Enhancement of renin and prorenin receptor in collecting duct of Cyp1a1-Ren2 rats may contribute to development and progression of malignant hypertension

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Prieto MC, Williams DE, Liu L, Kavanagh KL, Mullins JJ, Mitchell KD. Enhancement of renin and prorenin receptor in collecting duct of Cyp1a1-Ren2 rats may contribute to development and progression of malignant hypertension. Am J Physiol Renal Physiol 300: F581–F588, 2011. First published November 10, 2010; doi:10.1152/ajprenal.00433.2010.—To determine whether in the transgenic rat model [TGR(Cyp1a1Ren2)] with inducible ANG II-dependent malignant hypertension changes in the activation of intrarenal renin-angiotensin system may contribute to the pathogenesis of hypertension, we examined the gene expression of angiotensinogen (AGT) in renal cortical tissues and renin and prorenin receptor [(P)RR] in the collecting duct (CD) of the kidneys from Cyp1a1Ren2 rats (n = 6) fed a normal diet containing 0.3% indole-3-carbinol (I3C) for 10 days and noninduced rats maintained on a normal diet (0.6% NaCl diet; n = 6). Rats induced with I3C developed malignant hypertension and exhibited alterations in the expression of renin and (P)RR expressed by the CD cells. In the renal medullary tissues of the Cyp1a1Ren2 transgenic rats with malignant hypertension, renin protein levels in CD cells were associated with maintained renin content and lack of suppression of the endogenous Ren1c gene expression. Furthermore, these tissues exhibited increased levels of (P)RR transcript, as well as of the protein levels of the soluble (P)RR, gene expression in contrast to the chronic ANG

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II-infused rat model, in which the kidneys are exposed to elevated circulating ANG II levels and marked suppression of PRA and decreased kidney renin content (47, 51), the kidneys of hypertensive TGR(Cyp1a1Ren2) rats are exposed to elevated circulating levels of both (pro)renin/renin and ANG II (25, 38). In the TGR (Cyp1a1Ren2) rats even though renin is not constitutively expressed in the kidney (15), the renal tissue ANG II levels are enhanced and renal hemodynamic function is reduced (25, 27). Thus, it has been suggested that the elevated BP in the TGR(Cyp1a1Ren2) rats is at least partially due to increased intrarenal ANG II levels (25, 28).

Consistent with our previous findings demonstrating that total kidney ANG II content is elevated in Cyp1a1Ren2 rats with malignant hypertension (25, 36), the increases in the ANG II content in the kidney are prevented by AT1R blockade (25, 48). Interestingly, the intrarenal ANG II content measured in the Cyp1a1Ren2 rats induced with I3C is high in the cortex and even greater in the kidney medulla of these rats (48). Nevertheless, the inappropriate activation of AT1R by ANG II that contributes to the augmented BP and to increased kidney tissue ANG II levels, particularly in the renal medulla of Cyp1a1Ren2 rats with malignant hypertension, is due to mechanisms still uncertain (25). Thus, in the present study, we aimed to determine whether alterations in the expression of other components of the intrarenal RAS may help to explain the increases in intrarenal ANG II formation in this model. We used dissected renal cortical and inner medullary tissues from Cyp1a1Ren2 transgenic rats fed a diet with high content of I3C (0.3%) to induce ANG II-dependent malignant hypertension to determine whether changes in activation of intrarenal AGT and renin and (P)RR in the CD of the kidney contribute to the pathogenesis of hypertension in this model of ANG II-induced hypertension.

