Renin Enhancer Is Crucial for Full Response in Renin Expression to an In Vivo Stimulus

M. Andrea Markus, Christine Goy, David J. Adams, Frank J. Lovicu, Brian J. Morris

Abstract—We showed recently that deletion of a strong enhancer located 2.7 kb upstream of the renin gene in mice produces a strain with mild hypotension and salt-sensitivity. Here we set out to compare responses in renin expression in kidney and extrarenal tissues in these “REKO” mice. REKO and wild-type mice were placed on a low NaCl/enalapril regimen for 1 week, and then Ren-1c mRNA and renin enzyme activities were measured in tissues and plasma. In untreated REKO mice, renin and Ren-1c mRNA were reduced significantly in kidney, submandibular gland, adrenal, heart, and brain. In situ hybridization indicated a marked reduction in Ren-1c mRNA in juxtaglomerular cells and granular ducts of submandibular gland. After the chronic stimulus response in renal Ren-1c mRNA in REKO mice was blunted by 54% compared with wild-type mice, and was accompanied by almost complete exhaustion of renin stores. Response in plasma renin was blunted by 47%, this being mirrored in heart (54% decline), in which renin is derived mostly from the bloodstream. In adenral a 55% reduction was seen. These data are consistent with inability of REKO mice to adequately replenish renal renin stores during chronic stimulation of renin secretion. In conclusion, the renin enhancer is critical for replenishment of renin stores and response in renin to a chronic in vivo stimulus. (Hypertension. 2007;50:933-938.)

Key Words: renin gene expression ■ enhancer ■ knockout mouse ■ low sodium diet ■ angiotensin converting enzyme inhibitor ■ renin mRNA ■ renin enzymatic activity ■ kidney ■ extrarenal renin expression

Located far upstream of the renin gene of various species is a strong enhancer.1–5 It has been reported recently that deletion of the mouse6 or human7 enhancer in genetically engineered mice leads to a marked reduction in renin in renal juxtaglomerular (JG) cells, which are the classical source of circulating renin. Telemetry studies of mouse renin enhancer knockout (REKO) mice revealed normalized diurnal rhythm of blood pressure, but at a mean 9 mm Hg lower level.6 This was partly normalized by a classical source of circulating renin. The production of REKO mice was as described previously.5 The experiments described herein were approved by the University of Sydney Animal Ethics Committee. REKO and wild-type (WT; C57BL6) mice were housed in accordance with SPF regulations.

In Situ Hybridization

Renin mRNA expression patterns were examined by in situ hybridization using sense and antisense riboprobes labeled with digoxigenin. A 905-bp or a 386-bp renin fragment in pGEM and the DIG RNA Labeling kit was used to prepare the riboprobe according to the manufacturer’s protocol. Neutral buffered formalin-fixed paraffin sections (6 mm) were deparaffinized and pretreated as described previously.9 Prehybridization was at 55°C for 1 hour with hybridization buffer (50% formamide, 10% dextran sulfate, 0.05 mg/mL yeast tRNA, 0.5 mg/mL poly ribo A, 0.05 mol/L dithiothreitol, 0.3 mol/L NaCl, 10 mmol/L Tris, 10 mmol/L NaHPO4, 5 mmol/L EDTA, 0.2% Ficoll 400, 0.2% polyvinyl pyrrolidone) in a humidified chamber. Sections were hybridized overnight at 55°C with hybridization buffer containing approximately 200 ng/mL digoxigenin-labeled cRNA probe.

After hybridization, slides were washed at 65°C in decreasing concentrations of standard saline citrate (SSC), twice in 4×SSC for 20 minutes, once in 2×SSC, 1×SSC, and 0.5×SSC for 10 minutes each, and twice in 0.1×SSC for 20 minutes, with a final 0.1×SSC wash.

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933
washed for 5 minutes at room temperature. The distribution of the hybridized riboprobes was determined using an anti-digoxigenin alkaline phosphatase conjugated antibody (Roche) and 20 μg/mL of NBT/BCIP as instructed by the manufacturer. Sections were counterstained with 1.8 μg/mL bisbenzimide (Hoechst dye; Sigma) to label cell nuclei, rinsed with PBS, mounted, and examined using a Leica DMLB microscope. A technician examined kidney sections of WT and KO and systematically scored 87 glomeruli having a hybridization signal into degrees of intensity from 1+ (lowest) to 5+ (highest). The difference between WT and REKO was tested by χ² analysis with 2 degrees of freedom.

