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;40:1111-1117.)

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-HETEs contribute to the regulation of renal vascular tone and tubular sodium and water transport.5,8,9 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 NF-κB (NF-κB) 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-κB 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.
The human angiotensinogen construct made up the entire human angiotensinogen gene (5 exons and 4 introns), with 1.3 kbp of 5'-flanking and 2.4 kbp of 3'-flanking sequences. The rats were purchased from RCC Ltd (Füllinsdorf, Switzerland). The controls are nontransgenic SD rats from the same colony. The rats were allowed free access to standard 0.3%-sodium rat chow (SSNIFF Spezialitäten GmbH) and drinking water. All procedures were performed according to guidelines from the American Physiological Society and were approved by local authorities (permit No. G 408/97). Systolic blood pressure was measured by tail cuff under light ether anesthesia. Urine samples were collected over 24 hours. Urinary albumin was measured by enzyme-linked immunosorbent assay (CellTrend). Rats were killed at age 5 and 7 weeks. The kidneys and livers were washed with saline, blotted dry, and weighed. Plasma activities of alanine aminotransferase, aspartate aminotransferase, and glutamate dehydrogenase were determined by automated methods.

**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 150, EDTA 2, diethiothreitol 2, and phenylmethylsulfonyl fluoride 0.25. 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 dithiothreitol. [1-14C]AA (56 nCi/mmol) was purchased from Amersham Pharmacia-Biotech. The reaction mixtures contained (in a total volume of 0.1 mL) the following: 80 μg microsomal protein, 10 nmol AA (0.55×10^4 pmol), 50 nmol NADPH, 0.31 μmol glucose-6-phosphate, and 0.2 U glucose-6-phosphate dehydrogenase for regeneration of NADPH and (in μmol) Tris-HCl buffer 5 (pH 7.5), MgCl2 1, and KCl 15. Microsomes were preincubated for 10 minutes at 37°C with AA, and adding NADPH started the reactions. After shaking for 20 minutes at 37°C, the reactions were terminated by acidification to pH 3.5 to 4.0 with 0.4 mmol/L citric acid. The reaction products were extracted into ethyl acetate and resolved by reverse-phase high-performance liquid chromatography (RP-HPLC) (Shimadzu LC 10 Avp coupled to an online radioflow detector LB 509; Berthold). A Nucleosil 100-5C18 HD column (250×4 mm; Macherey-Nagel) was used with a linear solvent gradient ranging from acetonitrile (v)/water (v)/acetic acid (v) (50:50:0.1) to acetonitrile (v)/acetic acid (v) (100:0.1) over 40 minutes at a flow rate of 1 mL/min. AA hydroxylation activities were determined at the sum of 12- and 20-HETE (measuring unresolved at 16.1 minutes in RP-HPLC) formatted per mg microsomal protein. Total AA epoxygenase activities were calculated from the sum of the following EETs and corresponding dihydroxyeicosatetraenoids (DHTEs): 8,9-EET/DHET (retention times in RP-HPLC, 25.3 and 14.8 minutes, respectively), 11,12-EET/DHET (retention times, 24.7 and 13.8 minutes), and 14,15-EET/DHET (retention times, 23.4 and 12.7 minutes).

**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 antiserum against rat CYP4A1, rat CYP2C11, and rat CYP2E1 (Daiichi Pure Chemicals Co); rabbit antiserum against human CYP2J2 (generated by D.C. Zelden, National Institute of Environmental Health Sciences, NC); 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).

**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: 0, 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 (190±2.9 versus 117±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±11 versus 61±5.8 U/mL), aspartate aminotransferase (143.7±11.5 versus 149.8±20.6 U/mL), and glutamate dehydrogenase (10.2±2.8 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 8,9-EET in a ratio of about 1:4:1. Renal microsomes from dTGR metabolized AA at significantly lower rates. The hydroxylase activities were 215±12 pmol·min⁻¹·mg⁻¹ (corresponding to 76% of the control value; P<0.01), and the epoxygenase activities reached only 132±10 pmol·min⁻¹·mg⁻¹ (63% of control; P<0.001). In contrast to these alterations in renal microsomal P450 activities, liver microsomes of dTGR and control rats hydroxylated and epoxygenated AA with almost identical rates (Figure 1).

**Immunoinhibition of AA Epoxygenase Activities**

The antibody against CYP2C23 inhibited the AA epoxygenase activity of renal microsomes in a concentration-dependent manner without decreasing their ability to hydroxylate AA (Figure 2). Although slightly higher amounts of the antibody were required for 50% inhibition of EET formation with SD microsomes than with dTGR microsomes, the epoxygenase activities were almost completely abolished in both cases at higher antibody concentrations. The course of the inhibition curves and the epoxygenase activities remaining at the highest antibody concentration used were not significantly
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\%\( \pm \)4\%) of control values (Figure 3). A similarly prominent decrease was observed for CYP2J proteins (32\%\( \pm \)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\%\( \pm \)7\% and 71\%\( \pm \)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 epoxigenase 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 reduction 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 renal 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 down-regulation of CYP2C11, CYP2C23, and CYP2J. All these P450 isoforms may potentially serve as AA epoxygenases.5 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.3 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.

