preparations. Renin secretion from isolated afferent arterioles has been shown to be stimulated by furosemide only when the macula densa was included in the dissected specimen.\textsuperscript{13} In the isolated perfused juxtaglomerular apparatus (JGA), renin secretion is highly sensitive to changes in NaCl concentration at the macula densa.\textsuperscript{30,31} In this preparation luminal application of furosemide or bumetanide, which delivers the drug directly to the macula densa, stimulated renin secretion, whereas bath application of the diuretics, which provides direct access to the granular cells, was without effect.\textsuperscript{14} Furthermore, electrophysiological and optical studies of isolated perfused JGAs have provided direct evidence for a furosemide-sensitive transporter in the luminal surface of the macula densa cell.\textsuperscript{32–34} All this evidence suggests that inhibition of macula densa transport by furosemide results in acute stimulation of renin secretion. Since renin mRNA levels were also stimulated by administration of furosemide as early as 4 hours in the DOCA-salt-loaded animals, the results raise the possibility that a signal from the macula densa may also regulate renin mRNA synthesis, albeit with a lag of several hours. Certain previous observations are consistent with this hypothesis. For example, renal renin mRNA levels are increased in response to chronic salt deprivation,\textsuperscript{27,35} a condition where persistent activation

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**FIGURE 5.** Representative dot blot and Northern blot from studies performed to determine whether converting enzyme inhibition would potentiate response of renin messenger RNA (mRNA) to furosemide. All animals were pretreated with deoxycorticosterone acetate–salt. Converting enzyme inhibitor plus furosemide (CEI+Bur) group was given quinapril (2.5 mg/kg i.p.) 12 and 24 hours before receiving furosemide (10 mg/kg i.p.). Converting enzyme inhibitor (CEI) group was given quinapril alone and vehicle. All animals were killed 4 hours after diuretic or vehicle administration. Panel A: Dot blot. Serial twofold dilutions of total RNA from 10 to 0.625 ug were loaded on the filter. Figures below the graph indicate a comparison of the slopes of renin mRNA from the treated rats to that from the control (C) rat. Panel B: Northern blot. In each lane 12 ug total RNA from the kidney cortex was run. Filters were hybridized with rat al(IV) collagen complementary DNA (cDNA) (double arrows) and rat renin cDNA (single arrow). Figures below the graph indicate a comparison of the ratios of renin mRNA/al(IV) collagen mRNA in the treated rats to that of the control rat. Data indicate that pretreatment with CEI does not potentiate the response to furosemide.
of the macula densa signal is likely. Furthermore, during ontogeny, the localization of renin to the granular cells at the vascular hilum develops coincident with maturation of the loop of Henle and a fall in NaCl concentration at this site.36

In summary, the present studies demonstrate that furosemide can produce a rapid increase in renal renin mRNA levels, although the increase in renin mRNA lagged behind the renin secretory response by 2 hours. The stimulation was unmasked by pretreating animals with DOCA and high salt, which suppressed the baseline renin mRNA levels. The early stimulatory response was not detectable in animals that were not thus pretreated. Blockade of angiotensin II formation by converting enzyme inhibitor administration elevated baseline renin mRNA levels. The early stimulatory response of furosemide can produce a rapid increase in renal renin mRNA but did not potentiate the effect of furosemide. The observation that the increase in renin mRNA produced by furosemide lags 2-4 hours behind the secretory response suggests that the intracellular mechanisms controlling secretion do not immediately activate new renin mRNA synthesis and that renin mRNA may rise as a secondary response requiring either synthesis of other proteins or changes in renin mRNA stability.

Acknowledgments

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References

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