Renin–Angiotensin System

Estrogen Receptor α Is Required for Maintaining Baseline Renin Expression

Ko-Ting Lu, Henry L. Keen, Eric T. Weatherford, Maria Luisa S. Sequeira-Lopez, R. Ariel Gomez, Curt D. Sigmund

Abstract—Enzymatic cleavage of angiotensinogen by renin represents the critical rate-limiting step in the production of angiotensin II, but the mechanisms regulating the initial expression of the renin gene remain incomplete. The purpose of this study is to unravel the molecular mechanism controlling renin expression. We identified a subset of nuclear receptors that exhibited an expression pattern similar to renin by reanalyzing a publicly available microarray data set. Expression of some of these nuclear receptors was similarly regulated as renin in response to physiological cues, which are known to regulate renin. Among these, only estrogen receptor α (ERα) and hepatic nuclear factor α have no known function in regulating renin expression. We determined that ERα is essential for the maintenance of renin expression by transfection of small interfering RNAs targeting Esr1, the gene encoding ERα, in renin-expressing As4.1 cells. We also observed that previously characterized negative regulators of renin expression, Nr2f2 and vitamin D receptor, exhibited elevated expression in response to ERα inhibition. Therefore, we tested whether ERα regulates renin expression through an interaction with Nr2f2 and vitamin D receptor. Renin expression did not return to baseline when we concurrently suppressed both Esr1 and Nr2f2 or Esr1 and vitamin D receptor mRNAs, strongly suggesting that Esr1 regulates renin expression independent of Nr2f2 and vitamin D receptor. ERα directly binds to the hormone response element within the renin enhancer region. We conclude that ERα is a previously unknown regulator of renin that directly binds to the renin enhancer hormone response element sequence and is critical in maintaining renin expression in renin-expressing As4.1 cells. (Hypertension. 2016;67:992-999. DOI: 10.1161/HYPERTENSIONAHA.115.07082.) • Online Data Supplement

Key Words: enhancer ■ Esr1 ■ hormone response element ■ juxtaglomerular cells ■ renin ■ transcription

As the rate-limiting step of the renin–angiotensin system, expression of the enzyme renin by renal juxtaglomerular cells is tightly regulated by multiple physiological and molecular mechanisms. The renin-expressing As4.1 cell line has been extensively used to identify genomic regions essential for renin expression.1 Two cis elements have been found to be critical in regulating renin transcription: a proximal promoter (+6 to −117 bp) and a classic transcriptional enhancer (−2866 to −2625 bp).2,6 The proximal promoter enhancer contains binding sites for the HOXB9/D10-PBX1b-PREP1 protein complex, C-promoter binding factor 1, and Ets-1 transcription factor.4,5 The core of the ren enhancer is evolutionarily conserved and is required to fully activate renin promoter activity in renin-expressing cells.7–9 Two independent studies reported that mice carrying a deletion of the ren enhancer exhibited diminished baseline renin expression.10,11 Other transcription factors that have been shown to regulate renin in vivo have also been reported.7,12,13

Previous studies have identified many of the transcription factors that bind within the enhancer region. The promoter-proximal end of the enhancer (−2680 to 2625) is essential in maintaining the enhancer activity. This region comprises 4 elements: the cAMP response element (CRE), the E-box, the hormone response element (HRE), and the nuclear factor-Y (NF-Y)–binding site.2,14,15 cAMP response element–binding protein (CREB) and CRE modulator bind to the CRE to positively regulate renin expression.2 Both nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and vitamin D receptor (VDR) can compete with CREB and CRE modulator for the CRE, resulting in decreased renin expression.16,17 Upstream stimulatory factors 1 and 2 (USF-1 and USF-2) bind to the E-box to positively regulate renin expression.2 The HRE, located at the promoter-proximal end of the E-box, consists of 2 TGACCT repeats separated by an atypical 10 bp spacer (DR10). Like the CRE and E-box, the HRE is critical for maintaining enhancer function.14,18 The large spacer between the 2 half-sites suggests that multiple hormone nuclear receptors can potentially associate with that region to regulate renin expression. These characteristics of the HRE drew us to search for transcription factors that bind this element.

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Previous studies revealed that retinoic acid receptor and retinoid X receptor bind to the HRE.\textsuperscript{14} We also reported that 2 orphan nuclear receptors, Nr2f6 and Nr2f2, also bind to the HRE, but unlike retinoic acid receptor/retinoid X receptor, both of these nuclear receptors negatively regulated renin transcription.\textsuperscript{15} These studies led to the conclusion that both positive and negative regulators bind to the HRE. The most promotor proximal–binding site, Ea, matches the canonical binding sequence for NF-Y and partially overlaps with the HRE. Binding of NF-Y sterically prevents proteins from binding to the HRE, resulting in downregulation of renin expression.\textsuperscript{15} Artificially increasing the spacing between the HRE and the NF-Y-binding site restores renin promoter activity.