METHODS

Animals and Experimental Design

All experiments were performed using adult male transgenic TGR(Cyp1a1Ren2) rats with inducible expression of the Ren2 renin gene (15) with protocols approved by the Tulane Institutional Animal Care and Use Committee. All the transgenic rats used in this study were bred at Tulane University School of Medicine from stock animals supplied by Harlan UK Limited (Bicester, UK). Animals were divided into two groups as follows: group 1 (noninduced; n = 6) rats maintained on a normal diet [0.6% NaCl diet (diet TD 99414); Harlan-Teklad, Madison, WI] and group 2 (0.3% I3C; n = 6) Cyp1a1Ren2 rats fed a normal diet containing a I3C at a dose of 0.3% wt/wt (diet TD 05381; Harlan-Teklad) for 10 days to induce ANG II-dependent malignant hypertension, as described previously (8, 25, 27, 36, 37, 48). In all rats, body weight was measured every day. At the completion of the experimental protocol, the rats were anesthetized with pentobarbital sodium (IP, Nembutal Sodium Solution; Ovation, Deerfield, IL), and the abdominal cavity was opened to excise the left kidney immediately after unilateral renal ligature. This kidney was decapsulated under sterile RNAse-free conditions, renal inner medulla and renal cortex were dissected under stereomicroscope, and samples were divided to be either snap-frozen in liquid nitrogen and stored at −80°C for eventual determinations of renin content and Western blot analysis or were included in RNase later (Ambion) and stored at −80°C until be processed for total RNA extractions. The right kidney was used for immunohistochemistry studies after sequentially perfusion for 10 min with saline solution (0.9% NaCl at room temperature) and 20 min with cold 4% paraformaldehyde (PFH) by placement of a needle (18-gauge) into the right cardiac ventricle and opening a notch on the wall of the right atrium.

Intrarenal RAS Expression Studies

qRT-PCR studies. Twenty nanograms per well of total RNA were extracted from rat kidney cortex samples to amplify AGT mRNA and from the medulla samples to amplify Ren1c, Ren2, and (P)RR genes. The quantifications were performed using similar approaches as previously described (40). Following sequences were used for: 1) AGT: primers [sense 5'-GAGAAGTGAACATCTGGCATA GA-3'], probe [5'-6-FAM-TGGCTCTCCATGCCATGGACATCC-(BHQ1a-6FAM)-3'], and probe [5'-6-FAM-TGGCTCTCCATGCCATGGACATCC-(BHQ1a-6FAM)-3']; 2) Ren1c: primers [sense 5'-TGGCTCTCCATGCCATGGACATCC-(BHQ1a-6FAM)-3'] and probe [5'-6-FAM-TGGCTCTCCATGCCATGGACATCC-(BHQ1a-6FAM)-3']; 3) Ren2: primers [sense 5'-ACAGATCCCACACCAAGGAGGAAGAAG-3'; antisense 5'- GCACCAAGGACCCAGACA-3'] and probe [5'-6-FAM-TGGCTCTCCATGCCATGGACATCC-(BHQ1a-6FAM)-3']; and 4) (P)RR: primers [sense 5'-ATCCTTTGAGACGAAACAAAGA-3'; antisense 5'-AGCCAGTCATAATCCACAGT-3'] and probe [5'-6-FAM-ACACCCACAACTGCTTACACCTTG-BHQ1a-3']. All samples were assayed in triplicate in addition to coamplification of the β-actin gene labeled with 5'-6-HEX and 3'-black hole quencher-2.

Western blot analysis. Protein expression levels were determined in 20 µg/well of protein extracted from renal cortices, as previously described (19). We used a primary rabbit anti-rat/mouse AGT antibody (Code no. 28101, Immuno-Biological Laboratories; St. Louis, MO) at a 1:400 dilution. Results were expressed as fold of change of the analyzed specific bands of the gene of interest normalized against β-actin (Santa Cruz Biotechnology).

Immunohistochemistry. Three-micrometer paraffin-embedded, PFH-perfused kidney sections were immunostained using a peroxidase technique and either a polyclonal rat renin antibody raised in rabbit (Santa Cruz Biotechnology, Santa Cruz, CA; cat. ID scH-105) at a 1:100 dilution normalized against β-actin, as previously described (40). For (P)RR, the protein expression levels were separately quantified in tissue samples from renal cortex and medulla using a polyclonal rabbit anti-(P)RR (ATP6AP2, cat. ID HPA003156; Sigma, St. Louis, MO) at a 1:400 dilution. Results were expressed as fold of change of the analyzed specific bands of the gene of interest normalized against β-actin (Santa Cruz Biotechnology).

Statistical Analyses

Results are expressed as means ± SE. Data were evaluated by Grubb’s test followed when appropriate by paired and unpaired Student’s t-test or by one-way ANOVA with Tukey post hoc test. The significance of differences among groups was defined at a value of P < 0.05.