Renin Enzymatic Assay

Tissues were collected immediately on ice, stored frozen at −80°C, then homogenized in dH₂O (1:10, wt/vol) at 4°C. Various volumes of homogenate, diluted as appropriate for each tissue, were made up to 25 μL with dH₂O and incubated at 37°C with 25 μL of nephrectomized sheep plasma, and 25 μL of an angiotensinase inhibitor cocktail containing 30 mmol/L Na₂EDTA, 3 mmol/L 2,3-dimercaptopropanol, and 6 mmol/L 8-hydroxyquinoline buffered in 150 mmol/L sodium phosphate buffer, pH 6.2. In preliminary experiments, varying volumes of homogenate from each tissue were incubated for 0, 5, 30, and 90 minutes in duplicate at 37°C to ensure reaction kinetics were linear and not substrate-limited. Subsequent incubations at 37°C were for 1 hour. Reactions were terminated by the addition of 925 μL dH₂O and placing the tubes into boiling water for 4 minutes. Ang I generated was measured by RIA. Values were calculated for the linear range of the reaction curve (zero order kinetics) and expressed as pmol Ang I·h⁻¹·mg⁻¹ for tissues and pmol Ang I·h⁻¹·μL⁻¹ for plasma. Ren-1 mRNA values (n=6) are expressed relative to WT, taken as 1.0. Thus relativities are shown only between WT and REKO, not between tissues. Shown in parentheses is each value for REKO expressed as a percentage of WT.

Renin mRNA Quantification

Ren-1 mRNA was measured in kidneys, SMG, adrenal, brain, and heart from 2 to 12-month-old WT and REKO mice using glyceral-dehyde-3-phosphate dehydrogenase (GAPDH) as internal control. Total RNA was extracted with a SV Total RNA Isolation System (Promega), and reverse transcription was performed using SuperScript II RNase H-Reverse Transcriptase (Invitrogen) according to the manufacturer’s instructions. Briefly, a mixture of 1 μg RNA, 0.5 μg oligo(dT), 50 ng random hexamers, 10 mmol/L each dNTP, and 0.5 μL Superscript II was used per reaction. To ensure similar loading, the cDNAs generated were diluted before polymerase chain reaction (PCR) as follows: kidney cDNA 1:100, SMG 1:10, and other tissues undiluted, in accordance with known relativities in renin concentrations between these tissues. Semi-quantitative real-time reverse transcriptase (RT)-PCR was performed using 0.5 μL cDNA and the SybrGreen PCR kit (Invitrogen) according to the manufacturer’s instructions. Specific primers used for PCR were, for Ren-1, 5’-CCTCTACCTGGTTGAGGGATT-3’ and 5’-CTGGCTGAGGAAACCTTGGACT-3’ (which yielded a 123-bp amplicon) and GAPDH, 5’-GGT GAA GGT CGG TGT GAA CG-3’ and 5’-GCT TTG ACT GTG CCG TTG AA-3’ (178 bp amplicon). PCR conditions were 2 minutes at 95°C, followed by 40 cycles of 15 s at 94°C, 30 s at 61°C, 30 s at 72°C. Data were normalized by reference to GAPDH and the relative expression level of Ren-1 mRNA was calculated using the software tool REST.12

Low Salt + Enalapril Treatment

Mice were placed randomly on a normal (0.4% NaCl) diet or a low salt (0.05% NaCl) diet together with enalapril maleate, 1 mg/mL, in drinking water, provided ad libitum for a period of 1 week.

Results

Renin in Tissues and Plasma

Renin levels in tissues of male REKO mice were all significantly lower than WT, but in plasma the reduction was not significant (Table 1). Plasma renin (PRC) correlated with plasma aldosterone concentration (data not shown; r=0.83; n=6). In female mice kidney renin was 539±48 in WT and 351±15 pmol Ang I·h⁻¹·mg⁻¹ in REKO (P=0.010; n=3), ie, REKO was 65±3% of WT. In SMG renin was 15.0±3.5 and 2.5±0.59 (17±4%) in each respective strain (P=0.010; n=3).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>WT</th>
<th>REKO</th>
<th>(as % of WT)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>687±101</td>
<td>485±46</td>
<td>(71±6.7)</td>
<td>0.047</td>
</tr>
<tr>
<td>SMG</td>
<td>619±171</td>
<td>17±4.7</td>
<td>(2.7±0.75)</td>
<td>0.0021</td>
</tr>
<tr>
<td>Adrenal</td>
<td>0.20±0.039</td>
<td>0.12±0.011</td>
<td>(60±5.5)</td>
<td>0.034</td>
</tr>
<tr>
<td>Brain</td>
<td>0.017±0.0004</td>
<td>0.0150±0.0006</td>
<td>(88±4)</td>
<td>0.0083</td>
</tr>
<tr>
<td>Heart</td>
<td>0.19±0.027</td>
<td>0.099±0.017</td>
<td>(52±9)</td>
<td>0.0091</td>
</tr>
<tr>
<td>Plasma (PRC)</td>
<td>0.6±0.11</td>
<td>0.49±0.11</td>
<td>(80±18)</td>
<td>0.30</td>
</tr>
<tr>
<td>Plasma (PRA)</td>
<td>0.019±0.004</td>
<td>0.016±0.003</td>
<td>(84±16)</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Table 1. Renin Enzyme and Ren-1 mRNA Levels in WT and REKO Tissues
Ren-1c mRNA in Tissues

