P450-Dependent Arachidonic Acid Metabolism and Angiotensin II–Induced Renal Damage

Eva Kaergel, Dominik N. Muller, Horst Honeck, Juergen Theuer, Erdenechimeg Shagdarsuren, Alexander Mullally, Friedrich C. Luft, Wolf-Hagen Schunck

Abstract—Transgenic rats overexpressing both human renin and angiotensinogen genes (dTGR) develop hypertension, inflammation, and renal failure. We tested the hypothesis that these pathological features are associated with changes in renal P450-dependent arachidonic acid (AA) metabolism. Samples were prepared from 5- and 7-week-old dTGR and from normotensive Sprague-Dawley (SD) rats, ie, before and after the dTGR developed severe hypertension and albuminuria. At both stages, dTGR showed significantly lower renal microsomal AA epoxygenase and hydroxylase activities that reached 63% and 76% of the control values at week 7. Furthermore, the protein levels of several potential AA epoxygenases (CYP2C11, CYP2C23, and CYP2J) were significantly reduced. Immunoinhibition studies identified CYP2C23 as the major AA epoxygenase, both in dTGR and SD rats. Immunohistochemistry showed that CYP2C23 was localized in cortical and outer medullary tubules that progressively lost this enzyme from week 5 to week 7 in dTGR. CYP2C11 expression occurred only in the outer medullary tubules and was markedly reduced in dTGR compared with age-matched SD rats. These findings indicate site-specific decreases in the availability of AA epoxygenase products in the kidney of dTGR. In contrast to renal microsomes, liver microsomes of dTGR and SD rats showed no change in the expression and activity of AA epoxygenases and hydroxylases. We conclude that hypertension and end-organ damage in dTGR is associated with kidney-specific downregulation of P450-dependent AA metabolism. Because the products of AA epoxygenation have anti-inflammatory properties, this alteration may contribute to uncontrolled renal inflammation, which is a major cause of renal damage in dTGR. (Hypertension. 2002;40:273-279.)

Key Words: rats ▪ kidney ▪ cytochrome P450 ▪ arachidonic acid ▪ angiotensin II ▪ inflammation ▪ end-organ damage

Cytchrome P450 enzymes (P450/CYP) generate 2 major classes of arachidonic acid (AA) metabolites in the kidney, namely epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EETs) and hydroxyeicosatetraenoic acids (19- and 20-HETEs).1,2 In rat kidney, EET formation is catalyzed by P450 isoforms belonging to the CYP2C and CYP2J subfamilies.3–5 CYP4A subfamily members produce 20-HETE.6,7 Both EETs and 20-HETE contribute to the regulation of renal vascular tone and tubular sodium and water transport.3,8,9 Impaired renal hemodynamics and increased salt retention was associated with altered EET- and 20-HETE–generating P450 expression in a number of hypertensive rat and mouse models.10–17 A recent study indicates that EETs may be important by acting as anti-inflammatory mediators in endothelial cells.18 EETs were found to inhibit cytokine-induced activation of the nuclear transcription factor xB (NF-xB) and may thus prevent vascular inflammation at a very early stage. We have studied a double transgenic rat (dTGR) model, which harbors the human genes for renin and angiotensinogen.19,20 dTGR develop hypertension associated with impaired renal hemodynamics and tubular sodium reabsorption.21 They die of cardiac and renal failure at about the eighth week of age.20 In this model, end-organ damage is mainly caused by severe inflammation triggered by high angiotensin (Ang) II concentrations in the heart, kidney, and vasculature. Activation of the proinflammatory transcription factors NF-xB and activator protein (AP-1) plays a key role in the pathogenesis.20,22–26 To what extent P450-dependent AA metabolites are involved as mediators in this model is unknown. We therefore addressed the question whether or not P450-dependent renal AA metabolism is altered in dTGR compared with the nontransgenic parental Sprague-Dawley (SD) strain.

