Regulation of Renin Gene Expression by Oxidative Stress

Hana Itani, Xuebo Liu, Ehab H. Sarsour, Prabhat C. Goswami, Ella Born, Henry L. Keen, Curt D. Sigmund

Abstract—Increased arterial pressure, angiotensin II, and cytokines each result in feedback inhibition of renin gene expression. Because angiotensin II and cytokines can stimulate reactive oxygen species production, we tested the hypothesis that oxidative stress may be a mediator of this inhibition. Treatment of renin-expressing As4.1 cells with the potent cytokine tumor necrosis factor-α caused an increase in the steady-state levels of cellular reactive oxygen species, which was reversed by the antioxidant N-acetylcysteine. Exogenous H$_2$O$_2$ caused a dose- and time-dependent decrease in the level of endogenous renin mRNA and decreased the transcriptional activity of a 4.1-kb renin promoter fused to luciferase, which was maximal when the renin enhancer was present. The effect of H$_2$O$_2$ appeared to be specific to renin, because there was no change in the expression of β-actin or cyclophilin mRNA or transcriptional activity of the SV40 promoter. The tumor necrosis factor-α–induced decrease in renin mRNA was partially reversed by either N-acetylcysteine or panepoxydone, a nuclear factor κB (NFκB) inhibitor. Interestingly, H$_2$O$_2$ did not induce NFκB in As4.1 cells, and panepoxydone had no effect on the downregulation of renin mRNA by H$_2$O$_2$. The transcriptional activity of a cAMP response element-luciferase construct was decreased by both tumor necrosis factor-α and H$_2$O$_2$. These data suggest that cellular reactive oxygen species can negatively regulate renin gene expression via an NFκB-independent mechanism involving the renin enhancer and inhibiting cAMP response element–mediated transcription. Our data further suggest that tumor necrosis factor-α decreases renin expression through both NFκB-dependent and NFκB-independent mechanisms, the latter involving the production of reactive oxygen species. (Hypertension. 2009;53:1070-1076.)

Key Words: renin ■ hydrogen peroxide ■ oxidative stress ■ transcription ■ gene regulation

Renin is the rate-limiting step in the catalytic processing of angiotensinogen to angiotensin (Ang) I, which is further hydrolyzed to Ang II and Ang 1-7. Renin is tightly regulated at the transcriptional, posttranscriptional, and translational levels, and its secretion is controlled by a multitude of physiological cues. The cues are derived from both systemic and local signals, including the sympathetic nervous system, circulating and tissue Ang peptides, endocrine factors, NO, and cytokines. One hallmark of renin regulation in vivo is negative feedback, which serves to tightly regulate renin expression and release in response to Ang II, NaCl at the macula densa, and renal perfusion pressure.1 The mediators of negative feedback have been the subject of extensive investigation, but the mechanisms remain incompletely understood.

Ang II can stimulate cytokine production in a variety of cell types, and cytokines are among the inhibitory signals regulating renin transcription.2-5 The cytokine tumor necrosis factor (TNF)-α, was reported to be required to mediate the drinking and pressor responses to Ang II and is a strong negative regulator of renin expression.3,6-8 Like Ang II, cytokines can stimulate the production of reactive oxygen species (ROS) and cause oxidative stress.9 Interestingly, decreasing oxidative stress in the spontaneously hypertensive rat decreases blood pressure and increases plasma renin activity.10 On the basis of these observations, we considered the hypothesis that TNF-α may cause production of ROS and that ROS may negatively regulate renin expression. We used As4.1 cells, an in vitro model of juxtaglomerular cells that express endogenously renin mRNA, to test this hypothesis.11 A previous study reported that production of cellular ROS can be induced in As4.1 cells.12

We show that TNF-α stimulates ROS production, and its inhibitory effects on renin expression can be partially reversed by an antioxidant. We further show that H$_2$O$_2$ decreases renin expression and renin promoter activity through a nuclear factor κB (NFκB)–independent, but a cAMP response element (CRE)–dependent, mechanism.