These previous studies indicate that renin is tightly regulated by several transcription factors. However, these factors do not account for the entirety of renin transcriptional regulation. We hypothesize that there are additional nuclear receptors yet to be identified that are regulators of renin expression. We took an unorthodox approach of searching for nuclear receptors, which exhibit (1) a cell-specific pattern of expression similar to renin in the kidney and (2) a pattern of regulation similar to renin. This analysis leads us to identify estrogen receptor α (ERα) encoded by the Esr1 gene as a regulator of renin expression.

\section*{Methods}
Details of the experiments using mice, transfection of small interfering RNAs (siRNAs) in As4.1 cells, RNA isolation, DNA Affinity Precipitation Assay, Chromatin Immunoprecipitation, Western blotting, and analysis of previously published gene expression microarrays are shown in the expanded Methods section of the online-only Data Supplement.\textsuperscript{20} Care and use of mice followed the standards set forth by the National Institutes of Health guidelines. All procedures were approved by The University of Iowa Animal Care and Use Committee.

\section*{Data and Statistical Analysis}
Data were presented as mean±SEM. The Livak method was used to calculate gene expression fold change.\textsuperscript{21} Two-way repeated measures analysis of variance with Tukey post hoc analyses was used to calculate the statistics of concurrent transfection of 2 siRNAs. \textit{P}<0.05 was considered significant. Data were analyzed by use of SigmaStat (Systat Software).

\section*{Results}
To identify a candidate list of nuclear receptors that potentially regulate renin expression, we analyzed a set of microarray data from the GenitoUrinary Development Molecular Anatomy Project database.\textsuperscript{20} We compared the expression pattern of renin and the 48 nuclear receptors across various kidney cell types, including juxtaglomerular cells, glomerular mesangium, cortex vasculature, renal cortex, ureteric tip, capsule mesenchyme, and adult podocytes. Among all the screened kidney cell types, juxtaglomerular cells exhibited the most enriched renin expression followed by the glomerular mesangium. Similarly, expression of aldo-keto reductase family 1, member 7 (Akr1b7), which was reported to be coexpressed with renin in juxtaglomerular cells throughout murine development, exhibited the most similar pattern of expression to renin (row 2 in Figure 1).\textsuperscript{20}

Unexpectedly, like renin, some of the 48 nuclear receptors exhibited enriched expression in juxtaglomerular cells and had expression patterns similar to that of renin (Figure 1). Included in this set was Thrb, AR, Nr6a1, Esr1, Rorc, Hnf4g, Nr1l2, Ppara, Ppard, Nr1b4, Hnf4α, Esrra, vitamin D receptor (VDR), Nr3c2, and Nr4a1. Many of the set of 48 nuclear receptors are also expressed in As4.1 cells (see yellow column in Figure 1).

We next assessed which of these receptors were coregulated like renin. This was performed by pharmacologically altering renin expression in male C57BL/6 mice. The first cohort of mice underwent captopril treatment for 10 days. Captopril-treated mice exhibited increased fluid intake that peaked 7 to 11 days after treatment (Figure S1 in the online-only Data Supplement). The second cohort of mice was subcutaneously implanted with 50 mg deoxycorticosterone acetate (DOCA) pellet and was given 0.15 mol/L NaCl ad libitum in addition to regular chow and tap water for 21 days (DOCA salt). Kidney weight was elevated in DOCA salt–treated mice as previously reported (Figure S2). As expected, renin expression was markedly elevated (16.0±1.6-fold) in the kidney cortex of captopril-treated mice and was reduced by 80% (0.21±0.02-fold) in DOCA salt mice (Figure 2A).

Consistent with a previous report that Akr1b7 mRNA serves as a marker for renin expression in juxtaglomerular cells,\textsuperscript{20} Akr1b7 expression was positively correlated with renin mRNA level in both experimental cohorts (Figure 2B). Of the nuclear receptors examined, we observed suppressed expression of thyroid hormone receptor β (Thrb), VDR, Nr2f2, hepatocyte nuclear receptor alpha (Hnfα), and Esr1 encoding ERα in the kidney cortex of DOCA salt mice (Figure 2C–2G). The magnitude of the repression was remarkably similar to that of renin and Akr1b7. Unlike the change induced by DOCA salt, expression of these nuclear receptors only modestly increased in response to captopril. Nr2f6 expression exhibited an opposite pattern of expression (Figure 2H). There was no change in the expression of 8 other nuclear receptors in response to captopril or DOCA salt (Figure S3).

