Localization of cytochrome P-450 4A isoforms along the rat nephron

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Ito, Osamu, Magdalena Alonso-Galicia, Kathleen A. Hopp, and Richard J. Roman. Localization of cytochrome P-450 4A isoforms along the rat nephron. Am. J. Physiol. 274 (Renal Physiol. 43): F395–F404, 1998.—The expression of P-450 4A isoforms responsible for the formation of 20-hydroxyeicosatetraenoic acid (20-HETE) was examined using the reverse transcription and polymerase chain reaction in various nephron segments and preglomerular arterioles microdissected from the kidneys of Sprague-Dawley rats. Expression of cytochrome P-450 4A1, 4A2, 4A3, and 4A8 mRNA could be detected in RNA extracted from the whole kidney. The expression of P-450 4A1, 4A2, and 4A8 mRNA was similar in the kidney of male and female rats, whereas the expression of 4A2 mRNA was fourfold greater in the kidney of male vs. female rats. At the single-nephron level, P-450 4A1 mRNA could not be detected in either preglomerular arterioles or any nephron segments. P-450 4A2 mRNA was readily detected in preglomerular arterioles, glomeruli, proximal convoluted tubule (PCT), proximal straight tubule (PST), medullary thick ascending limb (MTAL), cortical thick ascending limb (CTAL), cortical collecting duct (CCD), outer medullary collecting duct (OMCD), and inner medullary collecting duct (IMCD). P-450 4A3 mRNA was also detected in every nephron segment, but the expression of this isoform was barely detectable in preglomerular arterioles. The expression of P-450 4A8 mRNA was detected in the glomerulus, PCT, PST, CTAL, and CCD. It was not detectable in preglomerular arterioles, MTAL, OMCD, or IMCD. Immunoblot analysis using a P-450 4A antibody exhibited a strong signal for P-450 4A protein in the proximal tubule. Smaller signals were also observed in glomerulus, MTAL, and preglomerular arterioles, but no signal could be detected in the IMCD. A similar pattern of P-450 4A protein expression was seen in kidney sections immunostained with this antibody. These results indicate that the expression of P-450 4A isoforms in the kidney of rats is sex dependent and that different P-450 4A isoforms are expressed throughout various nephron segments and the renal vasculature of rats.

20-hydroxyeicosatetraenoic acid; cytochrome P-450 4A isoforms; nephron segment; microdissection; renal hemodynamics; tubular transport

Recent studies have indicated that 20-hydroxyeicosatetraenoic acid (20-HETE) is a major metabolite of arachidonic acid produced in the kidney of humans (25), rat (20), and rabbit (4) and that this substance plays an important role in the regulation of both renal tubular (4, 20) and vascular function (8, 16, 19, 39). The proximal tubule (PT) (20), thick ascending limb (TAL) (4), and renal microvessels (16, 19) all have been reported to produce 20-HETE when incubated with arachidonic acid. 20-HETE is a potent constrictor of the renal arterioles (8, 16, 19, 39), and inhibition of 20-HETE production has been reported to block autoregulation of renal blood flow and tubuloglomerular feedback in the rat in vivo (37, 38). 20-HETE also inhibits Rb\(^{\text{+}}\) uptake in the medullary TAL cells of rabbits (7) and Na\(^{+}\)-K\(^{+}\)-ATPase in the PT of rats (22). Additional studies using P-450 inhibitors have revealed that the endogenous synthesis of 20-HETE regulates Cl\(^{-}\) transport in the TAL (36) and plays an important role in the long-term control of arterial pressure (23, 28).

The formation of 20-HETE from arachidonic acid is catalyzed by enzymes of the cytochrome P-450 4A family (26), which have been identified in rat, rabbit, and humans (21, 30). In the rat, four isoforms, P-450 4A1, 4A2, 4A3, and 4A8, have been cloned (14, 17, 18, 31). However, the high degree of homology between P-450 4A isoforms has limited the ability to develop antibodies or cDNA probes that can distinguish between the isoforms. Indeed, most of the antibodies and cDNA probes used in past studies cross-react between the isoforms. Thus considerable uncertainty remains regarding the expression of the isoforms in the kidney and the cell types in which these isoforms are expressed.

In the present study, we designed primers and reverse transcription and polymerase chain reaction (RT-PCR) protocols that could specifically amplify each of the P-450 4A isoforms expressed in the kidney of rats. These primers were then used to map the distribution of P-450 4A isoforms in various nephron segments and preglomerular arterioles microdissected from the kidneys of rats. Finally, the expression of P-450 4A isoforms was confirmed at the protein level by immunoblot analysis of bulk-isolated nephron segments and by immunohistochemistry using an antibody that recognizes each of the P-450 4A isoforms.

