Regular Article

**Contribution of CYP4A8 to the Formation of 20-Hydroxyeicosatetraenoic Acid from Arachidonic Acid in Rat Kidney**

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**Summary:** 20-Hydroxyeicosatetraenoic acid (20-HETE) has been shown to be an arachidonic acid metabolite of the cytochrome P450 (CYP) enzymes belonging to the CYP4A subfamily and is a predominant regulator of renal vascular tone and tubular ion reabsorption in rat kidney. CYP4A8 is one of the CYP4A enzymes expressed in rat kidney, but its contribution to 20-HETE formation has not been assessed. In order to clarify that the role of CYP4A8, we have developed bacterial expression systems for the expression of recombinant CYP4A8 (rCYP4A8). We also produced an antibody against rCYP4A8 which was used for immunoinhibition and immunohistochemical studies. In a reconstituted system, rCYP4A8 sufficiently catalyzed 20-HETE formation as well as prostaglandin A1 ω-hydroxylation, a marker activity for CYP4A8. In addition, anti-rCYP4A8 sera significantly inhibited prostaglandin A1 ω-hydroxylation and strongly inhibited arachidonic acid ω-hydroxylation in rat kidney microsomes. These observations suggested for the first time that CYP4A8 also contributed to 20-HETE formation in rat kidney. Furthermore, immunohistochemistry suggested that CYP4A8 is present in preglomerular arteries, where 20-HETE has been established to be a vasoconstrictor.

**Key words:** cytochrome P450; CYP4A8; 20-HETE; kidney; immunoinhibition; immunohistochemistry

**Introduction**

20-hydroxy-5,8,11,14-eicosatetraenoic acid (20-HETE) is a major metabolite of arachidonic acid (AA) in male rat kidney and has been shown to play an important role in the regulation of renal vascular tone and tubular ion reabsorption. 20-HETE inhibits the opening of a large conductance potassium channel in vascular smooth muscle cells, resulting in vasoconstriction and the regulation of arteriole vascular tone.1,2) In addition, both the proximal tubular Na⁺, K⁺-ATPase and the Na⁺-K⁺-2Cl⁻ cotransporter in the medullary thick ascending limb of kidney are inhibited by 20-HETE.1-3) In salt-sensitive Dahl rats, reduction of 20-HETE formation causes an increase in Cl⁻ reabsorption.4)

The formation of 20-HETE is catalyzed by cytochrome P450 (CYP) enzymes belonging to the CYP4 gene subfamily5,6) and its physiological actions can be effectively suppressed by inhibitors of the CYP4A enzymes,2,9,10) indicating that CYP4A enzymes are involved in the formation of 20-HETE. Four CYP4A enzymes (CYP4A1, 4A2, 4A3 and 4A8) have been isolated from rats and are present in rat kidney. CYP4A2 is the major constitutive CYP enzyme in rat kidney (approximately 70% of total CYP in rat kidney microsomes) and is expressed at significantly higher levels in spontaneous hypertensive rats than in normal rats.11) 20-HETE formation is also higher in spontaneous hypertensive rats when compared to normal11-14) these observations have lead to the theory that CYP4A2 may play a significant role in renal 20-HETE formation and the regulation of vascular tone in rats. However, this idea is inconsistent with results from previous reports. For example, Kroetz et al. demonstrated that 20-HETE formation by kidney microsomes and CYP4A2 expression levels increased in an age-depen-
dent fashion in spontaneous hypertensive rats, but they were not synchronized at each other. Another report showed that although CYP4A2 is expressed at a low level in female rat kidney, 20-HETE formation in both male- and female-derived kidney microsomes was similar. Additionally, pretreatment with antisense oligonucleotides of CYP4A2 does not significantly reduce 20-HETE formation in proximal tubules or microvessels of rat kidney. Collectively, these observations suggest that another CYP4A enzyme may be contributing to 20-HETE formation in rat kidney.

In the other members of the CYP4A enzyme family, CYP4A1 and CYP4A3 are expressed at very low levels in rat kidney. In contrast, CYP4A8 is constitutively expressed in rat kidney and likely in proglomerular arteries. In order to clarify that, we performed immunoinhibition and immunohistochemical studies and obtained the results suggesting that CYP4A8 also contributes to 20-HETE formation in rat kidney and likely in proglomerular arteries.