Because of their concordance with renin expression, we next examined the possibility of whether these nuclear receptors are regulators of renin expression. Thyroid hormone receptor β is a known positive regulator of renin mRNA.\textsuperscript{22} Multiple studies have shown that VDR, Nr2f2, and Nr2f6 are negative regulators of renin expression.\textsuperscript{16,19,23,24} To date, however, there is no report on the role of Hnf4α and ERα in regulating renin expression. We first determined whether these 2 nuclear receptors are expressed in the renin-expressing As4.1 cells. By real-time quantitative polymerase chain reaction, we were able to detect abundant Esr1 mRNA, but not Hnf4α (Table S1). This was consistent with our prior microarray-based analysis of the gene expression profile of As4.1 cells in which Esr1 displayed a higher expression level compared with Hnf4α (see arrowheads on right margin of Figure 1). Thus, Hnf4α was eliminated from further consideration.

To determine whether ERα is a regulator of renin expression, we used siRNA-mediated gene silencing and investigated whether interference with expression of the Esr1 gene would alter expression of endogenous renin mRNA. Esr1 mRNA expression was reproducibly knocked down by 70%
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(0.31±0.05 versus negative control; Figure 3A). Suppressing expression of Esr1 resulted in a 55% reduction of renin mRNA expression (0.44±0.21 versus negative control; Figure 3B). These data suggest that ERα is required for maximal expression of renin under baseline condition and suggests that ERα is a positive regulator of renin transcription. Surprisingly, treatment of As4.1 with β-estradiol had no effect on the level of Esr1 or renin mRNA after 6, 12, or 24 hours of incubation (Figure S4).

We next asked if ERα protein can bind to the HRE in the renin enhancer because the HRE is a partial inverted sequence of the canonical estrogen receptor response element. First, nuclear extracts from As4.1 cells were mixed with biotin-labeled HRE probes and subjected to a DNA affinity precipitation assay. Western blot was performed to detect eluted proteins. A probe where the critical nucleotides in the HRE were mutated was used as a control.19 ERα protein was detected in proteins eluted from the wild-type probe but not from the mutant probe, signifying that ERα is able to bind to the HRE sequence with high specificity (Figure 4A). Nr2f2 served as the positive control because we showed that it can bind to the HRE in previous studies.19 USF2 which binds to
the neighboring E-box but not the HRE was used as a negative control. Second, we tested whether ERα binds to the renin enhancer in chromatin in As4.1 cells. Chromatin immunoprecipitation showed that ERα antibody was able to pull down ≈30-fold (29.2±7.2) enrichment of the renin enhancer compared with IgG (Figure 4B). USF2, which binds to the neighboring E-Box sequence within the enhancer (included in this assay), was also enriched (9.0±5.6). Importantly, there was no binding of USF2 nor ERα to a region 10 kb upstream of the renin enhancer, providing confidence in the selectivity of the chromatin immunoprecipitation assay. To further validate the specificity of ERα enrichment within the enhancer and the siRNA-mediated knockdown of ERα at the protein level, we performed chromatin immunoprecipitation assay after siRNA-mediated Esr1 knockdown in As4.1 cells. In the negative control siRNA chromatin sample, USF2 antibody was able to pull down approximately a 20-fold (21.5±6.2) enrichment of the renin enhancer sequence; and ERα antibody was able to pull down ≈60-fold (59.7±19.7 of IgG) enrichment of the renin enhancer. However, the ERα chromatin immunoprecipitation signal was significantly reduced after small interfering RNAs targeting Esr1 (siEsr1; Figure 4C).

Because renin expression is tightly regulated by both positive and negative modulators, we considered the possibility that the significant reduction of renin followed by ERα suppression might be attributable to the altered expression of other transcription factors rather than only ERα per se. Thus, we assessed whether expression of other nuclear receptors were altered on siEsr1 transfection. Indeed, siEsr1 augmented expression of Nr2f6, VDR, and farnesoid X receptor (Nr1h4) in siEsr1-transfected cells (Figure 3C–3E). There was no consistent change in the expression of 10 other nuclear receptors in response to both siRNAs targeting Esr1 (Figure S5).
Nr2f6 and VDR are known negative regulators of renin, whereas there is no published information on the role of Nr1h4 in regulating renin expression. This finding led us to test whether the suppression of renin expression, caused by the loss of ERα, is because of the elevated expression of the negative regulators. To accomplish this, we simultaneously transfected siRNAs targeting both Esr1 and Nr2f6 into As4.1 cells, and asked whether suppressing Nr2f6 restored renin expression. In this experiment, the induction of Nr2f6 mRNA by siEsr1 was only modest and not statistically significant (Figure 5A). siRNA targeting Nr2f6 effectively knocked down Nr2f6 expression but did not significantly decrease Esr1 expression. As earlier, siRNA targeting Esr1 alone decreased renin mRNA. As we reported previously, there was a modest induction of renin by Nr2f6 knockdown alone.19 Contrary to our hypothesis, siRNA-mediated knockdown of both ERα and Nr2f6 preserved a decrease in renin expression. In fact, combined suppression of both ERα and Nr2f6 appeared to synergistically suppress renin expression (Figure 5A). Similar results were observed when we knocked down ERα and VDR simultaneously (Figure 5B). Thus, the reduction of renin expression mediated by the loss of ERα does not depend on the induction of Nr2f6 and VDR.