Materials and Methods

Cell Culture, Transient Transfection, and Luciferase Assay

As4.1 cells are available through the American Type Culture Collection (CRL2193). Cells were plated 24 hours before transfec-
tion in DMEM containing 10% FBS and penicillin-streptomycin (Gibco). Before transfection, cells were switched to 1% FBS, then transiently transfected with a master mix containing a luciferase (LUC) reporter vector using FuGENE-6 (Roche). After 5 hours, cells were split by trypsin and plated into 6-well plates. Increasing doses of H$_2$O$_2$ were added to the medium 24 hours after transfection. The cells were harvested after 48 hours, lysed, and LUC activity was determined using the Dual-Luciferase Reporter kit (Promega). RSV-LUC and pRLSV40 (Promega) were used as positive and internal controls, respectively. Luciferase activity was normalized to renilla and to total cellular protein and then calculated as a percentage of the RSV promoter activity. Luciferase activity assays in each experiment were performed in duplicate, and the average of the 2 readings represented 1 data point. As4.1 cells were treated with 0.15 mmol/L of xanthine and 0.2 U of xanthine oxidase (Sigma) in medium (1% FBS). Details on the transcriptional blockade are provided in the online data supplement (available at http://hyper.ahajournals.org).

**Plasmids and Vectors**

The LUC reporter vectors m2.6, mE2.6, 4.1, and 4.1-μ hormone response element (HRE) were described previously. Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis kit (Stratagene) and confirmed by DNA sequencing. Additional information is available in the online Data Supplement. TA-Luc and CRE-Luc reporter vectors were from Panomics. The vector Ad.NFκB.Luc was described previously.

Infection of Ad.NFκB.Luc was performed in DMEM containing 1% FBS at 200 and 500 multiplicities of infection in As4.1 and MCF-7 cells, respectively. Twenty-four hours postinfection, cells were stimulated with 1 ng/mL of interleukin (IL) 1β, 10 ng/mL of TNF-α, and 200 μmol/L of H$_2$O$_2$. Cells were lysed for measurement of LUC activity 6 hours posttreatment.

**RNA Isolation and RNase Protection Assay**

Total RNA was extracted using the Qiagen RNeasy kit. T3 RNA polymerase was used to prepare antisense RNA for RNase protection probes. The protected fragments were 326 nucleotides for mouse REN mRNA, 105 bases for cyclophilin, 249 bp for β-actin, and 80 bp for 18S. RNase protection was performed using the RPAIII kit (Ambion Inc). Protected fragments were quantified with a Phosphorlmager (GE Healthcare).

**Detection of Cellular ROS Levels**

Dihydrothidium (DHE) and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were used to measure the levels of cellular ROS. DHE fluorescence was used to measure the superoxide, whereas DCFH fluorescence was used to measure H$_2$O$_2$. As4.1 cells were washed with Hanks’ buffer salt solution and labeled with 10 μmol/L of DHE (Invitrogen) for 45 minutes and DCFH-DA (Invitrogen, Molecular Probes) for 15 minutes at 37°C in the dark. Cells were trypsinized with ice-cold phenol-free trypsin/EDTA. Trypsin was inactivated with medium containing 10% FBS. Cells were collected and resuspended in Hanks’ buffer salt solution. Samples were analyzed using flow cytometry with a 488-nm excitation laser. DHE was detected by a 585-nm band pass emission filter, and DCFH was detected using a 530-nm band pass emission filter, as described previously. Mean fluorescence intensity was analyzed using FlowJo software (Tree Star). Samples were corrected for autofluorescence using unlabeled cells with dimethyl sulfoxide.

**Microarray Analysis**

Microarray analysis was described in the online Data Supplement. The complete data set is available at the Gene Expression Omnibus at the National Center for Biotechnology Information (series accession: GSE14243).