Methods

Microdissection of nephron segments. Experiments were performed on 7-wk-old, male and female, Sprague-Dawley (SD) rats purchased from Harlan Sprague Dawley Laboratories (Indianapolis, IN). The rats were anesthetized with an injection of pentobarbital sodium (50 mg/kg ip) and the abdominal aorta was cannulated with a polyethylene PE-50 catheter below the left renal artery. The blood flow to the left kidney was interrupted, and the kidney was flushed with 10 ml of cold dissection solution (4°C), followed by 10 ml of the same solution containing 1 mg/ml collagenase (type II, 190...
the corticopapillary axis was made. The tissue section was placed in 10 ml of the dissection solution containing 1 mg/ml collagenase and hyaluronidase. The tissue was incubated for 30 min at 37°C on a rotary shaker while O2 gas was continuously blown over the incubation. After 30 min, the tissue was transferred to 20 ml of fresh dissection solution and was kept at 4°C. The following structures were microdissected under a stereomicroscope (>100) with fine forceps: glomeruli, proximal convoluted tubule (PCT), proximal straight tubule (PST), medullary TAL (MTAL), cortical TAL (CTAL), cortical collecting duct (CCD), outer medullary collecting duct (OMCD), inner medullary collecting duct (IMCD). The length of the tubules was measured using a calibrated eyepiece micrometer, and 30-mm length of tubules and/or 30 glomeruli were placed in 100 µl of phenol and guanidinium thiocyanate solution (TRIzol, Gibco-BRL; Life Technologies, Gaithersburg, MD) until RNA extraction. Kidney or liver tissue was resuspended in 10 ml of ice-cold dissection solution, and the integrity of the RNA was examined using a FluorImager SI (Molecular Dynamics, Sunnyvale, CA). Negative controls experiments included PCR amplification of RT reactions in which the reverse transcriptase was not added. Positive controls included amplification of plasmids (10 ng) containing full-length cDNA for each of the P-450 4A isoforms. The P-450 4A1, 4A2, 4A3, and GAPDH primer pairs were designed to amplify fragments of 902, 321, 321, 1956, and 970 bp, respectively, and they did not amplify genomic DNA because of large interspersed introns. In some experiments the PCR products were separated and sequenced using dideoxynucleotide method to confirm that the products obtained had the expected sequence. The structures of the primers used were P-450 4A1, 4A2, 4A3, 4A8, and GAPDH primer pairs were designed to amplify fragments of 902, 321, 321, 1956, and 970 bp, respectively, and they did not amplify genomic DNA because of large interspersed introns. Positive controls included amplification of plasmids (10 ng) containing full-length cDNA for each of the P-450 4A isoforms. The P-450 4A1, 4A2, 4A3, and GAPDH primer pairs were designed to amplify fragments of 902, 321, 321, 1956, and 970 bp, respectively, and they did not amplify genomic DNA because of large interspersed introns. In some experiments the PCR products were separated and sequenced using dideoxynucleotide method to confirm that the products obtained had the expected sequence. The structures of the primers used were P-450 4A1, 4A2, 4A3, 4A8, and GAPDH primer pairs were designed to amplify fragments of 902, 321, 321, 1956, and 970 bp, respectively, and they did not amplify genomic DNA because of large interspersed introns. Positive controls included amplification of plasmids (10 ng) containing full-length cDNA for each of the P-450 4A isoforms. The P-450 4A1, 4A2, 4A3, 4A8, and GAPDH primer pairs were designed to amplify fragments of 902, 321, 321, 1956, and 970 bp, respectively, and they did not amplify genomic DNA because of large interspersed introns. Positive controls included amplification of plasmids (10 ng) containing full-length cDNA for each of the P-450 4A isoforms. The P-450 4A1, 4A2, 4A3, 4A8, and GAPDH primer pairs were designed to amplify fragments of 902, 321, 321, 1956, and 970 bp, respectively, and they did not amplify genomic DNA because of large interspersed introns.
renal cortex of rats was sliced with a Stadie-Riggs microtome into 200-µm-thick sections and placed in 10 ml of the dissection solution containing 1 mg/ml collagenase (type II, 190 U/mg, Worthington Biochemical), 1 mg/ml soybean trypsin inhibitor (10,000 U/mg, Sigma Chemical), and 1 mg/ml BSA. The tissues were incubated for 60 min at 37°C on a rotary shaker while O₂ gas was continuously blown over the incubation. After 60 min, the supernatant was transferred to a tube and diluted with 10 ml of cold dissection solution. Ten minutes were allowed for the larger intact PT to settle, and the supernatant was discarded. The tubules were resuspended in a 50% solution of Percoll and dissection solution and centrifuged at 12,000 g for 30 min at 4°C. The bottom fraction, which was enriched with PT (>95%), was collected and rinsed several times with cold dissection solution. To ensure that the PT used in our experiments were not contaminated with other tissue types, this fraction was examined using a stereomicroscope, and individual PT were picked up by microdissection and placed in 100 µl of cold homogenization buffer.