**Discussion**

The main findings from the current study are that (1) contrary to expectation, several nuclear receptor transcription factors exhibit a pattern of gene expression in the developing and adult kidney that mirrors the expression of renin, (2) like renin, expression of 5 nuclear receptors exhibited a marked decrease in expression in response to DOCA salt, (3) unlike renin, none of the same nuclear receptors exhibited an increase in expression in response to angiotensin-converting enzyme inhibition, and (4) ERα, which binds to the HRE within the renin enhancer, is required for baseline expression of renin and acts independent of VDR and Nr2f6 and does not require exogenous estrogen.

Renin expression in juxtaglomerular cells is precisely regulated by both extracellular and intracellular stimuli through the actions of transcription factors, but the entirety of the mechanisms underlying this transcriptional regulation has not been fully elucidated. Detailed molecular studies have clearly defined 2 regions of the renin regulatory region that are required for maximal expression of the renin gene, a proximal promoter and a distal enhancer.1,25 Deleting the distal enhancer in vivo diminishes the level of renin expression and blunts the response to physiological cues regulating expression of the gene.1,25 The enhancer consists of a complex series of linked regulatory elements which both stimulate and inhibit expression of renin, but how these elements act in concert to regulate renin expression in response to physiological cues remains unclear. Major elements of the enhancer (proximal to distal) include a CRE, E-Box, HRE, and a binding site of NF-Y.2,14,15 We originally showed that retinoic receptor α and retinoid X receptor bind to the HRE and regulate renin expression in response to retinoids.14 However, electrophoretic mobility shift assays suggested that many proteins may bind to the sequence. In an effort to identify these proteins, we later performed yeast one hybrid and identified Nr2f2 and Nr2f6 as HRE-binding proteins with the capacity to antagonize enhancer activity.19,21 Attempts to identify other HRE-binding protein which may help explain the mechanisms of renin regulation have not been successful.

The publication of a comprehensive comparison of expression of renin to other genes across the genome in the kidney during development, in adults, and in response to physiological perturbations provided a novel opportunity to search for other transcription factors that are coexpressed, or enriched, in renin-expressing cells.26 Because several members of the nuclear hormone receptor family bind to the HRE to regulate renin, we focused on that family of transcription factors, and adopted a working yet unorthodox hypothesis that nuclear receptors coexpressed with renin, and coregulated like renin, may regulate renin. ERα was selected from among a group of nuclear receptors which appeared to exhibit enriched expression in juxtaglomerular cells and was highly expressed in As4.1 cells. None of these genes were exclusively expressed in juxtaglomerular cells, and each exhibited a varying cell-selectivity of expression. Expression of Esr1 exhibited only a modest increase in expression in the kidney in response to captopril, despite exhibiting increased...
expression in adult juxtaglomerular cells treated with captopril compared with untreated juxtaglomerular cells. It remains unclear whether the relative increase in \( \text{Esr1} \) expression in response to captopril reflects an increase in the level of expression per cell or reflects, like renin, an increase in the number of cells expressing \( \text{Esr1} \). Interestingly, like renin, expression of \( \text{Esr1} \) (and several other nuclear hormone receptors) decreased \( \approx 10 \)-fold in response to DOCA salt. DOCA salt was previously reported to induce the expression of over 2000 genes but decrease expression of only 50 genes in kidney from Wistar rats. 