**Statistical Analysis**

Data are presented as mean±SEM. Group comparisons of the data were accomplished by 1-way ANOVA with Bonferroni correction for multiple testing or by Student t test, as appropriate, using SigmaStat (SPSS Scientific).

**Results**

As reported previously, TNF-α markedly attenuates the level of endogenous renin mRNA and transcriptional activity of the renin promoter in As4.1 cells (Figure S1). We next measured the steady-state levels of cellular ROS in response to TNF-α in As4.1 cells (Figure 1). Antimycin A (10 μmol/L), an inhibitor of mitochondrial electron transport, augmented ROS (superoxide) levels in As4.1 cells. TNF-α resulted in a time-dependent increase in both DHE and DCFH fluorescence. The specificity of the DCFH assay for detection of cellular ROS was confirmed by the decrease in fluorescence caused by both H$_2$O$_2$ and TNF-α in the presence of the antioxidant N-acetylcysteine (NAC).

Because H$_2$O$_2$ is a diffusible ROS and, therefore, a potential paracrine factor in the regulation of renin expression, we focused on H$_2$O$_2$. There was no decrease in cell viability (measured by trypan blue exclusion) when As4.1 cells were treated for 24 hours with 200 μmol/L of H$_2$O$_2$. Treatment of As4.1 cells with H$_2$O$_2$ caused a dose-dependent (Figure 2) and time-dependent (Figure S2) decrease in the levels of endogenous renin mRNA. Notably there was no change in the level of cyclophilin or β-actin mRNA, indicating that the response was specific to renin.

**Figure 1.** TNF-α increases H$_2$O$_2$ in As4.1 cells. Mean fluorescence intensity of DHE (A) and DCFH (B and C) by flow cytometry. Samples were treated with TNF-α (10 ng/mL) for the time indicated. C, As4.1 cells were pretreated for 2 hours with the indicated amount of NAC before 16-hour incubation with H$_2$O$_2$ (200 μmol/L) or TNF-α (10 ng/mL). Control is nonstimulated, representing the background of the assay. V indicates vehicle; A, antimycin A; *P<0.05 vs control; †P<0.05 vs H$_2$O$_2$ or TNF-α without NAC.
We determined whether the renin mRNA response to H₂O₂ could be attributed to a decrease in renin promoter activity. As4.1 cells were transfected transiently with 7 constructs carrying different lengths of the mouse renin promoter, with or without the enhancer, or containing mutations in critical transcription factor binding sites (Figure 3). Constructs carrying the renin enhancer were much more active transcriptionally than constructs lacking the enhancer (compare 2.6LUC with mE2.6LUC and 4.1LUC). Mutation of the CRE, HRE, or the HoxD10 binding site markedly attenuated promoter activity (Figure 3A). H₂O₂ caused a greater reduction in transcriptional activity of the renin promoter in constructs carrying the enhancer (reduced to 18% to 21% of baseline) than in constructs lacking the enhancer (reduced to 43% to 47% of baseline) or carrying mutations in critical elements in the enhancer or promoter (reduced to 33% to 48% of baseline; Figure 3B). There was no effect of H₂O₂ on the activity of the renilla LUC control or the SV40 promoter/enhancer (pGL2C) validating the selectivity of the response.

The antioxidant NAC by itself had no effect on renin mRNA levels (Figure 4). However, NAC blunted the attenuation of renin mRNA by TNF-α, suggesting that a portion of the TNF-α response is attributable to ROS. Because TNF-α can inhibit renin expression through NF-κB, and H₂O₂ was reported to induce NF-κB, we determined whether H₂O₂ acted through NF-κB in As4.1 cells. NF-κB activity was assessed using an adenovirus containing a LUC reporter driven by 4 repeats of the NF-κB consensus site (Figure 5). TNF-α and H₂O₂ each induced NF-κB transcriptional activity in MCF7 cells, thus replicating previous results and validating the assay. TNF-α induced NF-κB transcriptional activity in As4.1 cells. Unexpectedly, H₂O₂ (200 μmol/L or 1 mmol/L) failed to induce NF-κB-mediated transcription in As4.1 cells, suggesting that the H₂O₂ portion of the TNF-α response may be NF-κB independent.