MTAL were isolated using an enzymatic digestion followed by a sieving technique (32). The kidneys of rats were flushed with 10 ml of cold dissection solution and then perfused with 10 ml of the same solution containing 1 mg/ml collagenase (190 U/mg), 1 mg/ml hyaluronidase (300 U/mg, Sigma Chemical), and 1 mg/ml soybean trypsin inhibitor (10,000 U/mg). The inner stripe of the outer medulla was carefully excised and dissected into small pieces along the corticomedullary axis using fine forceps. This tissue was incubated in 10 ml of the dissection solution containing 0.3 mg/ml collagenase, hyaluronidase, and trypsin inhibitor for three 15-min periods at 37°C on a rotary shaker while O₂ gas was continuously blown over the incubation. After each incubation, the supernatant was collected and placed on a 70-µm nylon sieve. The sieve was rinsed several times with cold dissection solution containing 1% BSA, and the retained tissue was washed off the sieve. This fraction was enriched with MTAL (>95%). To ensure that the MTAL used in our experiments were not contaminated with other tissue types, this fraction was examined using a stereomicroscope, and individual MTAL were picked up by microdissection and placed in 100 µl of cold homogenization buffer.

IMCD were isolated from inner medulla using the same procedure as that described for MTAL except that a higher concentration of hyaluronidase (9,000 U/ml) was used for enzymatic digestion for 60 min. This preparation was enriched with IMCD (>90%). To ensure that the IMCD were not contaminated with other tissue types, this preparation was examined using a stereomicroscope, and individual IMCD were picked up by microdissection and placed in 100 µl of cold homogenization buffer.

Bulk isolated glomeruli, PT, MTAL, and IMCD were homogenized by sonication for 15 s at moderate power. The homogenate was centrifuged at 9,000 g for 15 min, and the supernatant was transferred to a fresh microcentrifuge tube. The protein concentrations of the samples were measured using the Bradford method (3) with bovine γ-globulin (Bio-Rad Laboratories, Hercules, CA) as a standard. The samples were snap-frozen in liquid nitrogen, and stored at −80°C until the immunoblot experiments were performed.

Immunoblot analysis. Proteins were separated by electrophoresis on a 10 × 20-cm, 8.5% sodium dodecyl sulfate polyacrylamide gel for 1.5 h at 150 V. The proteins were transferred electrophoretically to a nitrocellulose membrane at 100 V in a transfer buffer consisting of 25 mM Tris·HCl, 192 mM glycine, and 20% methanol for 1 h at 4°C. The membrane was blocked overnight at 4°C by immersion into a TBST-20 buffer containing 10 mM Tris·HCl, 150 mM NaCl, 0.08% Tween 20, and 10% nonfat dry milk. The membrane was then incubated for 2 h with a 1:4,000 dilution of a rabbit polyclonal antibody raised against a synthetic peptide in the rat P-450 4A1 sequence that recognizes the P-450 4A1, 4A2, and 4A3 isoforms (11). The membrane was rinsed several times with TBST-20 buffer and then incubated with a 1:2,000 dilution of a horseradish peroxidase-coupled, goat anti-rabbit second antibody (Santa Cruz Biolaboratory, Santa Cruz, CA) for 1 h. Excess second antibody was removed by three to four washes in TBST-20, and the immunoblots were developed using an enhanced chemiluminescence kit (ECL; Amersham, Arlington Heights, IL).

Immunohistochemistry. The kidneys were flushed with 10 ml of ice-cold dissection solution and perfused with 10–20 ml of a fixative solution consisting of 2% paraformaldehyde and 15% picric acid in a 100 mM phosphate buffer. The kidneys were removed and placed overnight in the fixative solution at 4°C. The kidneys were transferred to a 10 mM phosphate buffer solution containing 1 M sucrose and kept overnight. The tissue was then embedded in OCT compound (Miles Scientific, Naperville, IL), and frozen 20-µm-thick sections were prepared. They were mounted on Vectabond-coated slides and air dried. Nonspecific binding sites were blocked by covering the section with a 5% solution of fetal bovine serum and normal goat serum in Tris-buffered saline (TBS) for 2 h. The slides were rinsed with TBS and incubated with a 1:200 dilution of a rabbit polyclonal P-450 4A antibody at room temperature for 60 min. The slides were rinsed with TBS and covered with a 1:200 dilution of a biotinylated-coupled, goat anti-rabbit second antibody for 30 min. The slides were rinsed with TBS, treated for 15 min with a 3% solution of hydrogen peroxide to inactivate endogenous peroxidase activity, and washed with TBS. The slides were lightly counterstained with hematoxylin and examined at ×400 using an Olympus BHT microscope (Tokyo, Japan). In every experiment, paired sections were incubated with preimmune serum or immune serum plus a high concentration (100 µg/ml) of the antigen peptide.