In Situ Hybridization

WT mice showed an expected strong hybridization signal in JG cells of kidney sections (Figure 1A) and SMG granular ducts (Figure 1E). In contrast, in REKO mice the signal was markedly reduced in JG cells (Figure 1B). Semiquantification according to signal strength assessed by eye by a trained technician, unaware of the objective of the experiment, into categories 1/100 to 5/100 demonstrated that the decrease was highly significant (Figure 2A). In SMG sections from REKO mice a Ren-1c mRNA signal was undetectable (Figure 1F).

qRT-PCR Results

Just as for renin, Ren-1c mRNA in whole kidney tissue of REKO mice was reduced by ~30% (Table 1), ie, was not reduced as much as in JG cells. In SMG, adrenal and heart Ren-1c mRNA levels were 93%, 91%, and 94% lower, respectively, in REKO compared with WT (Table 1). The reduction in Ren-1c mRNA in each were, moreover, comparable to the reduction seen in renin. Ren-1c mRNA levels in SMG, moreover, mirrored the in situ hybridization results for REKO and WT. Brain renin mRNA, as renin, was only slightly lower, however, in REKO mice.

Response to Stimulation

In the kidney, low NaCl enalapril increased Ren-1c mRNA and renin expression in WT mice by 11- and 3-fold, respectively (Figure 3; Table 2). In REKO mice, however, we saw a significantly blunted response in Ren-1c mRNA, and renin was markedly depleted (Table 2). In situ hybridization showed recruitment of Ren-1c mRNA in afferent arteriolar cells upstream of the glomerulus in both WT and REKO (Figure 1C and 1D). However, in REKO mice, Ren-1c mRNA signal was markedly reduced (Figure 1D), and semiquantification of the signal strength in WT and REKO indicated a similar differential as seen for the normal diet, but at a higher level (Figure 2B).

In other REKO tissues a highly significant blunting of the response in renin to low salt enalapril was seen, being 45%, 48%, and 46% of WT for adrenal, brain, and heart, respectively. In the case of Ren-1c mRNA, response was significantly blunted in adrenal, but no significant change in Ren-1c mRNA level was seen in heart, consistent with the increase in renin enzyme levels we observed having been from uptake from the circulation. Brain Ren-1c mRNA increased only slightly in response to treatment, and in SMG Ren-1c mRNA responded little to the stimulus, as expected, given the well-known difference in the nature of regulatory influences on SMG renin.

A blunted response to low-salt diet enalapril in REKO mice was also reflected in the plasma renin levels, where, in contrast to the 32-fold increase in WT mice, renin increased only 17-fold in REKO, ie, was blunted by 47% (Figure 3).

Discussion

The present study has demonstrated a crucial role for the renin enhancer in regulation of the response in renin expression to a conventional strong physiological stimulus in mice. Our study has also documented the extent of the reduction in renin expression in various tissues in mice lacking the renin enhancer.

In the kidney, under baseline conditions of a normal diet, we found marked depletion of Ren-1c mRNA in JG cells of
REKO mice, as determined by in situ hybridization. By immunohistochemistry, renin antibodies gave the usual JG staining in WT kidney, but were unable to detect renin in sections of REKO kidney. Curiously, however, at the whole tissue level, Ren-1 mRNA and renin enzyme were reduced by only 28% and 29%, respectively, and plasma renin was only 20% (PRC) and 16% (PRA) lower. Consistent with the marked loss of renin expression in JG cells, histopathology showed striking pseudostratification and hyperplasia of macula densa cells. The apparently contradictory findings for renin expression at the JG level versus the whole kidney level may, however, be reconciled by recognition of the fact that under extreme circumstances renin can be expressed in tubular cells. A separate, or additional, possibility is that a “short feedback loop” may operate, so that when renin promoter output is compromised and renin is rate-limiting there will be a net Ang II deficit which will tend to cause vascular recruitment (presumably even in the absence of a strong physiological challenge). The levels per cell would remain diminished because of the enhancer knockout, but the aggregate of cells responding would increase. Under severe physiological challenge this would be enhanced, and additional sites, eg, tubular or vascular, provoked. Because JG cells comprise <0.01% of the total renal cell population, low compensatory expression of renin in other renal cells would explain our findings. The renin antibody and hybridization probe we used are not sufficiently sensitive, however, to detect such low level expression. This response appears to distinguish kidney from other tissue sites. In this regard, the kidney appears to have a greater repertoire of cells capable of responding to a physiological challenge by ramping up renin production and secretion.