To gain additional evidence of specificity, we examined the global gene expression response to H₂O₂ by microarray analysis of As4.1 cells. Of 22 000 genes interrogated by the microarray, ~11 500 were expressed in As4.1 cells. There was no change in expression of any of the housekeeping genes, and few other genes displayed a change in expression.

**Figure 2.** Effect of H₂O₂ on endogenous Ren expression. Representative RNase protective assay of RNA from As4.1 cells treated with increasing doses of H₂O₂ (24 hours). The positions of the mouse renin, cyclophilin, and β-actin products are indicated. The graph quantifies multiple independent experiments, respectively (n=6). *P<0.05 vs nontreated. M indicates marker.

**Figure 3.** Effect of H₂O₂ on Ren promoter activity. A, Inset, Schematic of the constructs used. The extent of the promoter is indicated by the black line. The gray box indicates the proximal promoter, whereas the crosshatched box indicates the enhancer. Transcriptional activities of pGL2 basic (pGL2B), pGL2 control (pGL2C), or the indicated renin promoter construct transfected transiently into As4.1 cells and treated with increasing doses of H₂O₂. M indicates the presence of mutations in the CRE, HRE, and HoxD10 motifs. B, The effect of 200 μmol/L of H₂O₂ shown as the reduction to the percentage of baseline for each construct is shown (n=6). E2.6 indicates mE2.6; uC, μCRE; uH, μHRE; uX, μHoxD10; *P<0.05 vs 117P and 2.6P.
(43 and 86 genes were decreased or increased, respectively, ≥2-fold in response to 200 μmol/L of H₂O₂). Like TNF-α, IL-1β stimulated NFκB transcriptional activity in both MCF7 and As4.1 cells (Figure 5). We next queried genes of which the expression was induced strongly by IL-1β but not by H₂O₂. The expression of 34 genes satisfied these criteria; and 25 of them are known or proposed NFκB target genes (Tables S1 and S2). This supports the conclusion that, unlike IL-1β and TNF-α, H₂O₂ does not induce NFκB activity in As4.1 cells.

To functionally validate the independence of H₂O₂ from NFκB, we treated As4.1 cells with the NFκB inhibitor panepoxydone. Although panepoxydone partially reversed the effect of TNF-α on renin mRNA, it had no effect on H₂O₂-mediated downregulation of renin mRNA (Figure 6). A second independent NFκB inhibitor (6-amino-4-[4-phenoxyphenylethylamino]quinazoline) also failed to reverse H₂O₂-mediated downregulation of renin mRNA (data not

Figure 4. TNF-α–induced decrease in Ren expression involves H₂O₂. Top, Representative RNase protective assay using RNA isolated from vehicle and 10 ng/mL of TNF-α (16 hours)–treated cells. Cells were pretreated with the concentration of NAC indicated. The positions of the mouse renin and cyclophilin-protected products are indicated. Bottom, Independent assays were quantified (n=6). *P<0.01 vs untreated. †P<0.01 vs TNF-α.

Figure 5. H₂O₂ does not induce NFκB transcriptional activity in As4.1 cells. As4.1 cells and MCF-7 cells were infected with 200 multiplicities of infection and 500 multiplicities of infection of Ad.NFκBBLuc. Cells were treated with 1 ng/mL of IL-1β, 10 ng/mL of TNF-α, 200 μmol/L or 1 mmol/L of H₂O₂ (n=3) 24 hours postinfection. Cells were harvested 6 hours later to quantify transcriptional activity. The relative light units for TNF were multiplied by 50 to match the scale.
shown). TNF-α has been reported to block renin promoter activity by interfering with the interaction of cAMP response element binding (CREB) with the renin enhancer CRE, and, indeed, TNF-α blunts CRE-dependent transcriptional activity of a construct containing 3 copies of a CRE (Figure 7A). Similarly, H2O2 blunted both basal- and forskolin-induced, CRE-dependent transcriptional activity (Figure 7B).