Statistical analysis. Data are presented as means ± SE. The significance of differences in mean values was evaluated using analysis of variance and Duncan’s multiple range test.

RESULTS

Confirmation of specificity of PCR primers for P-450 4A isoforms. The specificity of each of the P-450 4A PCR primer pairs was tested by amplifying 10 ng of a full-length P-450 4A1, 4A2, 4A3, and 4A8 cDNA clone in a pcRII vector (Invitrogen, Burlingame, CA). The reactions were amplified for 35 cycles using an annealing temperature of 60°C. The results of these experiments are presented in Fig. 1. Each of the primer pairs amplified a product of the expected size (P-450 4A1, 902 bp; P-450 4A2, 321 bp; P-450 4A3, 321 bp; P-450 4A8, 1,956 bp) when reacted with the corresponding cDNA clone (Fig. 1). The P-450 4A1, 4A3, and 4A8 primers were all isofrom specific and did not amplify any products when reacted with the cDNAs of the noncorresponding P-450 4A isoforms (Fig. 1, A–C). In contrast, the P-450 4A2 primers cross-reacted with the P-450 4A3 cDNA clone and produced a product of the size expected for P-450 4A2 (Fig. 1D). Since the P-450 4A2 and 4A3 isoforms are highly homologous (>97%)
of 0.125–1.0 µg (Fig. 2).

Expression of P-450 4A isoforms in kidney and liver. The expression of P-450 4A isoforms was compared in the kidney and liver of 7-wk-old male SD rats. In these experiments, 0.5 µg of RNA was amplified by RT-PCR for 35 cycles with either of the P-450 4A or GAPDH primer pairs. The results of these experiments are presented in Fig. 3. The P-450 4A1, 4A2, and 4A3 primers produced strong bands from the liver samples, whereas the expression of P-450 4A8 mRNA could not be detected. In the kidney samples, each of the primers produced a band, although the P-450 4A1 bands was barely detectable. Amplification of GAPDH was similar in the kidney and liver samples. On a semiquantitative basis, the expression of P-450 4A1 mRNA was approximately fourfold greater in the liver than in the kidney, whereas the expression of P-450 4A2 mRNA was approximately twofold greater in the kidney than in the liver. Additionally, it should be noted that the P-450 4A1 primer amplified two different bands (902 and 827 bp) with similar intensity from the kidney and liver samples. These two bands correspond to the two alternate splice variants of P-450 4A1 that have been described, which are identical in the coding region but differ by a 75-nucleotide deletion in the 3′ noncoding region (10).

Comparison of expression of P-450 4A isoforms in kidney of male vs. female rats. We also compared the expression of the P-450 4A isoforms in the kidney of male and female 7-wk-old SD rats, since there are reports that the expression of these isoforms may be sex dependent (15, 31). In these experiments, 0.5 µg of RNA size from the P-450 4A2 cDNA clone, but they did not amplify a product when reacted with the P-450 4A3 cDNA clone.

Linearity of RT-PCR reactions. Numerous experiments were performed to determine the maximum number of PCR cycles that can be used to make semiquantitative comparisons of the expression of P-450 4A isoforms. The results of these experiments are presented in Fig. 2. Using 35 cycles, we found that there was a linear relationship between the fluorescent intensity of the RT-PCR products for each of P-450 4A isoforms and the amount of kidney RNA added to the RT-PCR reaction over the range of 0.125–1.0 µg (Fig. 2).

(18), it was impossible to design another P-450 4A2 primer pair that is more specific than the primer pair we already were using. Therefore, we tried a different approach to attain the required specificity. We repeated the experiments using a more stringent step-down PCR protocol, in which the reactions were first amplified for 10 cycles at an annealing temperature of 70°C, followed by 25 cycles at 60°C. As illustrated in Fig. 1E, under these conditions the P-450 4A2 primers are isofrom specific and amplify a single product of the expected