In the SMG of REKO mice we saw a clear-cut and consistent very marked reduction in Ren-1 mRNA and, as reported previously, renin protein, in granular duct cells by in situ hybridization and immunohistochemistry, respectively. Consistent with this, Ren-1 mRNA, measured by qRT-PCR, was 7% of WT, and renin, measured by enzyme assay, was 3% of WT. These findings demonstrate clearly the vital physiological role of the renin enhancer in renin expression in vivo.

In the REKO adrenal Ren-1 mRNA was 9% of WT. Renin, however, showed a lesser reduction, possibly attributable to contamination of tissue by extracellular fluid sources in this small gland in mice.

Table 2. Response in Ren-1 mRNA and Renin Enzyme Levels to Low NaCl+Enalapril Treatment for One Week

<table>
<thead>
<tr>
<th>Tissue</th>
<th>WT</th>
<th>REKO</th>
<th>REKO as % of WT Response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Renin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>2.76±0.60 (P=0.0073)</td>
<td>0.046±0.003 (P=2×10⁻⁷)</td>
<td>2±0.1% (P=0.0003)</td>
</tr>
<tr>
<td>SMG</td>
<td>1.6±0.3 (P=0.07)</td>
<td>1.6±0.2 (P=0.05)</td>
<td>100±12% (P=0.99)</td>
</tr>
<tr>
<td>Adrenal</td>
<td>30±5 (P=0.0001)</td>
<td>14±2 (P=2×10⁻⁵)</td>
<td>45±5% (P=0.0006)</td>
</tr>
<tr>
<td>Brain</td>
<td>2.6±0.03 (P=0.0001)</td>
<td>1.2±0.08 (P=0.011)</td>
<td>48±3% (P=0.0002)</td>
</tr>
<tr>
<td>Heart</td>
<td>32±4 (P=2×10⁻⁶)</td>
<td>15±2 (P=4×10⁻⁴)</td>
<td>46±6% (P=4×10⁻⁵)</td>
</tr>
<tr>
<td>Plasma (PRC)</td>
<td>32±4 (P=3×10⁻⁵)</td>
<td>17±2 (P=2×10⁻⁵)</td>
<td>53±5% (P=0.0007)</td>
</tr>
<tr>
<td><strong>Ren-1 mRNA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>11.4±2.71 (P=0.001)</td>
<td>5.3±0.66 (P=0.001)</td>
<td>46±5.7% (P=0.001)</td>
</tr>
<tr>
<td>SMG</td>
<td>0.75±0.13 (P=0.040)</td>
<td>0.71±0.27 (P=0.47)</td>
<td>95±36% (P=0.20)</td>
</tr>
<tr>
<td>Adrenal</td>
<td>2.0±0.48 (P=0.003)</td>
<td>1.5±0.40 (P=0.16)</td>
<td>72±20% (P=0.002)</td>
</tr>
<tr>
<td>Brain</td>
<td>1.3±0.1 (P=0.049)</td>
<td>1.4±0.2 (P=0.19)</td>
<td>110±17% (P=0.73)</td>
</tr>
<tr>
<td>Heart</td>
<td>0.91±0.04 (P=0.38)</td>
<td>1.0±0.3 (P=0.90)</td>
<td>113±29% (P=0.75)</td>
</tr>
</tbody>
</table>

*P* values are shown in brackets.
In the heart, the small amounts of Ren-1 mRNA present were similarly reduced markedly, REKO levels being 6% of WT. It is well-established that most of the renin present in heart is from uptake of circulating renin of renal origin.\textsuperscript{5,13,14,21} Nevertheless, despite only a 20% reduction in plasma renin in untreated REKO mice, cardiac renin was reduced 53%, suggesting that some of the renin in mouse heart could come from synthesis in this tissue in this mouse strain.