RESULTS

The rats induced with 0.3% I3C demonstrated severe lethargy, assumption of a hunched posture, and piloerection, which are manifestations of malignant hypertension in the rat (15, 25, 37). After 10 days of being on a normal diet containing a 0.3% I3C to induce ANG II-dependent malignant hypertension, the
induced rats exhibited a substantial decrease in body weight compared with noninduced rats (248 ± 2 vs. 349 ± 1 g; *P* < 0.01).

The renin content was similar in the Cyp1a1Ren2 hypertensive rats and in noninduced rats in both tissues, the renal cortex and renal medulla; however, the renin content in inner medullary tissues was markedly higher than in the cortex [hypertensive rats cortex: 659 ± 36; medulla: 3,067 ± 1,013 μg ANG 1·h⁻¹·g⁻¹ vs. noninduced (cortex: 470 ± 35; medulla: 3,398 ± 420 μg ANG 1·h⁻¹·g⁻¹); *P* < 0.05].

The expression levels of AGT mRNA and protein quantified in renal cortical tissues are shown in Fig. 1. AGT transcript and protein levels (52-kDa band) measured by qRT-PCR and Western blot were similar in both groups of rats [AGT mRNA: (0.8 ± 0.2 vs. 1.0 ± 0.1 AU); AGT protein (1.5 ± 0.4 vs. 1.3 ± 0.2-fold change); Fig. 1].

Renin immunostaining using a polyclonal rabbit anti-renin antibody showed no significant decrease in the positive immunoreactivity in JG cells of induced rats (Fig. 2, A and B). In contrast, renin immunoreactivity in CD cells was 3.8-fold higher in the induced Cyp1a1Ren2 transgenic rats compared with noninduced normotensive control rats (Fig. 2, C–E). Renin protein levels quantified in renal inner medullary tissues by Western blot analysis showed augmentation of prorenin and renin in hypertensive Cyp1a1Ren2 transgenic rats compared with noninduced normotensive control rats [prorenin: (1.29 ± 0.1 vs. 1.0 ± 0.0 AU; *P* < 0.05); renin: (1.34 ± 0.1 vs. 1.0 ± 0.1; *P* < 0.01); Fig. 2F]. To further assess the origin of prorenin/renin present in the CD of the Cyp1a1Ren2 transgenic rats, we also quantified the mRNA levels of the transgene, the mouse Ren2 renin gene, as well as the endogenous rat Ren1c renin gene in the renal medulla to avoid contribution of JG renin component. We did not find amplification whatsoever of the mouse Ren2 renin gene using as a control RNA extracted from a mouse strain 129 kidney, indicating that prorenin/renin is not derived from renal expression of the transgene (Fig. 3A).

However, we found detectable levels of the transcript for the Ren1c renin gene in the renal medullary tissues of both groups (0.3 ± 0.1 vs. 1.0 ± 0.1; *P* < 0.05; Fig. 3B), suggesting that the endogenous Ren1c gene is not completely suppressed in induced Cyp1a1Ren2 rats.

Intense (P)RR immunoreactivity was observed primarily in epithelial cells of the connecting tubules and CDs throughout the cortex, the outer medulla, and extending into the inner medulla (Fig. 4, top). Proximal tubules and thick ascending limbs of Henlé’s loop were negative. In sections in which the primary antibody was substituted with buffer only, no immunoreactivity was observed (data not shown). Cells of the CD exhibited decreased apical (P)RR immunoreactivity in the hypertensive rats induced with high I3C compared with noninduced rats (47.6 ± 2.6 vs. 100 ± 23.2%; *P* < 0.01; Fig. 4, bottom). Rarely, cells were observed that had diffused basolateral label in addition to apical label. In the CDs at the renal cortex, most of the cells expressed apical (P)RR immunoreactivity as well. In both, the outer and the inner stripe of the outer and inner medullary CDs, positive apical (P)RR immunoreactivity was predominantly observed in those cells with a rounded outline and bulging apical surface suggesting that they were intercalated cells as has been described previously (1).

