Renin knockout rat: control of adrenal aldosterone and corticosterone synthesis in vitro and adrenal gene expression

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Ralph H, Gehrand A, Bruder ED, Hoffman MJ, Engeland WC, Moreno C. Renin knockout rat: control of adrenal aldosterone and corticosterone synthesis in vitro and adrenal gene expression. Am J Physiol Regul Integr Comp Physiol 308: R73–R77, 2015. First published November 12, 2014; doi:10.1152/ajpregu.00440.2014—The classic renin-angiotensin system is partly responsible for controlling aldosterone secretion from the adrenal cortex via the peptide angiotensin II (ANG II). In addition, there is a local adrenocortical renin-angiotensin system that may be involved in the control of aldosterone synthesis in the zona glomerulosa (ZG). To characterize the long-term control of adrenal steroidogenesis, we utilized adrenal glands from renin knockout (KO) rats and compared steroidogenesis in vitro and steroidogenic enzyme expression to wild-type (WT) controls (Dahl S rat). Adrenal capsules (ZG; aldosterone production) and subcapsules (zona reticularis/fasciculata (ZFR); corticosterone production) were separately dispersed and studied in vitro. Plasma renin activity and ANG II concentrations were extremely low in the KO rats. Basal and cAMP-stimulated aldosterone production was significantly reduced in renin KO ZG cells, whereas corticosterone production was not different between WT and KO ZFR cells. As expected, adrenal renin mRNA expression was lower in the renin KO compared with the WT rat. Real-time PCR and immunohistochemical analysis showed a significant decrease in P450aldo (Cyp11b2) mRNA and protein expression in the ZG from the renin KO rat. The reduction in aldosterone synthesis in the ZG of the renin KO adrenal seems to be accounted for by a specific decrease in P450aldo and may be due to the absence of chronic stimulation of the ZG by circulating ANG II or to a reduction in locally released ANG II within the adrenal gland.

In the current study, we have begun this process by evaluating the aldosterone and corticosterone response to cAMP in vitro by the measurement of adrenal steroidogenic gene expression and by morphological examination of adrenal zonation by immunohistochemical analysis of the two major late-pathway steroidogenic enzymes: P450aldo (CYP11B2) that mediates the conversion of corticosterone to aldosterone in the ZG and P45011β (CYP11B1) that mediates corticosterone production in the zona fasciculata/reticularis (ZFR) (8).

MATERIALS AND METHODS

Generation of the renin KO rat. All experiments were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. The renin KO rat was created and characterized as described previously (13). Briefly, zinc-finger nucleases were used to induce a 10-bp deletion in exon 5 of the renin gene, causing a frame-shift mutation. KO rats were created from a wild-type (WT) background of SS/JrHsdMiw (SS) rats. Prepubertal (3- to 5-wk-old) female rats were used (N = 75).

Blood and tissue collection. Rats were anesthetized with isoflurane. After a laparotomy, adrenal glands were removed and quickly cleaned of adipose tissue. Adrenal glands used for in vitro analyses were decapsulated; subcapsules (primarily ZFR) and capsules (primarily ZG) were immediately placed in ice-cold buffer and pooled to make one batch of each cell type. Adrenal glands used for real-time PCR analysis were decapsulated and the capsules and subcapsules snap frozen in liquid nitrogen. Adrenal glands used for immunohistochemical analysis were snap frozen whole. In some rats, abdominal aortic blood was collected into a sterile syringe before the adrenal glands were removed. Blood samples were aliquoted for serum or plasma measurements into fresh tubes containing no additive (serum aldosterone and sodium-potassium), EDTA (serum renin activity; PRA), or phenathroline and EDTA (plasma ANG II). Serum sodium and potassium were measured using a flame photometer (model 943 Instrumentation Laboratory).