Methods

Experimental Animals

The model has been described in detail earlier.19 Briefly, the human renin construct used to generate transgenic animals made up the entire genomic human renin gene (10 exons and 9 introns), with 3.0 kB of the 5'-promoter region and 1.2 kB of 3' additional sequences.
Microsomal AA Metabolism

Microsomes were prepared from freshly dissected kidneys and livers of dTGR and SD rats. Homogenization was performed in 50 mmol/L Tris-HCl (pH 7.4) containing (in mmol/L) sucrose 0.25, KCl 150, EDTA 2, diethiothreitol 2, and phenylmethylsulfonyl fluoride 0.35. Microsomes were isolated by differential centrifugation and resuspended in 50 mmol/L Tris-HCl (pH 7.7) containing 20% glycerol, 5 mmol/L EDTA, and 1 mmol/L diethiothreitol. [1-14C]AA (56 nCi/mmol) was purchased from Amersham Pharmacia-Biotech. The respective peroxidase conjugated secondary antibodies were generated by J.H. Capdevila, Vanderbilt University, Nashville, Tenn.23 Rabbit antisera against rat CYP2C23 (generated by D.C. Zeldin, National Institute of Environmental Health Sciences, NC14); and rabbit IgG against rat CYP2C23 (generated by J.H. Capdevila, Vanderbilt University, Nashville, Tenn.15) were used. Plasma activities of alanine aminotransferase, aspartate aminotransferase, and glutamate dehydrogenase were determined by automated methods.

Immunohistochemistry

Ice-cold acetone-fixed cryosections (6 μm) were stained for CYP2C11 by immunofluorescence and for CYP2C23 by alkaline phosphatase/anti–alkaline phosphatase technique as described earlier.24,25 We used the same primary antibodies as for Western blotting. Preparations were analyzed under a Zeiss Axioplan-2 microscope (Carl Zeiss) and were digital photographed by use of the AxioVision-2 multichannel image processing system (Carl Zeiss). For quantification of CYP2C23, 10 different cortical view fields per rat (n = 5 per group) were analyzed. The sections were scored in the following categories: <60%, 60% to 80%, and 80% to 100% CYP2C23-positive tubules.

Statistical Analysis

All data are presented as mean±SEM and were analyzed by 1-way ANOVA followed by the Bonferroni multiple comparison test (InStat software; GraphPad Software Inc). A value of P<0.05 was considered statistically significant.

Results

dTGR featured hypertension and severe renal damage. At week 7, systolic blood pressure was increased by ∼80 mm Hg compared with that in SD rats (199±2.9 versus 119±5.5 mm Hg; P<0.001) and the 24-hour albumin excretion reached 16.98±2.42 versus 0.16±0.02 mg/d (P<0.001). In contrast, the livers of dTGR appeared normal. No significant differences were found in the activities of alanine aminotransferase (79.7 versus 61.5±8 U/mL), aspartate aminotransferase (143.7±11.5 versus 149.8±20.6 U/mL), and glutamate dehydrogenase (10.2±2.3 versus 8.9±0.8 U/mL) in blood samples from dTGR and control rats.

P450-Dependent AA Metabolism

At week 7, renal microsomes of control rats showed an AA hydroxylase activity of 278±16 pmol·min⁻¹·mg⁻¹ and a total AA epoxygenase activity of 209±16 pmol·min⁻¹·mg⁻¹ (Figure 1). The epoxygenase product consisted of 14,15-, 11,12-, and 12,13-EET (retention times, 23.4 and 12.7 minutes), respectively, and 14,15-EET/-DHET (retention times, 25.3 and 14.8 minutes, respectively), dihydroxyeicosatrienoic acids (DHETs): 8,9-EET/-DHET (retention times in RP-HPLC, 25.3 and 14.8 minutes, respectively), and glutamate dehydrogenase (10.2±2.3 versus 8.9±0.8 U/mL) in blood samples from dTGR and control rats.

Immunoinhibition

Microsomes were preincubated for 30 minutes at 37°C with 100 μg/mL rabbit IgG (125 μg IgG/mg microsomal protein) before substrate and NADPH were added to constitute the complete reaction mixture as described above for determining AA metabolism. The concentrations of anti-CYP2C23 IgG varied between 0 and 100 μg/mL, whereas the total amount of IgG was kept constant by appropriate additions of control rabbit IgG.

Western Blot Analysis

Microsomal protein (15 μg per lane) was separated on 10% SDS-PAGE and transferred to HybondECL nitrocellulose membranes (Amersham Life Sciences). Primary antibodies used were goat antisera against rat CYP4A1, rat CYP2C11, and rat CYP2E1 (Duichi Pure Chemicals Co); rabbit antisera against human CYP2J2 (generated by D.C. Zeldin, National Institute of Environmental Health Sciences, NC14); and rabbit IgG against rat CYP2C23 (generated by J.H. Capdevila, Vanderbilt University, Nashville, Tenn.). The respective peroxidase conjugated secondary antibodies were from Sigma. Blots were developed with the chemiluminescence substrate from Roche and evaluated with the Image Reader LAS-1000 (Fujifilm).