Fig. 1. Specificity of P-450 4A1- (A), P-450 4A2- (D and E), P-450 4A3- (B), and P-450 4A8-specific (C) polymerase chain reaction (PCR) primer pairs. Full-length P-450 4A1, 4A2, 4A3, and 4A8 cDNA clones (10 ng) were amplified by PCR for 35 cycles with each of a P-450 4A primer pairs. PCR products were separated by electrophoresis on 1% agarose gels containing ethidium bromide. Expected sizes of PCR products are 902 bp for P-450 4A1, 321 bp for P-450 4A2, 321 bp for P-450 4A3, and 1,956 bp for P-450 4A8. Lane 1, molecular weight marker (MW, 100-bp DNA ladder); lane 2, P-450 4A1 cDNA clone; lane 3, P-450 4A2 cDNA clone; lane 4, P-450 4A3 cDNA clone; lane 5, P-450 4A8 cDNA clone. A, B, and C: P-450 4A1, 4A3, and 4A8 primer pairs amplified a product of the expected size from the corresponding P-450 4A cDNA clone using an annealing temperature of 60°C. D: P-450 4A2 primers cross-reacted with the P-450 4A3 cDNA clone using an annealing temperature of 60°C and produced a product of the size expected for P-450 4A2. E: in this experiment, the PCR reaction was first amplified for 10 cycles at an annealing temperature of 70°C, followed by 25 cycles at 60°C. Under these conditions, the P-450 4A2 primers amplified a single product of the expected size from the P-450 4A2 cDNA clone, but they did not amplify a product from the P-450 4A3 cDNA clone.

Fig. 2. Relationship between the fluorescent intensity of the reverse transcription (RT)-PCR product for each of P-450 4A isoforms vs. the amount of kidney RNA added to the RT-PCR reaction over the range of 0.125–1.0 µg using 35 cycles.
Expression of 4A8 mRNA could not be detected in any of the medullary nephron segments studied, including the MTAL, OMCD, and IMCD (Fig. 5D).

The expression of P-450 4A mRNA was also examined in preglomerular arterioles. In each reaction, RNA that was extracted from a batch of afferent and interlobular arterioles was divided equally among the five tubes and amplified by RT-PCR for 40 cycles. Figure 6 presents the appearance of a typical gel illustrating the pattern of expression of the P-450 4A isoforms in the preglomerular arterioles of both male and female rats. P-450 4A2 mRNA was a major isoform expressed in the preglomerular arterioles of both male and female rats. A small amount of P-450 4A3 mRNA could be detected in preglomerular arterioles isolated from both sexes, whereas the expression of P-450 4A1 or 4A8 could not be detected in preglomerular arterioles isolated from either sex.

Localization of P-450 4A isoforms in the kidney and liver. The expression of P-450 4A proteins was studied by immunoblot analysis of glomeruli, PT, MTAL, IMCD, and preglomerular arterioles bulk isolated from the kidneys of 7-wk-old male SD rats. The results of these experiments are presented in Fig. 7. Microsomal protein (10 µg) prepared from the liver of a clofibrate-treated rat was used as a positive control. Two immunoreactive bands corresponding to P-450 4A1/4A2 and 4A3 isoforms were detected in microsomes prepared

Fig. 3. Expression of P-450 4A isoforms in kidney and liver of rats. RNA (0.5 µg) extracted from kidney and liver of male SD rats was reverse transcribed using random hexamers primer. cDNA was amplified by PCR for 35 cycles with either P-450 4A or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer pairs. Lane 1, molecular weight marker (MW, 100-bp DNA ladder); lanes 2–5, kidney samples; and lanes 6–9, liver samples. P-450 4A1, 4A2, and 4A3 primers produced strong bands from the liver samples, whereas the expression of P-450 4A8 mRNA could not be detected. Each of the primers produced a band from the kidney samples, although the degree of amplification of the P-450 4A1 bands was relatively weak compared with those seen with the other primers. Amplification of GAPDH was similar in the kidney and liver samples.

was amplified by RT-PCR for 35 cycles with either of the P-450 4A or GAPDH primer pairs. The results of these experiments are presented in Fig. 4. Each of the primers amplified a detectable product from the kidneys of both male and female rats. The P-450 4A2 primer produced strong bands from the kidneys of male rats, whereas the P-450 4A2 bands were weaker in the kidneys of female rats. The amplification of GAPDH was similar in each of the samples studied. On a semiquantitative basis, the expression of P-450 4A1, 4A3, and 4A8 mRNA was not significantly different in the kidneys of male and female rats, whereas the expression of P-450 4A2 mRNA was fourfold greater in the kidney of male vs. female rats.

Localization of P-450 4A isoforms mRNA along rat nephron. The expression of P-450 4A mRNA was also examined in microdissected nephron segments. In each reaction, RNA corresponding to a 5-mm length of tubule or 5 glomeruli was amplified by RT-PCR for 40 cycles with either of the P-450 4A or GAPDH primer pairs. The results of these experiments are presented in Fig. 5. Expression of P-450 4A1 mRNA could not be detected in 5-mm lengths of the microdissected nephron segments examined. In contrast, P-450 4A2 and 4A3 mRNA was readily detected in all of the nephron segments studied. Small but detectable signals for 4A8 mRNA were also found in all the cortical nephron segments examined, including glomerulus, PCT, PST, CTAL, and CCD. Expression of 4A8 mRNA could not be detected in 5-mm lengths of the microdissected nephron segments, including glomerulus, PCT, PST, CTAL, and CCD. Expression of 4A8 mRNA could not be detected in any of the medullary nephron segments studied, including the MTAL, OMCD, and IMCD (Fig. 5D).
from the liver of clofibrate-treated rat (Fig. 7, lane 7). Strong bands corresponding to 4A1/4A2 and 4A3 were detected in the lanes loaded with 50 µg of protein from PT (Fig. 7, lane 3). Smaller signals were detected in the lanes loaded with 100 µg of protein from glomeruli, MTAL, and preglomerular arterioles (Fig. 7, lanes 2, 4, and 6), whereas no signal was detected in 100 µg of protein from IMCD (Fig. 7, lane 5).