PRA, ANG II, and aldosterone. PRA was assessed by measuring the amount of ANG I produced in vitro (23). The minimum detectible limit for the PRA assay was 0.28 ng ml−1 h−1; the intra- and inter-assay coefficients of variation (CVs) were 5.8% and 11.0%, respectively. Plasma ANG II was measured by HPLC/RIA (21). The minimum detectible limit for the plasma ANG II assay was 1.7 pg/ml; the intra- and interassay CVs were 8.1% and 11.8%, respectively. Serum aldosterone was measured by direct radioimmunoassay (20). The minimum detectible limit for the serum aldosterone assay was 7.6 pg/ml; the intra- and interassay CVs were 1.8% and 5.7%, respectively.

Adrenocortical steroid synthesis in vitro. Adrenal steroidogenesis was assessed by measurement of basal and cAMP-stimulated (maximal stimulation) aldosterone release from dispersed capsular (primarily ZG) cells, and basal and cAMP-stimulated corticosterone release.

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from dispersed subcapsular (primarily ZFR cells), as described previously (3, 19). Briefly, tissue was digested with 4 mg/ml collagenase Type IV (Worthington Biochemical) in Krebs-HEPES buffer for 45 min on a shaker bath at 37°C. Cells were then prepared in fresh buffer, counted using a hemocytometer, and diluted to a final concentration of 100,000 cells/ml as described previously (3, 19). Cells were incubated for 2 h on a shaker bath at 37°C, in the presence or absence of dibutyryl-cAMP (0.01 mM, 0.1 mM, and 1.0 mM, samples run in triplicate). After the incubation, cell suspensions were centrifuged at 4°C, and supernatants were immediately frozen and stored at −20°C. Aldosterone and corticosterone accumulation was assessed with laboratory-developed radioimmunoassays (3). To account for different cell yields between preparations, data from each experimental day were normalized as a percentage of basal steroidogenesis in adrenal cells in the WT control (no cAMP).

Adrenal mRNA expression. Essential elements of the adrenal steroidogenic pathway were evaluated by real-time PCR (4). Total RNAs from capsules (ZG) and subcapsules (ZFR) were isolated using the RNasey Lipid Tissue Mini Kit with an on-column DNase digestion (QIAGEN). The concentration of RNA was quantified using a NanoDrop 2000, and all cDNA synthesis reactions were performed using the High-Capacity RNA-to-cDNA reverse transcription kit (Life Technologies). The final reaction volume of 20 μl consisted of 1× RT buffer, 1× RT enzyme mix, and 5 ng of previously isolated RNA. The concentration of cDNA was quantified using a NanoDrop 2000 and all cDNA synthesis reactions were diluted to 20 ng/μl in molecular biology grade water. Real-time PCR was performed using the Taqman Gene Expression Master Mix (FAM fluorophore) and premade primers and probes (Table 1) (Applied Biosystems, Foster City, CA). Renin real-time PCR was performed using the following custom oligos: renin forward 5'-TTACGGTTGTAACCTGAGCCA, renin reverse 5'-AGTATGCAAGGCTCATGCTTCC primers, renin probe 5'-[F6FAM]ACCACCGACCTTCACCATCTTGTC-3' (Integrated DNA Technologies). The final reaction volume of 20 μl consisted of 1× TaqMan Gene Expression Master Mix, 1× TaqMan primer/probe mix, and 100 ng (5 μl) of cDNA. Amplification/detection was performed with the ABI Prism 7900HT Sequence Detection System using the following thermal cycle conditions: 95°C for 10 min and 45 cycles at 95°C for 15 s, and 60°C for 1 min. Samples were assayed in triplicate. Gene expression was quantified by obtaining the number of cycles to reach a predetermined threshold value in the intensity of the PCR signal (C_T value). Ripl19 was used as the housekeeping gene for Taqman real-time qPCR for all mRNAs except for Ren, for which gapdh was used. If C_T changes were significant, relative changes in target mRNA expression (vs. baseline) were calculated using the 2^−ΔΔCt equation (11).

