Cytochrome P450–Dependent Renal Arachidonic Acid Metabolism in Desoxycorticosterone Acetate–Salt Hypertensive Mice

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Abstract—Cytochrome P450 (P450)-dependent arachidonic acid metabolites may act as mediators in the regulation of vascular tone and renal function. We studied arachidonic acid hydroxylase activities in renal microsomes from normotensive NMRI mice, desoxycorticosterone acetate (DOCA)-salt hypertensive mice, and DOCA-salt mice treated with either lovastatin or bezafibrate, both of which improve hemodynamics in this model. Control renal microsomes had arachidonic acid hydroxylase activities of $175 \pm 12$ pmol·min$^{-1}$·mg$^{-1}$. The metabolites formed were 20- and 19-hydroxyarachidonic acid, representing $\approx 80\%$ and $\approx 20\%$ of the total hydroxylation. Treatment with DOCA-salt resulted in significantly decreased hydroxylase activities (to $84 \pm 4$ pmol·min$^{-1}$·mg$^{-1}$) of the total microsomal P450 content and a decrease in immunodetectable Cyp4a proteins. Lovastatin had no effect on these variables, whereas bezafibrate increased arachidonic acid hydroxylase activities to $163 \pm 12$ pmol·min$^{-1}$·mg$^{-1}$. In situ hybridization with probes for Cyp4a-10, 12, and 14 revealed that Cyp4a-14 was the P450 isoform most strongly induced by bezafibrate. The expression was concentrated in the cortical medullary junction and was localized predominantly in the proximal tubules. In conclusion, these results suggest that the capacity to produce 20-hydroxyarachidonic acid is impaired in the kidneys of DOCA-salt hypertensive mice. Furthermore, bezafibrate may ameliorate hemodynamics in this model by restoring P450-dependent arachidonic acid hydroxylase activities. Lovastatin, on the other hand, exerts its effects via P450-independent mechanisms. (Hypertension. 2000;36:610-616.)

Key Words: mice ■ kidney ■ arachidonic acid ■ cytochrome P450 ■ hydroxyeicosatetraenoic acids

Several members of the cytochrome P450 (P450/CYP) superfamily1 hydroxylate or epoxygenate arachidonic acid (AA). The products include a series of regioisomeric hydroxyeicosatetraenoic acids (HETE) and epoxyeicosatrienoic acids. Some of these metabolites regulate vascular tone and renal function.2–6 20-HETE, which is produced by members of the P450 4A subfamily, has attracted particular attention because it may be important to both prohypertensive and antihypertensive mechanisms. For example, increased 20-HETE formation7 and enhanced P450 4A expression8,9 preceded the development of hypertension in spontaneously hypertensive rats (SHR). Inhibition of P450 enzymes prevented hypertension in SHR7,10 and in DOCA-salt–treated rats.11 On the other hand, Dahl salt-sensitive rats show deficient 20-HETE formation, and P450 4A induction by clofibrate prevented salt-induced blood pressure elevation and improved pressure natriuresis.12,13 Recently, we adapted methods with mice that allow measurements of total renal blood flow, pressure natriuresis and diuresis, and cortical and medullary blood flow.14 We found that DOCA-salt–induced hypertension in uninephrectomized mice was associated with reduced total renal blood flow and a rightward shift in the pressure-natriuresis-diuresis curve. Lovastatin and bezafibrate treatment lowered blood pressure and improved renal hemodynamics.14 Bezafibrate belongs to a group of compounds that induce P450 forms of the 4A subfamily.16 In the present study, we explored whether the P450-dependent AA metabolism is changed in the kidneys of mice after treatment with DOCA-salt, lovastatin, and bezafibrate.

Methods

Experimental Design and Statistical Analysis

Experiments were performed on male NMRI mice as described earlier.14,15 In brief, 4-week-old mice were treated for 3 weeks. Group 1 (controls with 2 kidneys) and group 2 (controls, uninephrectomized) received the vehicle Na-carboxymethylcellulose (1% solution, 2 $\mu$L/g body wt) daily via gavage. In groups 3, 4, and 5, the right kidney was removed and a DOCA pellet was subcutaneously implanted. Theretafter, the animal received 1% NaCl as drinking water. Group 4 mice also received lovastatin (40 mg/kg body wt in vehicle), and group 5 mice received bezafibrate (50 mg/kg body wt in vehicle). Each group consisted of $\approx 18$ animals, and microsomes

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were prepared after 3 kidneys were pooled, thus yielding ≥6 microsomal samples per group for enzymatic assays. All data are presented as mean±SEM and were analyzed by ANOVA followed by the Tukey-Kramer multiple comparison test (Instat software; GraphPad Software Inc). The significance level was taken at P<0.05.