Immunohistochemistry. Immunohistochemical experiments were also performed to better define the cell types responsible for the expression of P-450 4A protein in the kidney. The results of typical experiments are presented in Fig. 8. In the renal cortex, strong staining (brown to black) for P-450 4A protein was detected in the PT, TAL, glomeruli, and preglomerular arterioles (Fig. 8A). The expression of P-450 4A protein was also detected in macula densa cells and throughout the juxtaglomerular apparatus (Fig. 8B). In the outer medulla, P-450 4A protein was localized in the TAL but not in the collecting ducts (Fig. 8C). In the inner medulla, the collecting ducts and thin ascending and thin descending limbs were not stained, but there was strong staining of vasa recta capillaries between the collecting ducts (Fig. 8D). On higher power examination, it revealed that the intense staining is largely confined to pericytes on the outside of vasa recta capillaries. In control experiments in which the section were incubated with preimmune serum from the same rabbit or immune serum plus a high concentration of the antigen peptide, no visible staining of the sections could be detected (data not shown).

**DISCUSSION**

Recent studies using a variety of P-450 4A inhibitors have indicated that 20-HETE plays an important role in the regulation of both renal tubular (7, 22, 36) and vascular function (8, 16, 19, 37, 39) and in the long-term control of arterial pressure (23, 28). However, the P-450 4A isoforms responsible for the formation of 20-HETE in various cell types of the kidney have not been previously identified. Part of the problem has been that the high degree of homology between P-450 4A isoforms has limited the ability to develop antibodies or cDNA probes that can distinguish between the isoforms. For example, 4A1 shares 65% homology with 4A2 and 4A3 isoforms, whereas the 4A2 and 4A3 isoforms are 97% homologous with each other (17, 18). 4A8 exhibits 76% homology with 4A1 (30). In the present study, we designed and tested RT-PCR primers that could detect the expression of P-450 4A mRNA in an isoform-specific manner. We demonstrated that the P-450 4A1, 4A3, and 4A8 primers were isoform-specific, using an annealing temperature of 60°C (Fig. 1, A–C). However, at this temperature, the P-450 4A2 primers cross-reacted and amplified a 4A3 cDNA clone (Fig. 1D). This problem was overcome by starting at annealing temperature of 70°C and stepping down to 60°C for the later cycles (Fig. 1E).

The results of the present experiments indicated that mRNA for all of the P-450 4A isoforms can be detected in the kidney by RT-PCR and that P-450 4A1, 4A2, and 4A3, but not 4A8 mRNA can be detected in the liver (Fig. 3). Our results are consistent with a previous study using Northern blot analysis with oligonucleotide probes, which indicated that P-450 4A8 mRNA is expressed in the kidney but not in the liver of male Wistar rats (30). Our results also suggest that the expression of P-450 4A1 mRNA is very low but that P-450 4A2 and 4A3 mRNA are constitutively expressed throughout the kidney of male SD rats (Fig. 3). These results are consistent with the original finding of Kimura et al. (18) who reported, using Northern blot analysis with oligonucleotide probes, that P-450 4A2 mRNA is the only isoform that is constitutively expressed at a high level in the kidney of 4- to 6-wk-old male SD rats and that the expression of P-450 4A1 and 4A3 mRNA must be induced by clofibrate treatment to be detectable. Two other groups have reported, using Northern blot analysis, that P-450 4A2 and 4A3 mRNA are constitutively expressed in the kidney, whereas the expression of 4A1 mRNA is nearly undetectable in the kidney of 7-wk-old male SD rats (24) and 8- to 10-wk-old male Fisher 344 rats (31). In contrast, a different
pattern of P-450 4A expression has recently been reported in spontaneously hypertensive rats (SHR), which typically exhibit a higher level of 20-HETE production in the kidney than normotensive rats (20). In these animals, Schwartzman et al. (24) have reported, using immunoblot analysis, that P-450 4A1 and 4A3 but not 4A2 proteins were expressed in the kidney of 3-wk-old male SHR. The expression of P-450 4A1 and 4A3 proteins increased with age and peaked at 5- to 7 wk. Thereafter, the expression of these isoforms declined precipitously. The expression of P-450 4A2 protein was undetectable in the kidney of 5-wk-old SHR, but it gradually increased until it became the dominant isoform expressed in the kidney of adult animals (12–20 wk old). We have also reported that P-450 4A1, 4A2, and 4A3 proteins are all expressed in the kidney of neonatal, 3-wk-old male SHR and Wistar-Kyoto rats. In adult, 16-wk-old rats, however, P-450 4A2 protein was the only isoform that could be detected (29). Thus the available information indicates that the expression of P-450 4A isoforms in the kidney is age dependent and varies in different strains of rats. The factors that influence the regulation of P-450 4A expression and the significance of these differences for the growth and development of the kidney and renal function remain to be explored.