Table 1. TaqMan gene expression assays used for real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Symbol</th>
<th>Assay ID No.</th>
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</thead>
<tbody>
<tr>
<td>Angiotensin II receptor, type 1a</td>
<td>Agrp1a</td>
<td>Rn02758772_s1</td>
</tr>
<tr>
<td>Angiotensin II receptor, type 1b</td>
<td>Agrp1b</td>
<td>Rn02132799_s1</td>
</tr>
<tr>
<td>Cytochrome P450, family 11, subfamily a, polypeptide 1</td>
<td>Cyp11b1</td>
<td>Rn06072734_g1</td>
</tr>
<tr>
<td>Cytochrome P450, family 11, subfamily b, polypeptide 2 (aldosterone synthesis)</td>
<td>Cyp11b2</td>
<td>Rn01767818_g1</td>
</tr>
<tr>
<td>Cytochrome P450, family 11, subfamily c, polypeptide α</td>
<td>Cyp11b3</td>
<td>Rn00568733_m1</td>
</tr>
<tr>
<td>Melanocortin 2 receptor (ACTH receptor)</td>
<td>Mc2r</td>
<td>Rn02082920_s1</td>
</tr>
<tr>
<td>Scavenger receptor class B, member 1 (High-density lipoprotein receptor)</td>
<td>Scarb1</td>
<td>Rn00580588_m1</td>
</tr>
<tr>
<td>Steroidogenic acute regulatory protein</td>
<td>Star</td>
<td>Rn00580695_m1</td>
</tr>
<tr>
<td>Ribosomal protein L19</td>
<td>Ripl19</td>
<td>Rn00821265_g1</td>
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Immunohistochemistry (adenal enzyme expression). Adrenals (N = 6) from WT and renin KO rats were processed to assess the expression of P450 aldosterone synthase (P450aldo; a marker of the ZG) and P450 1β-hydroxylase (P4501B1; a marker of the ZFR) using immunofluorescence histochemistry as described previously (30). Frozen adrenals were sectioned (25 μm) and sections were incubated overnight with primary antibodies directed against P450aldo (1:100; rabbit host) and P4501B1 (1:125; sheep host) that were generously supplied by C. Gomez-Sanchez, University of Mississippi Medical Center. After overnight incubation, sections were incubated with secondary antibodies donkey anti-rabbit Alexa 488 (Molecular Probes) and donkey anti-sheep Cy3 (Jackson Immunoresearch) for 1 h, rinsed and cover-slipped in aqueous mounting media (Vectorshied). Optical images were collected using a fluorescence microscope (Leica DM4000B) using equivalent exposure times for all adenal sections, followed by overlapping using Adobe Photoshop CS5.1.

Statistical analyses. Data from each in vitro experiment were normalized and calculated as percentage of WT control (no cAMP added) and then were analyzed by one-way ANOVA. Hormone and real-time PCR data were analyzed by t-test. Post hoc analyses were performed by Student-Newman-Keuls method for multiple comparisons (Sigma Stat 2.03).

RESULTS

PRA and ANG II concentrations in the WT rats were 19.1 ± 1.1 ng·ml^−1·h^−1 and 58.1 ± 10.5 pg/ml (N = 6 for each mean ± SE), respectively, and <0.28 ng·ml^−1·h^−1 and <1.7 pg/ml, respectively, in the renin KO rats (below the detection limits of each assay). Although statistical significance was not reached, there was a trend toward a decrease in serum aldosterone concentrations in the renin KO rats (254 ± 28 pg/ml) compared with WT (323 ± 82 pg/ml; P = 0.14; N = 6 for each mean ± SE). Serum potassium was not different between WT (137.1 ± 0.4 mmol/l; N = 4) and renin KO (137.0 ± 0.5 mmol/l; N = 8) rats. Serum potassium was significantly increased in renin KO (6.5 ± 0.5 mmol/l) rats compared with WT (4.8 ± 0.2 mmol/l; P < 0.005).

Figure 1 shows the results of the cAMP-stimulated steroidogenesis experiments of aldosterone production from dispersed adrenal capsular (ZG) cells and corticosterone production from subcapsular (ZFR) cells. Capsular (ZG) cells from KO rats had significantly attenuated aldosterone response to cAMP, whereas corticosterone responses to cAMP from subcapsular (ZFR) cells were not different between WT and KO rats.