Microsomal Preparations, Enzymatic Assays, and Product Analysis

Homogenization of the kidneys, isolation of microsomes, NADPH-dependent conversion of lauric acid (LA) and AA, and HPLC analysis of the metabolites were performed as we described previously. For further characterization of the AA hydroxylation product, the initial product peak was collected and resolved with an isocratic solvent system of acetonitrile/water/acetic acid (48:52:0.1, v/v/v; flow rate 1 mL/min). The resulting 2 chromatographic peaks were identified with gas chromatography/mass spectrometry (GC-MS) under negative ion chemical ionization (NICI) and electron impact (EI) conditions. The pentfluorobenzyl ester (PFB), trimethylsilyl (TMS) ether derivatives required for NICI-GC-MS, and the methyl ester TMS derivatives for EI-GC-MS were prepared as described previously. Analyses were made with a GC-MS-QP5050A (Shimadzu) equipped with a 30-m/0.25-mm 6 fused silica capillary column (0.25-μm coating thickness; Macherey-Nagel) with helium used as the carrier gas and methane used as the reagent gas. For NICI spectra, oven temperatures were raised from 190° to 320°C (20°C/min) and held for 4 minutes at 290°, 300°, and 320°C. The ions at m/z 391 (M−PFB group of nonradioactive metabolite) and m/z 393 (M−PFB group of 14C-labeled metabolite) were monitored. For EI spectra, the oven temperature was raised from 50° to 250°C at 35°C/min and from 250° to 320°C at 10°C/min.

In Situ Hybridization

One half of the sagitally divided mouse kidneys were used. Unfixed tissues were frozen in isopentane at −35°C. Cryostat sections (14 μm) were mounted onto γ-aminopropyltriethoxysilane–activated slides and kept at −20°C until hybridization. Oligonucleotides specific for Cyp4a-10 (5′-AGC TTT CTC CAG TGA AAC TCT TCT CAG ACA TTG GAC CTC C-3′), Cyp4a-12 (5′-GAG GTG AAC AAG AGA AGA AAG AAT GAG ATG TGA GCA G-3′), and Cyp4a-14 (5′-GCC AGA TGG GAA ACA GGT ACA TGC ACA GGT TAG GAA GGT G-3′) were 3′-end labeled with [α-32P]dATP through terminal transferase and hybridized as described previously. On control sections, the unlabeled oligonucleotide was added in 100-fold excess compared with the labeled probe (displacement). Sections were exposed to imaging plates for 6 days at room temperature, followed by scanning with the Fujix Bio Imaging Analyzer BAS2000 (Fuji Photo Film Co). Slices were then dipped in Kodak NTB-2 autoradiography emulsion (Eastman Kodak) and exposed for 6 weeks at 4°C. Tissue was counterstained with hemalaun, and dark-field and differential interference contrast light microscopy was performed with an Axiosplan Universal Microscope (Zeiss).

Electrophoresis and Immunoblotting

Microsomal protein was separated on 10% SDS-PAGE and transferred to Hybond ECL nitrocellulose membranes (Amersham Life Sciences). The membranes were incubated with primary antibody (goat anti-rat CYP4A1; Daiichi Pure Chemicals Co) and anti-goat IgG peroxidase conjugate (Sigma Chemical Co) as secondary antibody. Blots were developed with the chemiluminescence substrate from Boehringer-Mannheim and evaluated with the Image Reader LAS-1000 (Fuji Photo Film Co).