The present study also evaluated whether there are sex-related differences in the expression of P-450 4A

![Fig. 6](image-url) - Expression of P-450 4A isoforms in preglomerular arterioles of male (A) and female (B) rats. RNA was extracted from a batch of afferent and interlobular arterioles was divided equally among the five tubes, reverse transcribed using random hexamers primer, and cDNA was amplified by RT-PCR for 40 cycles with either of a P-450 4A or GAPDH primer pairs. Lane 1, molecular weight marker (MW, 100-bp DNA ladder); lane 2, samples amplified with P-450 4A1 primers; lane 3, samples amplified with P-450 4A2 primers; lane 4, samples amplified with P-450 4A3 primers; lane 5, samples amplified with P-450 4A8 primers; and lane 6, samples amplified with GAPDH primers. P-450 4A2 mRNA was a major isoform expressed in preglomerular arterioles of both male and female rats. A small amount of P-450 4A3 mRNA could be also detected in preglomerular arterioles isolated from both sexes, whereas the expression of P-450 4A1 or 4A8 could not be detected in preglomerular arterioles isolated from either sex.

![Fig. 7](image-url) - Immunoblot analysis of P-450 4A protein in isolated glomeruli, proximal tubules (PT), MTAL, IMCD, and preglomerular arterioles using a polyclonal antibody raised against P-450 4A1 that cross-reacts with P-450 4A1, 4A2, and 4A3 proteins. Lane 1, 10 µg of microsomal protein prepared from the liver of a male rat; lane 2, 50 µg of protein from homogenates from glomeruli; lane 3, 30 µg of protein from homogenates from PT; lane 4, 100 µg of protein from homogenates from MTAL; lane 5, 100 µg of protein from homogenates from IMCD; lane 6, 100 µg of protein from homogenates from preglomerular arterioles; lane 7, 10 µg of microsomal protein prepared from the liver of a clofibrate-treated male rat. Two immunoreactive bands corresponding to P-450 4A1/4A2 and 4A3 isoforms were detected in microsomal protein prepared from the liver of a clofibrate-treated rat. Strong band was detected in the PT. Smaller signals were detected in glomeruli, MTAL, and preglomerular arterioles, whereas no signal was detected in the IMCD samples.
isoforms. P-450 4A2 mRNA was expressed in the kidneys of both male and female SD rats, but the levels of the expression were about fourfold greater in the kidney of male vs. female rats (Fig. 4). This finding is consistent with previous report by Sundseth and Waxman (31), who reported that mRNA and protein for the P-450 4A2 isoform could not be detected in the kidney and liver of 8- to 10-wk-old female Fisher 344 rats, but it could readily be detected in the kidney of male rats. Similarly, Imaoka et al. (15) found that the levels of P-450 4A2 protein are five times higher in the kidney of 10-wk-old male vs. female SD rats. These groups also reported that the expression of P-450 4A2 is regulated by testosterone (15, 31). Overall, there seems to be a consensus that there are sex differences in the expression of P-450 4A isoforms in the kidneys of the rats. However, the significance of this observation in regard to the well-described sex-related differences in renal vascular reactivity, tubular function, and susceptibility to the development of hypertension and glomerular disease remains to be explored.

We also examined the expression of P-450 4A isoforms in nephron segments and preglomerular arterioles microdissected from the kidney of rats. We were unable to detect the expression of P-450 4A1 mRNA in any nephron segment studied, even though this isoform could be amplified from RNA extracted from the whole kidney (Fig. 5). This suggests that the expression of P-450 4A1 mRNA is probably too low to be detected in the small amount (~1 ng) of RNA that could be extracted from a 5-mm length of a tubular segment. To estimate the minimal detectable amount of mRNA needed to obtain a detectable signal, we amplified various amounts of the P-450 4A1 cRNA transcribed from a full-length P-450 4A1 cDNA clone for 40 cycles. These studies indicated that a detectable band could be obtained under the present experimental conditions with as little as 20 copies of transcript. Since a 5-mm length of tubules has been reported to contain 1,500–3,000 cells (33), these results suggest that we should have been able to detect this isoform even if it is expressed as a single copy per cell.