Figure 3. Levels of P450 proteins in renal microsomes. A, Representative Western blots probed with antibodies against CYP4A1, CYP2C11, CYP2C23, CYP2J2, and CYP2E1. B, Chemiluminescence quantification. Statistically significant (*P<0.001) differences (dTGR, n=12 versus SD, n=6) were found for CYP2C11 and CYP2J2 and with P=0.05 for 2C23 and 2E1. CYP4A1 antibodies detected 2 proteins that represent CYP4A2 (lower band [lb]) and CYP4A3 (minor upper band [ub]) according to previous studies.7 Both bands did not differ between dTGR and SD. Note that for detection of CYP2J proteins, antibodies against human CYP2J2 were used that recognize all rat CYP2J isoforms.4

Figure 4. dTGR features at weeks 5 and 7 in comparison to those of age-matched SD control rats. A, dTGR (n=6) showed significantly (P<0.001) increased 24-hour albuminuria compared with that of SD controls (n=6) at week 7 but not at week 5 (n=9 and n=4 for 5-week-old dTGR and SD rats). B, Systolic blood pressure was significantly (P<0.001) higher in dTGR already at week 5, and further increased at week 7. C, AA hydroxylase (H) and epoxigenase (E) activities of renal microsomes isolated from dTGR at week 5 (n=9) and week 7 (n=11) expressed as percentage of the respective activities determined in parallel for samples prepared from SD rats (n=4 at week 5 and n=8 at week 7). Statistically significant differences were found for epoxigenase activities both at week 5 (P<0.01) and 7 (P<0.001) and for the hydroxylase activities only at week 7 (P<0.005). D, Western blot analysis of renal microsomes from 5-week-old dTGR (n=9) and SD control rats (n=4) showed significantly reduced levels for CYP4A (activities P<0.05) and CYP2C21 proteins (P<0.001). Values are the percentage of dTGR compared with SD control levels. Compare Figure 3 for a more comprehensive analysis of P450 isoform expression at week 7.

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. 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

Figure 5. Immunohistochemical analysis of CYP2C23 and CYP2C11 in dTGR and SD rats. A, CYP2C23 immunoreactivity was observed in cortical (c) and outer medulla (om) tubuli. Kidneys with minor damage (SD at weeks 5 and 7, as well as 5-week-old dTGR) showed a high percentage of positively stained tubules. In contrast, in 7-week-old dTGR with severe renal damage >50% of the tubules showed no staining. However, the dTGR tubules remaining to express CYP2C23 showed a stronger signal compared with that of nontransgenic tubules. B, Overview of CYP2C23 staining in 7-week-old SD rats with a smaller magnification. C, Semi-quantification of CYP2C23 expression in cortical tubules. The sections were scored in the categories <60%, 60% to 80%, and 80% to 100% CYP2C23-positive tubules. D, CYP2C11 immunoreactivity was exclusively observed in tubules of the outer medulla. The staining increased from week 5 to week 7 in both groups and was more intensive in SD tubuli compared with dTGR tubuli.
based on a NF-κB-binding negative regulatory element was proposed. 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. Increased NO concentrations may be also responsible for inactivation of CYP4A enzymes. Because NF-κB is strongly activated and NO synthase II is induced in the kidney of dTGR, both mechanisms may be important in our model.

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. Treatment of rats with a general P450 inhibitor was reported to sensitize the animals to subsequent inflammatory stimuli. 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 epoxide-nases was already significantly reduced in 5-week-old dTGR, and that the downregulation of systems involved in EET inactivation, such as soluble epoxide hydrolase, may represent interesting new targets to ameliorate hypertension and end-organ damage.

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 CYP 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.

Acknowledgments

This study was supported by the Deutsche Forschungsgemeinschaft (grant MU1467 to D.N.M. and grant Schu8223 to W.H.S.) and by the Klinischer-Pharmakologischer Verbund Berlin-Brandenburg. We acknowledge Dr. Darryl C. Zeldin (National Institute of Environmental Health Sciences, Durham, NC) for providing the antibodies against CYP2C23 and to Dr. Darryl C. Zeldin (National Institute of Environmental Health Sciences, Durham, NC) for providing the antibodies against CYP2J2.

References


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 and Wolf-Hagen Schunck

Hypertension. published online July 29, 2002;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2002/07/29/01.HYP.0000029240.44253.5E.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/