Materials and Methods

Materials

[1-14C]-AA (1.89 GBq/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). Prostaglandin A1 (PGA1), lauric acid (LA), AA, dilauroylphosphatidylcholine (DLPC) and dithiothreitol (DTT) were purchased from Wako Pure Chemicals (Osaka, Japan). 20-Hydroxy-PGE1 was a kind gift from Dr. Masami Tsuboshima, Ono Pharmaceuticals, Osaka, Japan. Phosphatidylserine (PS), phosphatidylcholine (PC), 20-HETE, and 12-hydroxylauric acid were kindly supplied from Kao Co., Ltd. (Tokyo, Japan). Bacterial Expression and Purification of CYP4A8

CYP4A8 cDNA (1527-bp) was cloned from a rat kidney cDNA (Stratagene, La Jolla, CA) using PCR with the specific primers designed from a previously reported sequence. Two different expression systems were developed for recombinant CYP4A8 (rCYP4A8) using pCW vector, a kind gift from Professor F. P. Guengerich (Vanderbilt University, Nashville, TN). The first expression system of rCYP4A8 required base modifications at the N-terminal sequence identical to those used in the expression of rCYP3A4 in Escherichia coli (E. coli). These modifications were done by PCR mutagenesis in two stages. The forward PCR primer for the first stage was 5’-TT-ATTAGCAGTTTTTCTCAGTGTCCTGCTG-3’ and that for the second was 5’-CGGCGATGGCT-CGTATTAGCAGTTTTTCTC-3’. The reverse primer in both rounds of PCR was 5’-CAGCCGCA-CAAGCTTAGATTGGAGCTTT-3’. The rCYP4A8 PCR product was inserted into the pCW vector according to the method of Gillam et al. The second expression system involved modifications to the one above to include a (His)6-tag at the C-terminus of rCYP4A8.

DH5αF’IQ E. coli competent cells (Gibco-BRL, Gaithersburg, MD) were transformed with pCW-rCYP4A8 or pCW-(His)6-rCYP4A8 plasmids and these recombinant enzymes were expressed as previously described. E. coli cells were harvested and preparation of the spheroplast and membranes was done by slight modification to a method previously described. rCYP4A8 was principally purified according to the published methods. In a case of (His)6-rCYP4A8, bacterial membranes were solubilized and mixed with TALON metal affinity resin (CLONTECH Lab. Inc., Palo Alto, CA). (His)6-rCYP4A8 was eluted with 100 mM imidazole and further purified with a KB Type-S column.

The purity of the isolated enzymes was judged by SDS-PAGE using an acrylamide concentration of 7.5% (w/v). Proteins were visualized with Coomassie Brilliant Blue R250.

Animals and Tissue Preparation

Male and female Sprague-Dawley rats were obtained from Japan Clea Laboratories (Tokyo, Japan) and sacrificed at 8 weeks of age for tissue preparation. Both kidneys were homogenized with 50 mM tris-HCl buffer (pH 7.4) containing 20% (w/v) sucrose and 0.1 mM EDTA, and microsomal fractions were obtained by differential centrifugation as previously reported. For immunohistochemistry, rat tissues were perfused with 0.1 M phosphate buffer (pH 7.2) containing 4% (w/v) paraformaldehyde and 0.5% (w/v) picric acid. Kidneys were removed and fixed in the above solution for 2 h at room temperature. After washing and overnight incubation in Holt’s gum sucrose solution, 10 μm frozen tissue sections were prepared on poly-L-Lysine coated slides and stored at −40°C prior to immunohistochemical staining.

Preparation of Antibodies Against rCYP4A8

Antisera against rCYP4A8 were prepared according to the method of Kaminsky et al. Anti-IgG fractions were partially purified with Hi-trap protein G column (Pharmacia LKB Biotech AB, Uppsala, Sweden). Antiserum against CYP4A2 was that as previously reported. Cross-reactivity of this antibody fraction was deter-
mained with CYP4A1, CYP4A2, CYP4A3 and CYP4A8 purified from rat liver or kidney microsomes by immuno blot analysis.

**Determination of PGA1 and Fatty Acid ω-Hydroxylation Activities**

NADPH-CYP reductase and cytochrome b5 were purified from rat liver microsomes as previously described. Recombinant CYP4A8 (50 pmol) was sequentially mixed with NADPH-CYP reductase (0.1 μmol cytochrome c reduced/min), phospholipids (30 μg), and cytochrome b5 (50 pmol). Either these reconstituted systems or rat renal microsomes (0.1 mg protein) were incubated at 37°C for 10–15 min in 0.5 ml potassium phosphate buffer (pH 7.4) containing 0.9% NaCl, and incubated overnight at 4°C with a 1:2000 dilution of preimmune, anti-rCYP4A8 or anti-CYP4A2 rabbit sera. The primary antibody was detected by using Vectastain Elite ABC kit with horseradish peroxidase and peroxidase substrate solution (Vector Laboratory).