In contrast to the results obtained with P-450 4A1 isoform, the expression of P-450 4A2 and 4A3 were readily detected in microdissected preglomerular arterioles, glomeruli, and in every nephron segment examined (Fig. 5 and 6). Smaller detectable signals for P-450 4A8 mRNA were also found in all cortical nephron segments examined but in none of the medullary nephron segments studied. The expression of P-450 4A isoforms was confirmed at the protein level by the immunoblot analysis on bulk-isolated nephron segments. These studies revealed the presence of two strong P-450 4A protein bands in the PT, likely P-450 4A2 and 4A3, with lesser detectable amounts of P-450 4A protein in glomeruli and MTAL. Moreover, the PT, glomeruli, preglomerular arterioles, and TAL all exhibited staining for P-450 4A protein in the immunohistochemical experiments. Overall, the present results provide the first RT-PCR results on the distribution of
P-450 4A isoforms at the single-nephron level using isoform-specific primers. In general, the results are consistent with recent RT-PCR (24), in situ hybridization (13), and immunoblot experiments (24) using less-specific probes that indicated that the PT was a major site of the expression of P-450 4A mRNA and protein in the kidney of rats. P-450 4A2 was the major isoform expressed in the preglomerular arterioles of both male and female rats (Fig. 6). Because the P-450 4A mRNA is highly expressed in renal tubules, the possibility that the signal seen in vessels is due to contamination with adherent PT fragments cannot be excluded in any RT-PCR study. However, this possibility seems less likely, because 4A2, 4A3, and 4A8 mRNA was detected in cortical nephron segments but only 4A2 was consistently amplified in the microvessel samples. Moreover, the finding that 4A2 seems to be the isoform preferentially expressed in the renal vasculature is consistent with our previous results, which investigated expression of P-450 4A protein using immunoblot analysis (16). It is somewhat surprising that the pattern of the expression of P-450 4A isoforms was similar in preglomerular arterioles of male and female rats, since the expression of P-450 4A2 is so much greater in the kidney of male vs. female rats. This finding is also important because P-450 4A2 is thought to be the constitutively expressed isoform, whereas P-450 4A1 and 4A3 are known to be induced by clofibrate and a number of hormones in the kidney of rats (18). This would suggest that the expression of P-450 4A protein in the renal vasculature may be less likely to be influenced by hormones and drugs than the expression of these isoforms in renal tubules. In contrast to the present findings in renal arterioles, our group has recently reported that P-450 4A1, 4A2, and 4A3 mRNA are expressed in the microvasculature of the crenal muscle (12) and that P-450 4A1 and 4A2 mRNA are expressed in the cerebral vasculature (R. J. Roman and D. R. Harder, unpublished observations) of male SD rats. These studies suggest that the expression of P-450 4A isoforms may differ in vessels obtained from various vascular beds. Since each of the isoforms appear to have different catalytic activities in regard to the production of 20-HETE (M. L. Schwartzman, personal communication), these differences may have some bearing on regional differences in the regulation of vascular tone.

In the present study, the immunohistochemical experiments indicated that P-450 4A protein is also expressed in the macula densa cells and in the pericytes surrounding vasa recta capillaries. We have previously reported that inhibition of 20-HETE production blocks tubuloglomerular feedback responses (38) and increases medullary blood flow (39) in the rat in vivo. Therefore, the present findings further support the view that 20-HETE produced in the macula densa cells and vasa recta capillaries may serve as a locally generated paracrine factor important in regulation of tubuloglomerular feedback and medullary blood flow.

In summary, we designed primers and RT-PCR protocols that could specifically detect expression of P-450 4A mRNA in an isoform-specific manner in the kidney of rats. P-450 4A2, 4A3, and 4A8 mRNA are constitutively expressed in the kidney of male SD rats, whereas the expression of 4A1 mRNA is barely detectable in this tissue. The expression of P-450 4A1, 4A3, and 4A8 mRNA is similar in the kidney of male and female rats, whereas the expression of P-450 4A2 mRNA was fourfold greater in the kidney of male vs. female rats. P-450 4A2 mRNA is constitutively expressed in glomeruli, preglomerular arterioles, and in every nephron segment examined. P-450 4A3 mRNA is also constitutively expressed in all of nephron segments examined. P-450 4A8 mRNA is detectable in the cortical but not in medullary nephron segments nor preglomerular arterioles. Immunoblot analysis and immunohistochemistry indicate that the P-450 4A protein is strongly expressed in the PT and is also expressed in glomeruli, TAL, macula densa, renal arterioles, and vasa recta capillaries.

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