Adrenal mRNA expression (C_T) results are shown in Table 2. When compared with WT, Ren mRNA was 8-fold downregulated in Ren KO capsules (ZG) and 22-fold downregulated in Ren KO subcapsules (ZFR). Cyp11b2 (P450aldo) mRNA was 79-fold downregulated in Ren KO capsules and 40-fold downregulated in Ren KO subcapsules. No other components of the steroidogenic pathway showed changes in mRNA expression. Ren mRNA expression in adrenals from Sprague-Dawley (SD) rats was also analyzed for comparison to the WT controls. C_T values (± SE) for SD capsules and subcapsules were 27.30 ± 1.19 and 27.91 ± 0.56, respectively, and were significantly lower (i.e., higher Ren mRNA levels) compared with the WT rat.

There were no clear morphological differences in adrenocortical zonation between WT and renin KO rats (Fig. 2). Adrenal sections expressed P4501B1 in the inner cortex denoting the zona fasciculata, P450aldo in the outer cortex denoting the ZG, and the intervening zona intermedia was devoid of
Cyp11b2 in Ren KO subcapsules†/H11005/H11021

P450aldo labeling (30). However, when compared with adrenals from wild-type rats, the expression of P450aldo was markedly reduced (but not absent) in the majority of renin KO rats (5 of 6). Since only a few P450aldo-labeled cells could be identified in most renin KO adrenals, the loss of renin resulted in reduced expression of the glomerulosa cell phenotype.

Table 2. Adrenal Real-Time PCR Analyses in WT and KO rats

<table>
<thead>
<tr>
<th></th>
<th>Capsule (ZG)</th>
<th>Subcapsule (ZFR)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>Ren KO</td>
</tr>
<tr>
<td>Ren</td>
<td>30.70 ± 0.20</td>
<td>34.01 ± 0.29†</td>
</tr>
<tr>
<td>Agr1a</td>
<td>30.63 ± 0.73</td>
<td>30.11 ± 0.67</td>
</tr>
<tr>
<td>Agr1b</td>
<td>30.19 ± 0.48</td>
<td>30.59 ± 0.85</td>
</tr>
<tr>
<td>Cyp11b1</td>
<td>22.95 ± 0.47</td>
<td>22.33 ± 0.53</td>
</tr>
<tr>
<td>Cyp11b2</td>
<td>26.50 ± 0.90</td>
<td>31.67 ± 0.80*</td>
</tr>
<tr>
<td>Cyp11al</td>
<td>21.80 ± 0.80</td>
<td>21.16 ± 0.71</td>
</tr>
<tr>
<td>Mc2r</td>
<td>26.47 ± 0.78</td>
<td>25.80 ± 0.67</td>
</tr>
<tr>
<td>Scarb1</td>
<td>25.90 ± 2.04</td>
<td>24.34 ± 0.78</td>
</tr>
<tr>
<td>Star</td>
<td>25.13 ± 0.71</td>
<td>23.68 ± 0.84</td>
</tr>
<tr>
<td>Rpl19</td>
<td>24.44 ± 0.92</td>
<td>23.30 ± 0.72</td>
</tr>
<tr>
<td>Gapdh</td>
<td>20.47 ± 0.12</td>
<td>20.77 ± 0.09</td>
</tr>
</tbody>
</table>

Values are means of cycles to threshold (Ct) ± SE (N = 6 samples per experimental group). ZG, zona glomerulosa; ZFR, zona reticularis/fasciculata. Ct analysis (2-ΔΔCt) to determine fold changes in mRNA was calculated for significant changes in mRNA using the housekeeping genes Rpl19 for Cyp11b2 and Gapdh for Ren. When compared with wild-type (WT), Ren in Ren knockout (KO) capsules = eightfold decrease; Ren in Ren KO subcapsules = 22-fold decrease; Cyp11b2 in Ren KO capsules = 79-fold decrease; Cyp11b2 in Ren KO subcapsules = 40-fold decrease. *P = 0.002 from WT; †P = <0.001 from WT.