Whether renin and \( \text{Esr1} \) were among those that were not reported.

siRNAs targeting \( \text{Esr1} \) blunted baseline renin expression. This along with data showing that ER\( \alpha \) has the capacity to bind to the HRE in the renin enhancer in vitro and binds to the HRE under baseline conditions in chromatin in vivo would generally support a conclusion that ER\( \alpha \) is among the many transcriptional regulators of renin. However, this regulation could be indirect, for example, through another transcription factor. Indeed, silencing ER\( \alpha \) also simultaneously increased expression of 2 known negative regulators of renin expression, VDR and Nr2f6. This led us to assess if the decrease in renin mRNA by siEsr1 was mediated by an increase in Nr2f2 or VDR. However, this mechanism was effectively ruled out because simultaneous silencing of ER\( \alpha \) and Nr2f2, or ER\( \alpha \) and VDR, did not restore expression of renin to baseline levels. The only other report we could find in the literature directly linking ER\( \alpha \) to renin gene expression was a study of ER\( \alpha \) receptor deletion in the subfornical organ of female mice, which results in a 1.4-fold increase in renin expression in the lamina terminalis. This suggests that regulation of renin by ER\( \alpha \) may be cell-specific, its expression blunted by ER\( \alpha \) in the brain and stimulated by ER\( \alpha \) in juxtaglomerular cells.

Given our findings, it is interesting to note that estrogen-related receptor \( \alpha \), an orphan member of the nuclear receptor superfamily, was previously reported to regulate renin expression. However, despite previous data showing that inhibition of estrogen-related receptor \( \alpha \) caused a 3-fold increase in renin expression in As4.1 cells, our studies revealed that inhibition of estrogen-related receptor \( \alpha \) with 2 different siRNAs (which decreased expression of estrogen-related receptor \( \alpha \) to

![Figure 5](http://hyper.ahajournals.org/). Esr1 does not regulate renin expression through Nr2f6 or vitamin D receptor (VDR). Expression of the indicated genes after small interfering RNAs targeting Esr1 (siEsr1), Nr2f6 (siNr2f6), or both (n=4, A) or after siEsr1, small interfering RNAs targeting VDR (siVDR), or both (n=3, B). \( * \)P<0.05 compared with negative control (NC) siRNA by 2-way repeated measures analysis of variance (ANOVA; siEsr1 X siNr2f6, or siEsr1 X siVDR). Ren indicates renin.
The identification of ERα as a potential regulator of renin is interesting in light of the effect of estrogen on renin–angiotensin system activity and the long held hypothesis that estrogen deficiency in menopause may play a role in increasing renin–angiotensin system activity. Therefore, it was notable that 17β-estradiol treatment of As4.1 cells had no effect on expression of Esr1 or renin mRNA. Similarly, there was no change in expression of renin mRNA in primary chorionic cells exposed to exogenous estrogen, although the importance of ERα itself was not experimentally tested. This suggests that unless an endogenous ERα ligand is synthesized in As4.1 cells, its activity on renin expression does not require ligand binding. Although ERα-bound ligand is the classical model for transcriptional activation in response to endogenous or exogenous estrogen, estrogen-independent activation of ERα transcriptional activity was reported to occur in breast cancer cells with increased levels of ERα. It was hypothesized that the activation of ERα function in the absence of ligand (termed concentration-inducible) is explained by a conformational change in ERα when the concentration of ERα is high, that looks similar to the conformation of active ERα in the presence of estrogen. Of course, it is unclear if the level of ERα in As4.1 cells, a fully transformed cell line derived from a renal tumor, reaches that level.

In addition to the classical ligand, many nuclear receptors, including ERα, can be activated by a variety of extracellular signals in a ligand-independent manner. For example, ERα can be activated through the cAMP/protein kinase A pathway. cAMP signaling is able to stimulate ERα transcriptional activity via protein kinase A–mediated phosphorylation of coactivator-associated arginine methyltransferase 1, which then associates with ERα, resulting in the recruitment of ERα coactivators and transcriptional activation of ERα target genes. This is interesting when one considers that there is a CRE directly adjacent to the HRE in the renin enhancer, which when mutated causes a complete loss of enhancer activity, coupled with data suggesting that protein kinase A may be constitutively activated in As4.1 cells. Thus, it remains possible that constitutive activity of protein kinase A in these cells signals constitutive activity of ERα to maintain renin expression.

Perspectives

Females usually exhibit a lower blood pressure compared with their age-matched males, at least until menopause at which time blood pressure rises. The link between estrogen levels and renin–angiotensin system activity has been studied extensively. Indeed, it is well established that plasma renin activity increases in women during the luteal phase of the menstrual cycle, a time when plasma estrogen levels are high. Because expression of hepatic and renal angiotensinogen is induced by 17β-estradiol, it comes as no surprise that plasma Ang-II levels also increase with increasing circulating estrogen. It is therefore notable and surprising that despite the known relationship between estrogen and plasma renin activity and Ang-II, and the importance of the postmenopausal increase in blood pressure in women, that there is little information on the regulation of renin gene expression in response to estrogen. Our identification of ERα as a potential regulator of renin gene expression, at least in As4.1 cells, should spark new studies directly assessing the importance of this transcriptional pathway, the relative importance of ligand-dependent and ligand-independent ERα-mediated renin transcription, and whether mechanisms of renin expression through ERα differ before and after menopause when the levels of estrogen markedly differ. These would provide a more comprehensive assessment to determine whether our identification of ERα as a regulator of renin expression plays a role in the cardiovascular protection offered by estrogen before menopause and the increased cardiovascular risk after menopause.