Results

P450-Dependent AA Conversion

Renal microsomes from control mice exhibited a specific P450 content of ~400 pmol/mg protein, which was not changed significantly by uninephrectomy: 380±17 (group 1, n=6) versus 410±18 (group 2, n=7). LA was converted in an NADPH-dependent reaction to 12- and 11-hydroxylauric acid as judged with reversed phase HPLC. The 12-11-OH product ratio was ≈2:1, and the total hydroxylation rate ranged from 1.2 to 1.5 nmol · min⁻¹ · mg⁻¹. Incubation of renal microsomes with [1-14C]AA and NADPH resulted in the formation of radio labeled metabolites that eluted from the reversed phase HPLC column as an almost single product peak. It had a retention time of 15.9 minutes (Figure 1A) and corresponded to 1 of the major products obtained with mice liver microsomes (Figure 1B). Rechromatography of the renal product peak revealed 2 metabolites formed in a 20:80 ratio of the total products (Figure 2A). Subsequent GC-MS analysis identified the major product (P₂) as 20-HETE and the minor (P₁) as 19-HETE. In the NICI modus, the TMS ether derivative of both metabolites were eluted in the GC at different retention times (P₁ at 9.4 minutes, P₂ at 10.1 minutes), and both metabolites showed the expected predominant peak at m/z 391, corresponding to M⁻181 (loss of the PFB group) and peaks of lower intensity at m/z 393, characteristic for the 14C-labeled AA metabolites. EI mass spectra were recorded for the TMS ether methyl ester derivatives of the metabolites. The fragmentation patterns obtained (Figures 2B and 2C) agree with published EI mass spectra of 19- and 20-HETE and correspond to the 14C-labeled AA metabolites. EI mass spectra were analyzed by ANOVA followed the Tukey-Kramer multiple comparison test (Instat software; GraphPad Software Inc). The significance level was taken at P<0.05.
Figure 2. Identification of AA metabolites produced by mouse renal microsomes. The product peak obtained in the initial reversed phase HPLC (see Figure 1A) was collected and resolved in a second HPLC as described in Methods (A, fractions P₁ and P₂). The GC-MS-EI spectra of the methyl ester, TMS ether derivatives of P₁ and P₂ are shown in B and C, respectively. Ions of [1-¹⁴C]AA acid metabolites were not detected because their total amount was <7%. Structures are given and prominent ions are indicated.
no significant epoxygenase activity, which would result in the formation of epoxyeicosatrienoic acids and the corresponding dihydroxyeicosatrienoic acids. However, epoxygenase products were clearly found in hepatic microsomes prepared from the same mice under identical conditions (Figure 1B).

**Effects of DOCA-Salt, Bezafibrate, and Lovastatin**

DOCA-salt treatment had a significant effect on renal microsomal AA hydroxylase activities, which were ≈48% of control mice (Figure 3). CO-difference spectra showed that there also was a loss of total P450 content that ranged between 200 and 250 pmol/mg for all microsomes prepared from DOCA-salt–treated mice: 242±7 (group 3, n=7), 228±21 (group 4, n=7), and 245±13 (group 5, n=6) compared with 410±18 (control group 2, n=7) (P<0.001). Moreover, Western blots probed with anti–P450 4A1 antibodies revealed that the specific content of P450 4A proteins was strongly diminished on DOCA-salt treatment (Figure 4). Bezafibrate treatment increased AA hydroxylase activities, thereby restoring the low AA hydroxylase activities in DOCA-salt hypertensive mice from ≈48% to >90% of control levels (Figure 3). In contrast, lovastatin did not influence the renal microsomal AA activities compared with DOCA-salt mice (Figure 3). LA hydroxylase activities, which ranged between 1.0 and 1.2 nmol·min⁻¹·mg⁻¹ for groups 3 and 4, increased to 1.5 to 1.6 nmol·min⁻¹·mg⁻¹ with bezafibrate. Bezafibrate treatment induced an additional, slightly lower-molecular-weight protein that reacted with antibodies against rat P450 4A1 (Figure 4A) in the Western blot.

**P450 4A Isozyme Localization in Renal Structures**

We next examined the known mouse members of the P450 subfamily 4A (P450s 4a-10, 4a-12, and 4a-14) with respect to their intrarenal localization. Isoform-specific oligonucleotides were designed and tested in Southern blots with the individual P450 cDNAs for their specificity (data not shown) and were used for in situ hybridization. P450 4a-10 and 4a-12 mRNAs were detectable in the cortical-medullary junction of control mice (Figures 5A and 5B). A clear expression of both isoforms is indicated on phosphorimaging (insets) and by the presence of numerous silver grains on autoradiography. P450 4a-14 was not detectable in control animals (Figure 5C), but was strongly expressed after bezafibrate treatment (Figure 5D). In all cases, the P450 4a isoforms were predominantly expressed in tubular structures. Grain distribution in the medulla (Figures 5A and 5B), as well as on control sections (Figure 5D), did not exceed the background level. A higher magnification showed the grains localized to the proximal tubular structures (Figure 6). The glomeruli and blood vessels showed no specific labeling (Figure 6).