**Discussion**

The major findings of our study are as follows: (1) TNF-α induces the production of ROS; (2) H2O2 can negatively regulate endogenous renin mRNA and renin promoter activity through a mechanism requiring transcription factor–binding sites in the renin enhancer and promoter; and (3) the negative influence of H2O2 on renin expression occurs independent of NFκB activation but may act by modulating the activity of CREB. Our data further suggest that TNF-α inhibits endogenous renin expression and renin promoter activity in As4.1 cells and blunts cAMP-mediated induction of renin expression in isolated native juxtaglomerular cells.8 The physiological importance of TNF-α as a regulator of renin expression is evidenced by the observation that renal renin expression is significantly increased in TNF-α-deficient mice.8 Todorov et al3,7 have reported that the mechanism of TNF-α–mediated inhibition of renin expression involves an NFκB-dependent decrease in the binding of CREB to the renin enhancer and a decrease in NFκB p65 transcriptional activity at the CRE, which the authors proposed is a noncanonical NFκB binding site. Our data suggest there is also an NFκB-independent component to renin expression with concomitant increases in several cytokines; and adenoviral overexpression of oncostatin M suppresses renal renin mRNA.4 Several cytokines blunt the activity of the renin promoter in As4.1 cells via a mechanism involving the renin enhancer.19 The renin gene enhancer consists of a close clustering of evolutionarily conserved transcription factor binding sites that strongly stimulates transcriptional activity of the renin promoter in vitro and is required for the activity of the renin promoter in vivo.20–23 Like other cytokines, TNF-α inhibits endogenous renin expression and renin promoter activity in As4.1 cells and blunts cAMP-mediated induction of renin expression in isolated native juxtaglomerular cells.8

It is known that cytokines are potent modulators of renin expression (reviewed in Reference 18). Systemic inflammation caused by lipopolysaccharide results in decreased renal

![Figure 8. Converging pathways regulating renin expression by CREB. A schematic of the renin gene showing the location of the proximal promoter (PP) and the mouse enhancer (mE). An expansion of the distal 55 nucleotides of the enhancer is shown identifying the major transcription factor binding sites identified through functional studies. TNF-α, H2O2, and vitamin D3 (through the vitamin D receptor [VDR]) all repress transcription through mechanisms converging on CREB.](http://hyper.ahajournals.org/attach/hypertension/64/1/1074H.pdf)
the TNF-α response that acts through production of ROS and oxidative stress. This is consistent with our data showing a partial reversal of TNF-α-mediated inhibition of renin expression after either panepoxydone (NFκB dependent) or NAC (NFκB independent) and the production of ROS by TNF-α. Further support for an NFκB-independent effect comes from our data showing that H₂O₂ decreases renin expression (and renin promoter activity) but does not activate NFκB activity in As4.1 cells. This is not unique to As4.1 cells, because H₂O₂ does not activate NFκB or the expression of the NFκB target gene ICAM-1 in endothelial cells.²⁴

Our data also suggest that H₂O₂ may interfere with CREB/CRE-mediated transcription in As4.1 cells. CREB activity has been reported to be increased by ROS, and there is evidence that the DNA binding activity of CREB can be modulated by the redox state of cysteine residues in its DNA binding domain.²⁵ H₂O₂ activates CREB activity in some cells types but not in others. In HEK293 cells, H₂O₂ increases phosphorylation of CREB at sites other than the canonical Ser-133, causing a loss of transcriptional activity and decreased binding of CREB with CREB-binding protein.²⁶ Similarly, in cultured neurons, oxidant stress-induced increases in lipid peroxidation caused an increase in phosphorylated CREB but a concomitant decrease in CREB-dependent activity of the brain-derived neurotrophic factor promoter.²⁷ H₂O₂-induced Ser-133 phosphorylation of CREB in T cells was correlated with decreased transcriptional activity in response to activation of the T-cell receptor.²⁸ Therefore, oxidative stress–mediated decreases in CREB transcriptional activity are not unique to renin-expressing As4.1 cells.