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Disclosures

None.

References

Novelty and Significance

What Is New?

• Several nuclear hormone receptor transcription factors exhibit a pattern of gene expression in the developing and adult kidney that mirrors the expression of renin.
• Like renin, expression of 5 nuclear receptors exhibited a marked decrease in expression in response to deoxycorticosterone acetate salt, but none exhibited an increase in expression in response to angiotensin-convert-
ing enzyme inhibition.
• Estrogen receptor α, which binds to the hormone response element within the renin enhancer, is required for baseline expression of renin and acts independent of vitamin D receptor and Nr2f2 and does not require exogenous estrogen, at least in As4.1 cells.

What Is Relevant?

• Nuclear hormone receptors are known to regulate expression of the renin gene, but which receptors bind to the renin enhancer remains unclear.

Summary

Our current studies identify that ESR1 encoding estrogen receptor α exhibits an expression pattern enriched in renin-expressing cells in the kidney and is required for baseline expression of renin in As4.1 cells, a cellular model for juxtaglomerular cells.
Estrogen Receptor α Is Required for Maintaining Baseline Renin Expression
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Supplemental Materials

1. Detailed Methods
2. Supplemental Figures S1-S5
3. Supplemental Table S1
Supplemental Methods

Animal: Two cohorts of male, C57BL/6 mice, six-weeks of age underwent the following treatments: angiotensin converting-enzyme inhibitor (captopril) and deoxycorticosterone acetate (DOCA)-salt, respectively. In the first cohort, the experimental group received 100g/kg/day captopril in drinking water for 10 days. Both groups were maintained at standard chow. The second cohort of mice were anesthetized by isoflurane inhalation to have 50-mg of DOCA subcutaneously implanted (21-day release). During this time, they were maintained on standard chow and had ad libitum access to both tap water and 0.15 mol/L NaCl drinking solution.

Gene Expression Microarray Analysis: To compare the expression pattern of renin and the 48 nuclear receptors across various kidney cell types, we downloaded the publicly available Mouse Developing Kidney ST microarray dataset from the GenitoUrinary Development Molecular Anatomy Project (GUDMAP). To compare a particular gene across different cell types and to detect genes displaying similar expression patterns, the raw microarray signals were normalized for each gene by conversion into z-scores. The resulting data was then clustered using Cluster 3.0 and visualized by creating a heatmap with the matrix2png program. To determine if a nuclear receptor gene from the above analysis was also expressed in As4.1 cells, a microarray dataset GSE14243 (available from NCBI-GEO) previously generated in our laboratory was used. The presence or absence of gene expression in this dataset was determined using the Affymetrix MAS5.0 algorithm.

siRNA Transfection and RNA isolation: ERα experiments: As4.1 cells (ATCC CRL2193) were sub-cultured and were allowed at least 12 hours to attach and grow in 37°C, 5% CO2 incubator. A mixture of siRNA (100 μmol/L, Integrated DNA Technologies, Coralville, IA), HiPerfect reagent (310705, Qiagen) and DMEM serum-free media was added to the cells. After 8 hours of transfection, complete DMEM (10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin) was added. Forty-eight hours following the initial siRNA incubation total RNA was isolated by using spin columns (RNeasy Plus Kit, Qiagen). In some experiments, two siRNA duplexes were used at 100 μmol/L each; thus, a total of 150 μmol/L of siRNA duplexes was transfected into the cells. The catalog numbers for the siRNAs used are as follows: siEsr1 #1 (MMC.RNAI.N007956.12.1), siEsr1#2 (MMC.RNAI.N007956.12.2), siNr2f6 #1 (MMC.RNAI.N010150.12.1), siNr2f6 #2 (MMC.RNAI.N010150.12.2), and siVDR (MMC.RNAI.N009504.12.1). Using this high concentration of siRNA, we did not observe any morphological change or cell death. 500ng of RNA was reversed transcribed using 1U of Superscript III (Invitrogen) in 20μl reactions. Reactions were incubated at 50°C for 5 minutes, 4°C for 3 minutes, 55°C for 45 minutes and finally at 70°C for 15 minutes to inactivate the reaction. The cDNA was diluted at 1:40, and gene expression was measured using SYBR Green Gene Expression Master Mix (4309155, Applied Biosystems). Renin primers (For – 5’-TGA AGA AGG CTG TGC GGT AGT-3’; Rev- 5’- TCC CAG GTC AAA GGA AAT GTC-3’), Esr1 primers (For- 5’-CCA CAC ATT TAC CTT GAT TCC TG-3’; Rev- 5’–TGG CTG GAG ATT CTG ATG ATT G-3’), VDR primers (For- 5’- CCG GTT GTC CTT GGT GAT G-3’; Rev- 5’- TCA ACG CTA TGA
CCT GTG AAG-3') were used to assess their level of expression after siRNA transfection. GAPDH primers (For- 5’-CAT GGC CTT CCG TGT TCC TA-3’; Rev- 5’-GCG GCA CGT CAG ATC CA-3’) were used as the internal control. Data were analyzed using the 2-ΔΔCt method to calculate fold-changes relative to the negative control sample.\(^5\)