**Other Assays**

CYP concentrations were measured spectrophotometrically according to the method of Omura and Sato. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL), using bovine serum albumin as the standard.

**Results**

*Expression and purification of rCYP4A8 and (His)6-linked rCYP4A8 from E. coli.*

The harvested bacterial cells expressing rCYP4A8 showed a typical CO-reduced difference spectrum with the maximum absorbance at 452 nm (data not shown). Expression levels were approximately 200 nmol CYP per 1 liter of culture medium. Following purification, the recovery of rCYP4A8 was 1.9% from whole cells; the comparative CYP content was 2.53 nmol CYP/mg protein. Some contaminated proteins were detected by SDS-PAGE, but the partially purified rCYP4A8 was judged to be acceptable for evaluating metabolic activities in a reconstituted system because no endogenous CYP enzymes were present in E. coli.

(His)6-rCYP4A8 was highly purified using metal affinity chromatography, but its CYP content was lower than that of rCYP4A8 (1.82 nmol CYP/mg protein).

Final preparations of rCYP4A8 and (His)6-rCYP4A8 exhibited a characteristic and identical reduced CO-difference spectra, with maximum absorbance at 452 nm and very little absorbance at 420 nm, indicating the presence of little or no inactive enzyme (data not shown).

*PGA1 and AA ω-hydroxylation by rCYP4A8 in a reconstituted system.*

As previously reported, CYP4A8 possesses PGA1 ω-hydroxylation activity; which is not catalyzed by CYP4A1, CYP4A2 and CYP4A3. Therefore, PGA1 ω-hydroxylation activity was measured using the partially purified rCYP4A8. The PGA1 ω-hydroxylated metabolite was formed by rCYP4A8 in the reconstituted system and its formation was significantly enhanced by the addition of b5 [0.25 nmol/min/nmol CYP vs. 2.73 nmol/min/nmol CYP (DLPC:PS = 1:1)]. Furthermore, various combinations of phospholipids produced a large range of PGA1 ω-hydroxylation activities as following; 0.13 (DLPC:PC :PS = 1:1), 0.52 (PC), 0.78 (DLPC:PC:PS = 1:1:1) and 3.72 (PC:PS = 1:1) nmol/min/nmol CYP. PS was likely to be an important component for the high activity of CYP4A8.

20-HETE formation activity by rCYP4A8 was also measured in the reconstituted system. Reconstituted rCYP4A8 formed 20-HETE from AA even at a physiologically relevant concentration (AA = 5 μM), and the activity was strongly enhanced by the addition of b5, similar to that seen with PGA1 ω-hydroxylation activity. The turnover rate of 20-HETE formation by rCYP4A8 was 0.78 and 2.97 nmol/min/nmol CYP in the absence of b5 and 2.55 and 13.8 nmol/min/nmol CYP in the presence of b5 at 5 and 30 μM of AA, respectively.

*Effects of anti-rCYP4A8 on LA ω-hydroxylation and 20-HETE formation (AA ω-hydroxylation) by rat kidney microsomes.*

In order to assess the contribution of CYP4A8 to 20-HETE formation in rat kidney microsomes, an immunoinhibition study was performed using antibodies.
Effects were 77% inhibition of PGA1 \( \omega \)-hydroxylation activity in kidney microsomes from male and female rats, respectively. The maximum inhibitory effect of anti-CYP4A8 antibody was much higher in female rats than in male ones. Therefore, we compared the effects of anti-rCYP4A8 IgG on LA \( \omega \)-hydroxylation and 20-HETE formation activities (Table 1). Kidney microsomes were pre-incubated for 30 min at room temperature with 0.25 mg IgG protein per 0.1 mg microsomal protein and then incubated with 200 \( \mu \)M of LA (A) or 5 \( \mu \)M of AA (B). Activity values represent the mean ± S.D. from 4 or 3 animals. The relative values against control condition are shown in the parentheses. *Significantly different from control group \((p<0.05, \text{Dunnett’s } t\)-test\).

Fig. 1. Immunoblot analysis of purified rat CYP4A enzymes and rat kidney microsomes with anti-rCYP4A8 serum. Purified CYP4A enzymes (lane 1, CYP4A1; lane 2, CYP4A2; lane 3, CYP4A3; lane 4, CYP4A8; 0.5 pmol CYP/well) and kidney microsomal protein from male rats (lane 5; 5 \( \mu \)g microsomal protein/well) were transferred to nitrocellulose membrane after SDS-PAGE and immunostained using anti-rCYP4A8 serum.