In relation to renin expression in the brain, the cardiovascular and behavioral changes we have noted in REKO mice, in the face of a negligible change in plasma renin, provide clear evidence in support of reduction in at least certain regions of the CNS of REKO mice.\textsuperscript{6,8} Renin levels in brain tissue are, however, well known to be extremely low, and for decades even its very existence was questioned.\textsuperscript{4} Although we found little change in Ren-1 mRNA and renin at the whole brain level, our previous studies demonstrate alteration in vasomotor and other responses involving CNS pathways,\textsuperscript{6,8} so that follow-up studies are required to discover the cells and CNS nuclei involved.

An important finding in the present study was the response in renin expression to a potent in vivo stimulus. REKO mice treated with low NaCl+enalapril exhibited a remarkably consistent blunting of the response to this stimulus in tissues that such a stimulus usually affects. In kidney and adrenal response in Ren-1 mRNA was, respectively, 46% and 72% of WT. In plasma and adrenal, renin response was 53% and 45% of WT, but, remarkably, in kidney, renin was only 5% of that seen in REKO mice on a normal diet. Not only is this consistent with our failure to detect renin immunohistochemically in low NaCl REKO mice,\textsuperscript{6} but suggests marked depletion of renin stores. We suggest that the blunted response in Ren-1 mRNA has meant failure to supply adequate amounts of renin to the protein synthesis machinery for replenishment of renin stores in the kidney. In the face of the strong stimulus to renin secretion, renin secretory granules may have become exhausted as secretion attempts to maintain adequate levels of renin in the bloodstream, albeit, achieving only half the WT level in response to the stimulus. This phenomenon was, moreover, observed in experiments performed on 2 separate occasions (data not shown).

In line with the argument that most of the renin in the heart is from uptake from the bloodstream, we saw in WT mice an identical 32-fold increase in renin in heart and plasma in response to the stimulus, and in REKO mice an increase of 15-fold and 17-fold in heart and plasma, respectively. The fact that Ren-1 mRNA did not change is consistent with all of the additional renin having come from the circulation.

In the SMG, renin expression in the SMG is controlled by testosterone and, as expected, there was little change in Ren-1 mRNA and renin in response to the stimulus used.

The data indicate that in tissues known to respond to low salt+enalapril, the renin enhancer is necessary for about half of the response to this conventional stimulus. One or more of the over 11 transcription-factor binding sites that have been identified in the 241-bp enhancer region (see review\textsuperscript{5}) would therefore appear to be involved in this portion of the response. The precise molecular pathways and mechanisms that transduce signaling consequent to low dietary NaCl and pharmacological reduction in angiotensin II by an ACE inhibitor will require extensive investigation. Our finding also implies that other DNA elements, such as those in the proximal promoter DNA,\textsuperscript{5,22} or other elements or factors such as ones involved in renin mRNA stability,\textsuperscript{23} are responsible for the remainder of the response. Whereas there was no change in blood pressure in WT mice on a low salt diet, REKO mice were salt-sensitive, a 2.0 mm Hg increase in mean arterial pressure being seen (where on a normal diet, REKO mice have a 9.9 mm Hg lower MAP than WT).\textsuperscript{6} Our present results now show that this rise involves a 17-fold increase in plasma renin, rather than the 32-fold increase seen in WT mice.

Our findings are in apparent contrast to recent data from the Sigmund Laboratory, which show that Ang II reduced (human) REN mRNA by 3-fold in strains of mice they retrofitted with a 160-kb human construct containing, or lacking, the homologous human renin enhancer.\textsuperscript{7} This could, however, either be (1) an effect of the enhancer itself, which in human lacks a 23-bp sequence at the 3' end that confers 5-fold higher enhancer activity than the sequence conserved between each species,\textsuperscript{2,3} and therefore contributes, in part, to the over 100-fold higher renin expression and plasma levels in mouse as compared with human, or (2) the possibility that mouse transcription factors do not confer similar actions on human renin promoter and enhancer elements as they do on these DNA sequences in the mouse.

In conclusion, the present investigation has established that a strong enhancer 2.7 bp upstream of the renin gene is an important determinant of renin expression, in particular in effecting a full and adequate response to a well-known in vivo stimulus.

**Perspectives**

The renin enhancer has long been touted as vital for renin expression. But this was based on in vitro experiments. Now we have provided the important in vivo confirmation of this and show that it mediates approximately half the response to a common stimulus. The low renin model we have produced has been engineered to remove an important primary target of signaling molecules at the earliest point of contact in the renin expression cascade. As such it provides a very much more physiological scenario than models in which the entire renin gene is deleted with drastic consequences. Thus our more subtle model that still expresses renin will be a valuable resource for all kinds of studies. Accordingly, we are happy to make REKO mice available to other researchers on request.

**Acknowledgments**

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**Disclosures**

None.
References
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