ERR\(\alpha\) experiments: As4.1 cells (ATCC CRL2193) were sub-cultured and were allowed at least 12 hours to attach and grow in 37°C, 5% CO2 incubator.\(^4\) A mixture of ERR\(\alpha\) siRNA (30 nmol/L, MMC.RNAI.N007953.12.1 or MMC.RNAI.N007953.12.1, Integrated DNA Technologies, Coralville, IA), 8 μl HiPerfect reagent (310705, Qiagen) and DMEM serum-free media was added to the cells. After 8 hours of transfection, complete DMEM (10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin) was added. Forty-eight hours following the initial siRNA incubation total RNA was isolated by using spin columns (RNeasy Plus Kit, Qiagen). We did not observe any morphological change or cell death. 500ng of RNA was reversed transcribed as previously described. The cDNA was diluted at 1:40, and gene expression was measured using SYBR Green Gene Expression Master Mix (4309155, Applied Biosystems). Renin primers (For – 5’-TGA AGA AGG CTG TGC GGT AGT -3’; Rev- 5’-TCC CAG GTC AAA GGA AAT GTC-3’), ERR\(\alpha\) primers (For- 5’-GGC CAC TCT CTG TGA CCT TTT-3’; Rev- 5’-CAG CAC TTC CAT CCA CAC AC-3’) were used to assess their level of expression after siRNA transfection. Mouse \(\beta\)-actin was used as the internal control. Data were analyzed using the 2-ΔΔCt method to calculate fold-changes relative to the negative control sample.\(^5\)

\(\beta\)-Estradiol Treatment: As4.1 cells were sub-cultured in DMEM/F12 Phenol Red-free media (because phenol red may act as a weak estrogen) and were allowed at least 12 hours to attach and grow in 37°C, 5% CO2 incubator.\(^6\) Cells were starved for an hour before treatment. \(\beta\)-Estradiol (Sigma, E2758) was solubilized in 100% ethanol, as recommended by the manufacturer. Cells were treated at the final concentration 100 nmol/L.\(^7\) RNA was isolated using spin columns (RNeasy Plus Kit, Qiagen) after 6 hours, 12 hours, and 24 hours of treatment, respectively. Reverse transcription reaction was performed on 500 ng of the isolated RNA as described before. Expression of Esr1 and renin was then assessed using the primers stated above. Data were analyzed using the 2-ΔΔCt method to calculate fold-changes relative to the negative control sample.

DNA Affinity Precipitation Assay: DNA affinity purification assays (DAPA) were carried out using two biotin-TEG 5’-labeled double stranded DNA probes (WT/5’-BioTEG/CAA AAC TGC AGA CGA CGA GTG TGA CCT GGC TGT ACT CTG ACC TCT CAG AT-3’; \(\mu bc\)/5’-BioTEG/ CAA AAC TGC AGA CGA GTG GTC TAC GGC TGT ACT CGT CTA CCT CAG AT-3’).\(^4\) Eighty pmol/L of double-stranded probe was mixed with 50μl of streptavidin-conjugated Dynabeads MyOne C1 (Invitrogen) for at least 3 hours at room temperature. The streptavidin-conjugated beads were blocked with 0.05 mg/ml BSA and 0.05mg/ml glycogen for at least 1 hour at room temperature to reduce non-specific binding. Nuclear extracts from As4.1 cells (100μg) were then incubated with the double-stranded labeled probes while rotating at 4°C overnight. Beads were collected using a DynaMag-2 magnet (12321D, Invitrogen) and washed three times with binding buffer. The eluted extracts were subjected to Western blotting.
**Western Blotting:** Proteins were separated using 10% SDS-PAGE gel and then transferred to PVDF membrane (Millipore) overnight at 4°C, 37 Volts. The membrane was then blocked with 5% non-fat milk for 1 hour at room temperature. Blots were probed with antibodies detecting Esr1 (sc-543X, Santa Cruz Biotechnology), Nr2f2 (PP-H7147-00, Perseus Proteomics), and USF2 (sc-861X, Santa Cruz Biotechnology). All antibodies were diluted at 1:5000 in 5% non-fat milk. The blot was washed with TBS with 0.05% Tween-20 for three times. Anti-rabbit IgG Horseradish Peroxidase (NA-934, GE Healthcare Life Science) was diluted at 1:10000 in 5% non-fat milk for 1 hour at room temperature for both Esr1 and USF2 blots. On the other hand, secondary anti-mouse IgG Horseradish Peroxidase (NA931, GE Healthcare Life Science) was used for Nr2f2 blots. The signal was visualized using chemiluminescence (GE Healthcare Life Science) to develop the blot. The blot was exposed for 2 minutes in the dark room before processing.