Table 1. Effect of anti-rCYP4A8 IgG on LA \( \omega \)-hydroxylation and 20-HETE formation by male and female rat kidney microsomes.

<table>
<thead>
<tr>
<th>Condition</th>
<th>LA ( \omega )-hydroxylation activities (nmol/min/mg protein, ( n = 4 ))</th>
<th>20-HETE formation activities (pmol/min/mg protein, ( n = 3 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Control</td>
<td>1.00 ± 0.05 (100)</td>
<td>1.09 ± 0.14 (100)</td>
</tr>
<tr>
<td>Preimmune</td>
<td>0.97 ± 0.04 (97)</td>
<td>0.99 ± 0.13 (92)</td>
</tr>
<tr>
<td>Anti-rCYP4A8</td>
<td>0.63 ± 0.06* (62)</td>
<td>0.50 ± 0.14* (46)</td>
</tr>
</tbody>
</table>

Kidney microsomes were pre-incubated for 30 min at room temperature with 0.25 mg IgG protein per 0.1 mg microsomal protein and then incubated with 200 \( \mu \)M of LA (A) or 5 \( \mu \)M of AA (B). Activity values represent the mean ± S.D. from 4 or 3 animals. The relative values against control condition are shown in the parentheses. *Significantly different from control group \((p<0.05, \text{Dunnett’s } t\)-test\).
Contribution of CYP4A8 to 20-Hydroxyeicosatetraenoic Acid Formation

Fig. 2. Immunohistochemical localization of CYP4A8 and CYP4A2 in male rat kidney. Frozen tissue segments of whole kidney from male rats were immunostained with preimmune (A, negative control), anti-rCYP4A8 (B), and anti-CYP4A2 (C) sera and a positive immunostaining was shown in brown color (A-C; bar = 1 mm). A cortex area in (B) and (C) was partially magnified (D, anti-CYP4A8 and E, anti-CYP4A2) and including the following regions; proximal straight tubule (PST), proximal convoluted tubule (PCT), cortical collecting duct (CCD), and glomerulus (Gm) sections (D and E; bar = 0.1 mm). Additional magnified pictures from those of whole kidney showed preglomerular arteries in outer stripe of outer medulla immunostained with preimmune (F, negative control), anti-rCYP4A8 (G), and anti-CYP4A2 (H) sera, respectively (F-H; bar = 20 μm).
ner medulla. These immunohistochemistry observations are consistent with the results of Stromstedt et al. who did in situ hybridization using a specific oligonucleotide probe for CYP4A8. Magnification of the strongly stained region showed that rCYP4A8 immunoreactive proteins were localized in the proximal straight tubules and were weakly distributed in proximal convoluted tubules (Fig. 2D). In contrast, there was no detectable immunostaining in the distal convoluted tubules, cortical collecting ducts or glomerulus. Magnification of the outer stripe of the outer medulla clearly showed immunostaining with anti-rCYP4A8 in the preglomerular arteries, although the extent of staining was lower than in other positive regions (Fig. 2G). There was no immunostaining with anti-rCYP4A8 serum in afferent arterioles or arcuate arteries (data not shown).

The regions that showed positive staining for CYP4A2 were similar to those for CYP4A8, but were slightly expanded to the cortex (Fig. 2C) due to the fact that the immunostaining was distributed more strongly in the proximal convoluted tubules (Fig. 2E). Unlike anti-rCYP4A8 staining, anti-CYP4A2 serum produced no immunostaining in the preglomerular arteries (Fig. 2F and 2H); however, like with anti-rCYP4A8, the afferent arterioles and arcuate arteries were not stained by anti-CYP4A2 (data not shown).

In female rats, the distribution of immunostained proteins was similar to that in male rats both with the anti-CYP4A2 and anti-rCYP4A8 sera (data not shown). The staining density with anti-CYP4A2 sera was much lower in female rats than in males, but no significant difference was observed in immunodensity with anti-rCYP4A8 between males and females.