Immunoassay

Concentrations of anti-CYP2C23 IgG varied between 0 and 100 μg/mL.
different in renal microsomes isolated from 5- and 7-week-old animals (Figures 2A and 2B).

### Alterations in P450 Protein Levels

The total P450 content of renal microsomes determined by carbon monoxide difference spectra averaged at 75 and 55 pmol/mg of protein for dTGR and SD rats and was not significantly different between the groups. Western blot analysis showed that the level of CYP2C11 protein was significantly decreased in renal microsomes of 7-week-old dTGR to $\approx 30\%$ (29% to 4%) of control values (Figure 3). A similarly prominent decrease was observed for CYP2J proteins (32% to 6% of the control values) as detected with antibodies against human CYP2J2 (Figure 3). The levels of P450 proteins reacting with antibodies against rat CYP2C23 and rat CYP2E1 (Figure 3) were moderately decreased in dTGR to $\approx 70\%$ of the control values (69% to 71% and 71% to 6%, respectively). No significant differences were found in the contents of CYP4A proteins when comparing renal dTGR and SD microsomes (Figure 3). Liver microsomes prepared from the same animals did not show significant changes in CYP2C11, CYP2C23, and CYP2J protein levels when comparing dTGR and SD control samples (data not shown).

### Comparisons at 5 and 7 Weeks

Further studies indicated that the alterations in renal P450-dependent AA metabolism described above for 7-week old dTGR were already apparent at week 5, before the development of albuminuria and severe end-organ damage (Figure 4A and 4B). Compared with SD rats of the same age, in dTGR the AA epoxgenase activity was reduced by 30% (Figure 4C). Western blot analysis demonstrated a significant downregulation of renal microsomal CYP2C11 protein. In addition, there was a tendency to reduced CYP2C23 levels (Figure 4D). In contrast to week 7, there was a significant decrease of CYP4A levels by 30%, comparing renal microsomes from 5-week-old dTGR and SD rats.

### Localization of CYP2C23 and CYP2C11

CYP2C23 protein was expressed in tubules in the renal cortex and outer medulla (Figures 5A and 5B). In contrast, CYP2C11 immunoreactivity was restricted to tubules of the outer medulla. Glomeruli and blood vessels showed no immunoreaction for either isoform. Semiquantification of kidney sections from 5- and 7-week-old dTGR and SD rats revealed progressive and significant changes in the cortical tubular CYP2C23 expression. At week 5, most of the transgenic and nontransgenic cortical tubules expressed CYP2C23. At week 7, >50% of analyzed dTGR tubules showed no CYP2C23 immunoreactivity, whereas >90% of SD tubules were positive for CYP2C23 (Figure 5C). Nevertheless, the few tubules remaining to express CYP2C23 in 7-week-old dTGR exhibited significantly increased labeling compared with SD tubules (Figure 5A). CYP2C11 was localized in tubules of the outer medulla (Figure 5D). No expression was observed in cortical tubules. The CYP2C11 immunostaining increased from weeks 5 to 7 in both groups. At all time points, CYP2C11 immunoreactivity was significantly increased in nontransgenic SD tubules compared with dTGR tubules (Figure 5D).
Discussion

The P450-dependent AA metabolism was significantly diminished in the kidney but not in the liver of dTGR compared with SD control rats, even before end-organ damage occurred. The capacity to epoxygenate AA to EETs was most severely affected. Western blot analysis indicated a downregulation of CYP2C11, CYP2C23, and CYP2J. All these P450 isoforms may potentially serve as AA epoxygenases. We found that CYP2C23 was responsible for the majority of total renal EET production, both in dTGR and SD rats, as judged by almost complete inhibition of microsomal epoxygenase activities with antibodies against CYP2C23. CYP2C23 was also shown to be the predominant renal AA epoxigenase in SD rats after induction by dietary salt loading in an earlier study. Our finding that CYP2C23 plays the major role in renal EET production in both dTGR and SD rats was unexpected. At first glance, the finding seems to exclude a significant contribution of CYP2C11 and CYP2J, the enzymes that were the most strongly downregulated AA epoxygenases. Although the activity of CYP2C23 in microsomes from whole kidney was predominant, the data do not exclude specific contributions to local EET production by CYP2C11 and CYP2J. In fact, our immunohistochemical studies revealed a different intrarenal localization of CYP2C23 and CYP2C11.