**Chromatin Immunoprecipitation:** As4.1 cells were fixed for 8 minutes with 1% formaldehyde and quenched with 0.125 mol/L glycine for 5 minutes. Cells were then washed twice with phosphate buffered saline (PBS), collected by scraping and centrifugation, then lysed with 3 ml of lysis buffer (0.15 mol/L NaCl, 0.01 mol/L HEPES pH 7.4, 0.0015 mol/L MgCl2, 0.01 mol/L KCl, 0.5% NP-40, 0.0005 mol/L DTT). Nuclei were collected by centrifugation and were then resuspended in nuclear lysis buffer (0.05 mol/L NaCl, 0.0167 mol/L Tris pH 7.5, 0.0033 mol/L EDTA, 1% SDS). Nuclei were diluted with 2 volumes of chromatin immunoprecipitation (ChIP) dilution buffer (0.15 mol/L NaCl, 0.0167 mol/L Tris pH 7.5, 0.0033 mol/L EDTA, 1% Triton X-100, 0.1% SDS, 0.5% Na-Deoxycholate). Chromatin was sonicated using the model 250 Branson Scientific Sonic Dismembrator at amplitude of 30% for 20 cycles of a 10-s pulse with 20-s rest between each pulse. 5μg of USF2, Esr1, and IgG antibody was incubated respectively with Protein G magnetic beads (10003D, Invitrogen). Chromatin was then subjected to immunoprecipitation to the antibodies bound with Protein G magnetic beads. Precipitated chromatin was eluted by resuspending the cells in Elution Buffer (100 μl of 1.0 mol/L Tris pH 8.0, 40 μl of 0.5 M EDTA pH 8.0, 200 μl of 10% SDS, 1660 μl Autoclaved DI H2O) and incubating at 65°C for 30 minutes. The eluent was then incubated at 65°C overnight to reverse the crosslinks. Chromatin was treated with RNaseA and Proteinase K. DNA was column purified (PCR Purification Kit, Qiagen). qPCR was performed with the purified DNA using primers amplifying renin enhancer region (5’- TTG GAC CCT CTC CAT TCC TTC ACG-3’, 5’-ATG CGC TAT CAC AAC CAG CCA CTC-3’) and a region 10 kb upstream of the enhancer as the negative control (5’-ACA GAA GGA GGT CGG AAG AC-3’, 5’-ACA GAA GGA GGT CGG AAG AC-3’). Data were analyzed using the 2-ΔΔCt method to calculate fold-changes relative to IgG immunoprecipitated samples.
Supplemental References


**Figure S1:** Daily fluid intake of captopril-treated and control mice throughout the 10-day treatment period (n = 5, * = p < 0.05, p < 0.001 = Treatment x Day, Two-Way Repeated Measure ANOVA Analysis).
Figure S2: Kidney weight and body weight ratio of DOCA-salt treated and control mice (n = 6, * = p < 0.05, One-Way ANOVA Analysis).
**Figure S3:** Nuclear receptors expression in both captopril-treated and DOCA-salt mice.
Figure S4: Esr1 and renin expression after 17-β Estradiol treatment 6, 12, and 24 hours respectively (n =3). There were no significant differences comparing vehicle to Estradiol.
Figure S5: Expression of nuclear receptors in response to the suppression of Esr1 expression. *, P<0.05 vs NC.
<table>
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<th>Genes</th>
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**Table S1**: Quantitative PCR and microarray data assessing the expression level of Esr1 and HNF-alpha in As4.1 cells. Values are CT shown in the presence and absence of reverse transcriptase (RT).