A functional CRE sequence is located 5′ of the closely linked E-box and HRE in the renin gene enhancer, the mutation of which abolishes enhancer activity (Figure 8).²⁹ Forskolin increases the association of acetylated histone H4 with chromatin at the renin enhancer CRE.²⁹ Recent studies show that coactivators of CREB are required for the maintenance of renin cell identity and renin expression.³⁰ Although the CRE is required for enhancer activity, mutation of the CRE markedly attenuates the negative effect of cytokines on renin enhancer/promoter function, suggesting that this essential transcription factor–binding site is also important for cytokine-mediated inhibition.³¹ Vitamin D3 has also been shown to decrease renin expression, and targeted expression of vitamin D receptor in juxtamedullary cells in vivo decreases renin mRNA.³²,³³ It was reported that the ligand-occupied vitamin D receptor interacts with CREB, thus blocking its ability to bind to the renin enhancer CRE.³³ Our data suggest that ROS may interfere with CRE-mediated transcription in As4.1 cells. Consequently, all of these data suggest that CREB may be a convergence point for physiological signals that regulate renin synthesis (Figure 8).

Perspectives

Increased Ang II and arterial pressure can each decrease renin expression and renin release in animal models. Although seemingly paradoxical, decreased plasma renin activity is not uncommon in essential (nonrenovascular) hypertension, presumably because mechanisms remain intact that cause feedback inhibition of renin synthesis and release.³⁴ Inflammatory cytokines and Ang II can promote ROS formation, and inflammation is a likely contributor to end organ damage in hypertension. Secretion and expression of TNF-α in peripheral blood monocytes in response to inflammation are higher in hypertensive than in normotensive subjects.³⁵ Supporting the hypothesis that ROS may be one of the mediators of feedback inhibition of renin expression are data from the spontaneously hypertensive rat model of hypertension. In that model, a Tempol-induced decrease in oxidative stress reduced blood pressure but increased plasma renin activity.³⁶ Although it was not directly tested, we propose that this may occur in response to relief of negative feedback inhibition on renin. Our study provides a potential molecular link between oxidative stress, caused by cytokines, and vasopressor substances, eg, Ang II in hypertension, with the regulation of renin gene expression. However, proving this will require additional in vivo studies where the effects of oxidative stress and confounding variables, eg, arterial pressure, can be carefully controlled.

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Disclosures

None.

References

9. De Keulenaer GW, Alexander RW, Uschio-Fukai M, Ishizaka N, Griendling KK. Tumor necrosis factor alpha activates a p22phox-based...


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DATA SUPPLEMENT

Regulation of Renin Gene Expression by Oxidative Stress

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Supplemental Methods

Transcriptional Blockade. To block transcription, As4.1 cells were independently treated with 200 µM \( \text{H}_2\text{O}_2 \) or 2 µM actinomycin D (AMD). The rate of renin mRNA turnover in response to transcriptional inhibition by AMD was measured. Cells were pretreated with or without \( \text{H}_2\text{O}_2 \) for 8 hours prior to the addition of AMD and renin mRNA abundance was quantified at 0, 3, 6, 9, 12 and 24 hours.