The (P)RR transcript levels were significantly greater in the renal medulla than in the cortex, regardless of the group (data not shown). (P)RR mRNA levels were significantly augmented in the renal medullary tissues of hypertensive rats compared with the noninduced controls (1.97 ± 0.2 vs. 1.0 ± 0.1-fold increase; *P* < 0.01; Fig. 5A). In these tissues, the (P)RR immunoblot using a purified rabbit IgG against an epitope of the ectodomain and small portion of the cytoplasmic domain (cat. ID HPA003156; Sigma) showed that the Cyp1a1Ren2 rats induced with a high dose of I3C and that exhibit malignant hypertension presented a slightly decreased expression of the full-length (P)RR (37-kDa band; Cyp1a1Ren2: 0.7 ± 0.1 vs. noninduced: 1.0 ± 0.3-fold change; *P* < 0.05; Fig. 5B). However, the soluble form of the (P)RR (28-kDa band) was significantly augmented in the renal medulla of these rats (Cyp1a1Ren2: 1.3 ± 0.1 vs. noninduced: 1.0 ± 0.1-fold change; *P* < 0.05; Fig. 5B). In this experiment, protein extracted from lysate of neuronal cells overexpressing (P)RR generously given by Dr. Y. Feng (Tulane Univ., New Orleans, LA) was used as a positive control.
DISCUSSION

Induction of the Ren2 renin gene by dietary administration of 0.3% I3C for 10 days resulted in a marked decrease in body weight, extreme lethargy, hunched posture, and piloerection, which are clinical manifestations of malignant hypertension previously described in these model rat (15, 25, 37). Most importantly, the results of the present study demonstrate that in Cyp1a1Ren2 transgenic rats fed a diet containing 0.3% I3C to induce ANG II-dependent malignant hypertension, there are alterations in the expression of renin and (P)RR expressed by the CD cells. These alterations may contribute to increased intrarenal ANG II de novo formation and thus, to the pathogenesis of hypertension in this model. In the renal medullary tissues of the Cyp1a1Ren2 transgenic rats with malignant hypertension, the expression of renin and prorenin was altered compared to noninduced rats.
hypertension, we found increased renin protein levels in CD cells associated with maintained renin content and lack of suppression of the endogenous Ren1c gene expression. Furthermore, these tissues exhibited increased levels of (P)RR transcript, as well as of the protein levels of the soluble form of this receptor, the s(P)RR. Intriguingly, although previous findings demonstrated that urinary AGT excretion is augmented in Cyp1a1Ren2 transgenic rats fed a diet with high content of I3C (24), in the present study we did not find changes in the gene expression of AGT in renal cortical tissues of these rats.

Previous studies demonstrated that despite the marked elevation of arterial pressure, the renal tissues of rats with ANG II-dependent hypertension such as the nonclipped kidney of the 2K1C Goldblatt hypertensive rat and the kidneys of ANG II chronically infused rats, TGR(mRen2)27 transgenic rats, and Cyp1a1Ren2 transgenic rats exhibit a marked augmentation of intrarenal ANG II content beyond its levels in the circulation (9, 26, 52). The increases in intrarenal ANG II content in the Cyp1a1Ren2 rats with malignant hypertension may be secondary to AT1R-mediated stimulation uptake of circulating ANG II (25, 52) given that AT1R are located on the luminal and the basolateral membranes of the proximal tubule and CD cells (10). However, de novo generation of ANG II may also occur in this model such as has been reported in the chronic ANG II-infused rat model (20, 52). Augmented tissue ANG II formation in the kidneys of chronic ANG II-infused rats and in the nonclipped kidney of 2K1C Goldblatt rats has been associated with upregulation of tubular renin in the CD (40–42). Renin synthesis in principal cells of the CD is stimulated by ANG II (40) in ANG II-dependent hypertension via an AT1R mechanism (41). In the present study, the demonstration that JG renin expression is maintained in Cyp1a1Ren2 rats with malignant hypertension together with the findings that the renal medullary tissues of these rats, which are devoid of JG renin, exhibit augmented renin immunoreactivity in the CD cells, increased renin protein levels, and proven amplification of the endogenous Ren1c, indicates that the kidneys of Cyp1a1Ren2-hypertensive rats are not depleted of renin. In contrast to the kidneys of chronic ANG II-infused rats, in which the kidneys are exposed to elevated systemic ANG II levels and marked suppression of plasma renin activity (47, 50, 51); the kidneys of hypertensive Cyp1a1Ren2 rats are subjected to elevated systemic levels of both renin and ANG II due to the extrarenal induction of the Ren2 transgene. Therefore, although it cannot be ruled out that part of renin found in the kidneys of Cyp1a1Ren2 rats is from systemic origin, the presence of the transcript of the endogenous renin gene (Ren1c) indicates that renin synthesis is still able to occur in the kidneys of Cyp1a1Ren2 rats regardless of the marked elevation of arterial BP. The findings that renin immunoreactivity is augmented in the principal cells of I3C-induced hypertensive rats and that the renin activity in renal medullary tissues of these rats is similar to those values quantified in noninduced rats also indicate that the activity of renin in the CD of these rats is not suppressed even in conditions of severe hypertension. This observation is consistent with our previous findings demonstrating that the ANG II contents in the kidney medulla and cortex are elevated in Cyp1a1Ren2 rats with malignant hypertension (48). Accordingly, it is apparent that the kidneys of Cyp1a1Ren2 transgenic rats exhibit an impaired ability to suppress intrarenal JG renin, and most importantly, display stimulation of renin in the CD.