Fig. 2. Adrenal sections collected from control (A) and renin KO rats (B) and immunolabeled for P450 aldosterone synthase (P450aldo; green) and P450 11β-hydroxylase (P45011β; red), denoting the zona glomerulosa (ZG) and zona fasciculata (ZF), respectively, separated by the zona intermedia (ZI). Reduced expression of P450aldo was observed in adrenals collected from renin KO rats. Scale bar = 50 μm. cap = adrenal capsule.

**DISCUSSION**

This study evaluated adrenocortical function and steroidogenic pathway gene (mRNA) and protein expression in adrenal glands from renin KO rats compared with their WT background (Dahl S rats). We found that PRA and ANG II concentrations were unmeasurable in the KO rat, but that the KO rat was able to generate a significant serum aldosterone level under isoflurane anesthesia and after laparotomy. The KO rats were normonatremic but did exhibit hyperkalemia. The production of aldosterone in vitro from ZG cells in response to stimulation with cAMP was significantly attenuated, but still present, in the adrenal glands from KO rat. The corticosterone response from ZFR cells was unaffected in the KO rat compared with the WT controls. There was a dramatic decrease in the expression of Cyp11b2 mRNA and P450aldo protein in the ZG of the renin KO adrenal.

In the KO adrenals, the expression of Cyp11b2, one of the genes required for the normal development and expression of aldosterone synthase activity in the rat adrenal, is markedly reduced (22-fold decrease; *P = 0.002 from WT; †P = <0.001 from WT). This is supported by studies showing a decrease in aldosterone production and P450aldo expression with decreased stimulation of the adrenal gland by circulating plasma ANG II (8, 14, 15, 17).
The second is that the local expression of adrenal renin is required for normal ZG function (7, 9, 10, 14, 16, 26, 28, 29, 31). One of the theories is that local generation of adrenal ANG II mediates the ZG response to increases in potassium and that this is mediated by AT1-type angiotensin receptors (14, 26, 29). It is interesting to note, however, that the expression of AT1a and AT1b mRNAs was not altered in the renin KO rat.

These results are consistent with our finding of hyperkalemia in the renin KO rats. The serum potassium concentrations in the WT controls agree with previous results in the same animal model (24). In fact, one could consider the renin KO rat a model of hyporeninemic relative hypoaldosteronism (6). Regardless of which mechanism is correct, it is clear that corticosterone synthesis in the ZFR is not dependent on either circulating plasma ANG II or adrenal renin expression. This confirms that circulating ACTH is the primarily controller of ZFR function (18, 20).

It is interesting to note that the WT rat had lower renin mRNA levels in the adrenal compared with the outbred Sprague-Dawley rat. Future experiments are warranted to compare and contrast the adrenal RAAS between different outbred and inbred strains of rats.

One interesting finding was that, despite unmeasurable plasma ANG II concentrations, serum aldosterone levels were not markedly suppressed. This can be explained by the fact that the blood was obtained from the abdominal aorta after laparotomy under isoflurane anesthesia, and the rats were likely stressed by the blood sampling (18, 27). The serum aldosterone levels may represent maximal ACTH-stimulated aldosterone release as well as the extent hyperkalemia utilizing the reduced remaining P450aldo expression in the adrenal gland. The ability to produce aldosterone despite decreased P450aldo expression is similar to the effect of chronic administration of late-pathway enzyme inhibitors during which, because of the closed-loop nature of feedback control, substrate levels for the inhibited enzyme are increased leading to “break-through” of the enzyme blockade (1, 12, 22).

**Perspectives and Significance**

The global absence of normal renin expression in the rat leads to a marked decrease in the ability to synthesize aldosterone in vitro. This seems to be due to a specific attenuation of the expression of the late-pathway enzyme aldosterone synthase in the ZG. This suggests that the lower basal blood pressure in the renin KO rat (13) may be due, at least in part, to the decrease in steroidogenesis in the ZG. It is possible that therapy targeted to the adrenal renin-angiotensin-aldosterone system may prove useful in the management of hypertension.

**ACKNOWLEDGMENTS**

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**REFERENCES**