Mutant Constructs. The mutants 4.1-µCRE and 4.1-µHOXD10 were generated with the oligonucleotides: (mutated nucleotides underlined), 

\[
5' - CTGTAATCCCTCCCAATG\underline{TGT}TCACTAAACCACGCAGATG\underline{T}GACC-3' \text{ and } 5' - CCCACAGG\underline{C}CCCTGGGGGTA\underline{A}GAACG\underline{C}AAAGCAGGAGCCTTG\underline{A}TAC-3',
\]

Microarray Analysis. For the microarray hybridizations, two separate biological replicates from each experimental group were used. All the microarray procedures were conducted at the University of Iowa DNA Core facility using standard Affymetrix protocols. In brief, approximately 5 µg of total RNA was used as input to a one-step amplification procedure to generate biotin-labeled RNA fragments for hybridization to the Affymetrix GeneChip Mouse Genome 430 2.0 array. This whole-genome expression array contains 45,101 probe sets interrogating approximately 22,000 distinct genes. Raw microarray data was normalized using the Robust Multi-array Average (RMA) method as described by Irizarry et al {1}, and present/absent calls were made using the Affymetrix MAS 5.0 algorithm (http://www.affymetrix.com). Probesets considered “absent” were excluded from further analysis. For genes associated with multiple probesets, the probeset having the highest intensity value during vehicle treatment was selected. The complete dataset from the microarray study has been submitted to the Gene Expression Omnibus (GEO) at NCBI (series accession: GSE14243).

Reference:

Supplementary Table S1. NFκB Target Genes in As4.1 Cells

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NFκB target genes strongly induced by IL-1β treatment that show minimal change in expression in As4.1 cells treated with H₂O₂. The changes indicated are fold-change relative to vehicle treated As4.1 cells. PMID is the reference to a journal article at NCBI-Pubmed providing experimental evidence that the corresponding gene is a NFκB target gene. The complete set of gene expression profiling data has been deposited at NCBI-GEO (accession number GSE14243).
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Genes strongly induced by Interleukin (IL-1\(\beta\)) treatment that show minimal change in expression in As4.1 cells treated with \(H_2O_2\). For these genes, there was insufficient evidence in the literature that they were NF\(\kappa\)B target genes. For 4 of these genes, there was evidence for an association with NF\(\kappa\)B in the corresponding journal article indicated by the NCBI-Pubmed identifier (PMID). The changes indicated are fold-change relative to vehicle treated As4.1 cells. The complete set of expression data has been deposited at NCBI-GEO (accession number GSE14243).
Figure S1. Effect of TNFα on Ren Expression and Promoter Activity in As4.1 cells. 
A. RPA of RNA isolated from As4.1 cells incubated with TNFα (10 ng/ml) for the indicated time. B. Transcriptional activities of pGL2 basic (promoterless Luciferase vector) and 4.1-luc transfected into As4.1 cells. Cells were treated with vehicle (Gray bars) or 10ng/ml TNFα (filled bars). *, P<0.05 vs vehicle (n=4).
Figure S2. Effect of H$_2$O$_2$ on Endogenous Ren Expression.
Representative RPA of RNA from As4.1 cells treated for increasing time with 200 µM H$_2$O$_2$. The positions of the mouse renin mRNA, cyclophilin, and β-actin products are indicated. B. Quantification of multiple independent dose and time response experiments, respectively (n=6). * p<0.05 vs. non-treated. M, marker.
Figure S3. *ROS produced by Xanthine and Xanthine Oxidase Decreases Ren Expression.*

A. Total RNA isolated from 0.15 mM xanthine (X) and 0.2U xanthine oxidase (XO) treated As4.1 cells was analyzed by RPA using probes specific for mouse renin mRNA and normalized for expression of cyclophilin. B. Quantification of independent experiments (n=4). *, P<0.01 vs. vehicle. M, marker.
Figure S4. Effect of Actinomycin D and H₂O₂ on Ren Expression.

A. As4.1 cells were treated with 2 μM actinomycin D or 200 μM H₂O₂ for 24 h. RNA was analyzed by RPA using probes specific for mouse renin mRNA and normalized for the expression of 18S. B. Cells were treated with or without 200 μM H₂O₂ for 8h prior to addition of actinomycin D for the indicated time. Quantification was from RNase protection assays (N=6).