![Fig. 3. Renin mRNA levels quantified by real-time PCR (qRT-PCR) in inner renal medulla of Cyp1a1Ren2-hypertensive rats. A: inner medullary tissues from Cyp1a1Ren2 rats, noninduced (n = 6) and induced (n = 6) with a high-I3C diet, have no detectable mRNA levels of the mouse Ren2 gene (transgene). However, the mRNA levels of the endogenous rat renin 1c gene (Ren1c) are not completely suppressed in the renal medulla of Cyp1a1Ren2 rats subjected to a similar scheme of diet (B). RNA extracted from kidney tissues from a mouse 129 strain was used as control of Ren2 gene expression.](http://ajprenal.physiology.org/)

![Fig. 4. Semiquantitation of prorenin receptor [(P)RR] immunoreactivity in CD of paraffin-embedded kidney sections of Cyp1a1Ren2 rats. Densitometric analysis of the (P)RR intensity of Cyp1a1Ren2 rats shows reduced immunoreactivity in CD of Cyp1a1Ren2-hypertensive rats induced with I3C diet compared with noninduced normotensive rats. Values are means ± SE. *P < 0.05 vs. noninduced rats.](http://ajprenal.physiology.org/)
hypertension, we also examined the gene expression of (P)RR
ANG II in the Cyp1a1Ren2 transgenic rats with malignant

tained capability on the kidneys to continue forming intrarenal
the cortex (48).

contents which are actually greater in the renal medulla than in
which may contribute to the increased intrarenal ANG II
levels in Cyp1a1Ren2 rats. (Pro)renin receptor expression appears to be upregulated under pathological conditions, e.g., in the heart of spontaneously hypertensive rats on a high-salt diet (12). In this study, we also found upregulated levels of (P)RR transcript in the kidney medulla of Cyp1a1Ren2 transgenic rats with malignant hypertension. This finding contrasts with the decreased (P)RR immunoreactivity in cortical, outer, and inner medullary CDs, as well as of protein levels of the full-length (P)RR form (37-kDa band) and it is consistent with the existence of a short negative feedback loop proposed by Schefe et al. (45), by which high prorenin levels suppress the (P)RR gene expression. According to the concept of Schefe et al. (45), it is possible that high prorenin circulating levels such as occur in the hypertensive Cyp1a1Ren2 rats lead to diminished renal (P)RR expression. This seems to be the case in the Cyp1a1Ren2 rats in which high circulating and intrarenal prorenin/renin levels downregulate the (P)RR immunoreactivity in the medullary CD cells and the protein levels of the full-length (P)RR but not the s(P)RR in these tissues. Nevertheless, the mechanisms underlying the augmented expression of the soluble form of the (P)RR (28-kDa band) in the same renal tissues of Cyp1a1Ren2 rats are an issue that remains to be clarified. Recently, it has been proposed that a few aminocoids outside of the transmembrane domain serve as a putative cutting site for furin, a protease that participates in (P)RR processing and that might liberate the extracellular (pro)renin binding part (2, 4) into the circulation or other compartments where it may play a functional role (4, 33). In ANG II-dependent hypertension, the renal interstitial ANG II levels are greater than can be explained on the basis of equilibration with the plasma concentrations (34, 35, 46), suggesting that local regulation of ANG II formation in the renal interstitial compartment and enhanced production of interstitial ANG II might be secondary to specialized ANG II-forming pathways or accumulation mechanisms (34). The (P)RR immunorexpression on the basolateral side of distal tubular cells may be particularly significant in regulating interstitial ANG II levels (32). Given that the presence of (P)RR has also been localized to the basolateral side of distal tubular segments cells, it is likely that the secreted soluble form could contribute to the pool of ANG II in the interstitial renal compartment in Cyp1a1Ren2 rats with malignant hypertension by binding (pro)renin in the interstitium. Nonetheless, at the present, whether there is secretion of the s(P)RR into the renal interstitium or the tubular lumen or whether the s(P)RR plays a functional role in these compartments is an issue that remains uncertain.