**Discussion**

We demonstrated that 20-HETE is the major product of AA conversion catalyzed by microsomal P450 enzymes in mouse
Figure 5. In situ hybridization of P450 4a isoforms. Signals are black layers in phosphorimages (insets; arrowheads) and white dots with dark-field microscopy. c indicates inner cortex; m, medulla. A, P450 4a-10 oligo, water/vehicle (group 2). B, P450 4a-12 oligo, water/vehicle. C, P450 4a-14 oligo, water/vehicle. D, Controls (displacement). E, P450 4a-14 oligo, DOCA-salt/vehicle (group 3) (note absent signals). F, P450 4a-14 oligo, DOCA-salt/bezafibrate (group 5). Note the P450 4a-14 induction with bezafibrate. Magnification insets, ×2.3; micrographs, ×100; bar (C)=250 μm.
kidneys and suggest that a reduced capacity to produce this metabolite is linked to the development of DOCA-salt–induced hypertension.

Candidates for AA hydroxylation in mice are the P450 forms 4a-10, 4a-12, and 4a-14, 21–23 which were, however, not characterized yet in terms of their substrate specificity. In situ hybridization experiments revealed that the 3 mouse P450 4A forms were highly concentrated within tubular structures. mRNAs for P450s 4a-10 (detected in all groups) and 4a-14 (detected preferentially in bezafibrate-treated mice) were predominantly expressed in the proximal tubules, which is also the major site of P450 4A expression in rat kidneys.24,25 P450 4a-12 mRNA (detected only in control mice) showed a broader distribution, which also included other tubular structures. Vessels and glomeruli were not labeled; however, more sensitive methods, such as microdissection followed by reverse transcription–polymerase chain reaction, will be required to determine whether these renal structures express P450 4A isoforms, as recently shown in the rat.26–28

DOCA-salt treatment decreased microsomal AA hydroxylase activities, the total microsomal P450 content, and particularly the P450 4A proteins. Long-term treatment with bezafibrate, which we previously showed to improve renal hemodynamics in DOCA-salt hypertensive mice,15 almost completely restored microsomal AA hydroxylase activities. In addition, our in situ hybridization data indicated a strong P450 4a-14 induction in the proximal tubules. Lovastatin, which also increased renal blood flow, had no significant influence on the P450 system. However, lovastatin may act through entirely different mechanisms.12,29

Our results that show diminished AA hydroxylase activities and reduced P450 4A expression in DOCA-salt mice and improved renal hemodynamics on P450 4A induction by bezafibrate are in accord to findings reported in salt-sensitive Dahl rats.13,30 However, they are in contrast to a recent report that shows increased AA hydroxylase activity and prevention of hypertension with P450 enzyme inhibition in DOCA-salt–treated rats.11 In SHR, increased 20-HETE production was also reported. The reasons for these discrepancies are not immediately obvious. In addition to model, species, and strain differences, the heterogeneous role of 20-HETE at different sites within the kidney is becoming increasingly apparent. As reviewed recently, several different aspects of 20-HETE action must be considered in the kidney. First, in support of a prohypertensive role, 20-HETE was shown to be a locally formed vasoconstrictor in renal arterioles26 that acts by inhibiting a calcium-activated potassium channel31,32 and partially mediates the response to endothelin-1.32 Second, in favor of an antihypertensive role, 20-HETE formed in renal tubular structures promotes salt excretion and diuresis by inhibiting the Na$^{+}$,K$^{+}$-ATPase,33 the Na$^{+}$,K$^{+}$-2Cl$^{-}$ cotransporter,34 and HCO$_3^-$ reabsorption.35 Third, 20-HETE can be further metabolized by cyclooxygenase, yielding a prostaglandin analog with vasodilator function36,37 and other vasoactive metabolites.4 Thus, changes in 20-HETE generation in tubular structures may affect local blood flow after transcellular transport and metabolism.4 Interestingly, bezafibrate treatment improved renal hemodynamics in our DOCA-salt mice and induced a P450 form predominantly localized in tubular structures. However, much information on the mouse model is still missing, making a clear interpretation difficult. The precise identity, catalytic specificity, regulation, and site of action of the individual mouse P450 4a forms must first be defined. The present study provides the first evidence that changes in 20-HETE production are linked to hypertension in mice. Transgenic and knockout approaches may facilitate future attempts to reveal the complex mechanisms that affect P450 genes and the expression of their products and lead to disorders in renal blood flow and function.
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