**Discussion**

Bacterially expressed rCYP4A8 extensively catalyzed 20-HETE formation, as has been shown with purified enzyme from rat kidney microsomes. Our present study demonstrated that the turnover rate of reconstituted CYP4A8 was strongly affected by the presence of b5 and phospholipid composition. Recently, Hoch et al. succeeded in the expression of (His)_6-linked rCYP4A8 enzyme in E. coli, but formation of 20-HETE was ~1 nmol/min/nmol CYP even at a high substrate concentration (AA = 100 μM), much lower than formation rates here. Additionally, Nguyen et al. reported that rCYP4A8 expressed in Sf9 cell membranes could not form 20-HETE. These discrepant results might be due to the use of suboptimal b5 and phospholipid concentrations in these studies.

The antibody prepared against rCYP4A8 partially inhibited LA ω-hydroxylation as shown in Table 1. The antibody has little cross-reactivity against CYP4A2 (Fig. 1) and has a low inhibitory effect on CYP4A2 catalyzed reactions. This was supported by the different inhibitory effects between male and female rats (Table 1) which was consistent with the fact that CYP4A2 is a male-specific CYP isoform and mainly responsible for LA ω-hydroxylation in male rat kidney microsomes. Therefore, the ability of anti-rCYP4A8 to inhibit LA ω-hydroxylation activity was found to be due to inhibition of CYP4A8, and not due to the inhibition of CYP4A2. Accordingly, the prominent inhibition of 20-HETE formation with anti-rCYP4A8 IgG confirmed that CYP4A8 is contributing to 20-HETE formation in rat kidney. As the content of CYP4A8 was about 10-fold less than that of CYP4A2 in kidney microsomes from male rats, the turnover rate of CYP4A8 must be much higher than that of CYP4A2. The turnover rate of rCYP4A8 obtained here was 13.8 nmol/min/nmol CYP at 30 μM AA and is approximately 10-fold higher than rates obtained with purified CYP4A2 enzyme or recombinant CYP4A2 enzymes (0.9–2.0 nmol/min/nmol CYP at 30–100 μM of AA). The relative contribution of CYP4A2 and CYP4A8 to 20-HETE formation, estimated from their turnover rates and relative abundances, closely correlates with the results observed in the immuno-inhibition study here (Table 1). Moreover, as previously reported by Ma et al., 20-HETE formation activities in kidney microsomes were higher in females compared with males (Table 1), an indication that CYP4A8 was capable of the formation of physiologically relevant quantities of 20-HETE. As the antibody preparation raised against rCYP4A8 displayed high cross-reactivity with CYP4A1, there still remains the possibility of participation of CYP4A1 in renal 20-HETE formation, however CYP4A1 expression levels are very low in rat kidney. Collectively, these observations suggest that CYP4A8 also plays a significant role in 20-HETE formation in rat kidney.

When rat kidney segments were immunostained with anti-rCYP4A8 or antiCYP4A2 sera, there was distinct localization of stain in the proximal tubules, but not in the glomerulus or other regions (Fig. 2). These stained areas match reported renal distributions for the mRNAs for these enzymes as well as the distribution of 20-HETE formation. In addition, positive immunostaining in the preglomerular arteries was observed only with antisera raised against rCYP4A8, not with anti-CYP4A2 (Fig. 2G and 2H), strongly suggesting that CYP4A8 is directly involved in 20-HETE formation in this region. On the other hand, because the antisera raised against rCYP4A8 displays strong cross-reactivity with CYP4A1 (Fig. 1), this positive immunostaining might include CYP4A1 molecules. Specific oligonucleotide probes have demonstrated that CYP4A1 expression in rat kidney is very low, but pretreatment with an antisense oligonucleotide to CYP4A1 reduces both renal 20-HETE formation and blood pressure in rats. Although CYP4A1 might be
localized at preglomerular arteries, its low expression level in rat kidney supports the idea that CYP4A8 is one of the predominant enzymes responsible for 20-HETE formation and the regulation of renal vasoconstriction via inhibition of the Ca\(^{2+}\)-activated potassium channels.

Recently, Gebremedhin et al. reported that both CYP4A1 and CYP4A8 were expressed at a similar level in rat cerebral arterioles.\(^{40}\) Definitive clarification of the relative contribution of the CYP4A enzymes to 20-HETE formation in preglomerular arteries requires further investigation.

In this study, we expressed rCYP4A8 in \textit{E. coli} and prepared an antibody against this enzyme. Experimental evidence showed that CYP4A8 is one of the major contributors to 20-HETE formation in rat kidney, in spite of its low content in comparison to CYP4A2. The high formation rate of 20-HETE by CYP4A8 might be sufficient to produce enough 20-HETE to inhibit a Ca\(^{2+}\)-activated potassium channel in the preglomerular arteries.

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