which may contribute to the increased intrarenal ANG II
contents which are actually greater in the renal medulla than in the
cortex (48).

To investigate further the mechanisms underlying the main-
tained capability on the kidneys to continue forming intrarenal
ANG II in the Cyp1a1Ren2 transgenic rats with malignant
hypertension, we also examined the gene expression of (P)RR
in the renal tissues of these rats. Nguyen and associates (32)
cloned the (P)RR that equally binds renin and prorenin. Re-
ceptor-bound renin exhibits fivefold increased catalytic activity
and receptor-bound prorenin exhibits full enzymatic activity
comparable to that of active renin (32). We found that the
(P)RR is predominantly immunoexpressed on the apical
aspects of the bulging CD cells in kidneys from induced
Cyp1a1Ren2 rats. The presence of (P)RR on the cell apical
surface of the intercalated cells (1) in the CD might be of great
relevance in the light of new findings of renin synthesis and
secretion from the principal cells of the CD (14, 40, 42, 44).
We proposed that (P)RR located on the surface of the interca-
lated cells may play a pivotal role to increase the efficiency of
intratubular angiotensin peptide generation in the CD by an-
choring renin or prorenin synthesized and released by principal
cells (39). (Pro)renin receptor expression appears to be upregu-
lated under pathological conditions, e.g., in the heart of sponta-
naneously hypertensive rats on a high-salt diet (12). In this
study, we also found upregulated levels of (P)RR transcript in
the kidney medulla of Cyp1a1Ren2 transgenic rats with ma-
lignant hypertension. This finding contrasts with the decreased
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Finally, we determined the renal expression of AGT in the Cyp1a1Ren2 transgenic rats with malignant hypertension. In these rats, neither renal cortical AGT mRNA nor protein expression was increased. However, we recently reported that Cyp1a1Ren2 transgenic rats with inducible malignant hypertension exhibit enhanced urinary AGT excretion (24). It is possible that the increased urinary levels of AGT may reflect glomerular damage and subsequent filtration of systemic AGT. In this regard, we previously showed that during the development of malignant hypertension, the kidneys of hypertensive Cyp1a1Ren2 transgenic rats exhibit renal pathological changes characterized by increased inflammation and cellular proliferation in cortical vessels and tubulointerstitial and higher glomerulosclerosis than normotensive rats (8). These changes may help to explain the increases in the urinary excretion of AGT in Cyp1a1Ren2 transgenic rats and, thus, abundant substrate availability in the lumen of the CD.

In summary, the present findings demonstrate that Cyp1a1Ren2 rats with ANG II-dependent malignant hypertension exhibit upregulation of renin and (P)RR gene expression in the CD and maintained gene expression of AGT in the renal cortex. Evidence demonstrating that renin/prorenin can be secreted by the principal cells of the distal nephron segment (14, 44) along with the observations that Cyp1a1Ren2 rats exhibit increased urinary AGT excretion (24) and upregulation of renin and (P)RR gene expression in the CDs may constitute a leading mechanism for the elevation of kidney ANG II levels that occurs in Cyp1a1Ren2 rats with inducible ANG II-dependent malignant hypertension.

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