CYP2C23 was localized in the tubules of the renal cortex and outer medulla. Untreated 7-week-old dTGR showed severe renal damage with a dramatic reduction of CYP2C23-positive tubules. Nevertheless, the remaining CYP2C23-positive tubules in dTGR showed an increased immunoreactivity. Thus, it appears that the availability of EETs was strongly reduced in most cortical tubular structures, whereas it may have even been enhanced in others. Unexpectedly, reduced AA epoxygenase activities were already apparent in 5-week-old dTGR before a significant decrease in CYP2C23
protein levels had occurred. This state of affairs was revealed both by immunohistochemistry and Western blot analysis. The reasons for this discrepancy are unclear. Possibly, a portion of the immunodetectable CYP2C23 was present in an enzymatically inactive form.

CYP2C11 was detected in tubular structures of the outer medulla but not in cortical regions expressing CYP2C23. The tubular expression of CYP2C11 was clearly reduced in both 5- and 7-week-old dTGR, a finding that substantiates the results of Western blot analysis. CYP2C11 is known for its high catalytic activity and represents the predominant AA epoxygenase in the liver of male rats. Thus, without contributing much to overall renal EET formation, changes in CYP2C11 expression may be important for EET availability in specific tubular segments. CYP2J enzymes are further candidates for renal AA epoxygenases. Moreover, an increase in both EET formation and CYP2J2 immunoreactive protein levels was reported in the spontaneously hypertensive rat. CYP2J2 was shown to be a potential source of EETs in human endothelial cells. Whether or not one of the different rat CYP2J isoforms plays this role in the rat renal vasculature is an important question for future studies.

In contrast to our findings in dTGR, hypertension induced by long-term infusion of Ang II in rat was not associated with reduced renal AA epoxygenase activities and decreased CYP2C23 and CYP2C11 levels. The main difference of our model, compared with Ang II infusion, may be related to the fact that uncontrolled inflammation triggered by high local Ang II concentrations is responsible for renal damage in dTGR. Downregulation during inflammation appears to be a general phenomenon for a number of different P450 isoforms. For example, the present finding that dTGR show strongly reduced renal CYP2C11 levels is directly paralleled by results of hepatic CYP2C11 expression in models of hepatic inflammation.

In this case, a downregulation at the transcriptional level...
based on a NF-κB–binding negative regulatory element was proposed.30 A NO-dependent mechanism was held responsible for decreased endothelial CYP2C expression and for downregulation of the endothelium-derived hyperpolarizing factor in response to proinflammatory mediators.31 Increased NO concentrations may be also responsible for inactivation of CYP4A enzymes.32 Because NF-kB is strongly activated and NO synthase II is induced in the kidney of dTGR, both mechanisms may be important in our model.20,23,24

The relationships between inflammation and P450 expression were previously studied mostly in terms of P450-dependent hepatic drug metabolism without investigating the possible consequences for the metabolism of endogenous P450 substrates such as AA. Whether or not the downregulation reflects a deleterious or protective mechanism is unclear.33,34 Treatment of rats with a general P450 inhibitor was reported to sensitize the animals to subsequent inflammatory stimuli.33 Although the actual cause-and-effect relationships remain open, our results point in a similar direction. They show that the expression and activity of AA epoxygenases was already significantly reduced in 5-week-old dTGR, before the animals developed severe hypertension and albuminuria. Proinflammatory cascades both in vascular endothelial cells and in tubular epithelial cells play a central role in triggering progressive inflammation and renal damage.35 For this reason, the exact intrarenal localization of the different EET-producing P450 isoforms is of special interest. Because EETs were shown to have anti-inflammatory properties in the vasculature,19 the question of whether or not this mechanism extends to the tubular system is important. Ang II and albumin can both trigger NF-kB activation in the renal tubules.36

Perspectives
Decreased EET levels are expected to have negative effects on the regulation of vascular tone, tubular function, and control of inflammation. All these effects may have contributed to the renal damage observed in our Ang II–based model. In terms of future therapeutic interventions, both an induction of EET-generating P450 enzymes and an inhibition of systems involved in EET inactivation, such as soluble epoxide hydrolase, may represent interesting new targets to ameliorate hypertension and end-organ damage.

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References


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