Expression of Peroxisome Proliferator-Activated Receptor Isoform Proteins in the Rat Kidney

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Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors mediating ligand-dependent transactivation. Among the 3 isoforms, PPAR-α is involved in lipid metabolism in the liver, while PPAR-γ(-1 and -2) is involved in adipocyte differentiation. Recently, PPARs have been suggested to be involved in renal electrolyte metabolism as well as atherosclerosis. PPAR-δ is known to regulate cytochrome P450 gene expression, and may possibly affect sodium retention in the kidney. Moreover, PPAR-β/δ is involved in the transcription regulation of blood pressure regulatory genes, including thromboxane and angiotensin II type 1 receptors. In the kidney, although expression of PPARs has been reported, detailed immunohistochemical analyses have not been performed. We here generated isoform-specific anti-PPAR antibodies to localize their proteins in the kidney. Anti-PPAR antibodies were raised against synthetic peptides. Their isoform specificity was confirmed by immunoblot analyses, immunoprecipitations, and antibody supershift experiments by electrophoretic mobility shift assay. We therefore studied the protein expression of PPARs in the kidney of adult Sprague-Dawley rats using these antibodies. Immunoblot analyses demonstrated protein expression of PPAR-α and -γ1, but not of -γ2, in the kidney nuclear extracts. Immunohistochemical analyses demonstrated that both PPAR-α and -γ1 proteins were widely expressed in the nuclei of mesangial and epithelial cells in glomeruli, proximal and distal tubules, the loop of Henle, medullary collecting ducts, and intima/media of renal vasculatures. PPAR-α and -γ1 proteins are thus widely expressed along the nephron segments, and may affect gene expression at these segments. Further studies will be needed to identify additional target genes for PPARs along the nephron segments. (Hypertens Res 2004; 27: 417–425)

Key Words: nuclear hormone receptor, nephron, antibody
PPAR-ISH using rabbit and human kidneys, and showed that localization of PPAR expression, and the reported function therefore, there exists a discrepancy between the reported expression of PPARs in the restricted nephron segments. Thus, while the results of these reports are generally incompatible, they all demonstrate a limited expression of PPARs in the restricted nephron segments.

### Methods

#### Preparation of Synthetic Peptides and Immunization

Synthetic peptides containing the following mouse PPAR amino acid residues were used in the immunizations: PPAR-α (1–18) (2), PPAR-γ1 (60–79) (3), and PPAR-γ2 (14–30) (3) (Fig. 1). These particular amino acids were chosen as epitopes because of their location in regions unique to each PPAR isoform and the high antigenicity index of their amino acid sequences, as determined by computer analysis. As indicated in Fig. 1, the antibody raised against PPAR-γ1 (60–79) may recognize both PPAR-γ1 and -γ2. Moreover, their sequences were identical between the mouse and rat. These peptides were synthesized, conjugated to Keyhole Limpet Hemocyanin via m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester, and used to immunize rabbits as previously described (14). The titer of each antiserum was screened using an enzyme-linked immunosorbent assay containing immunizing peptide as previously described (14).

#### Plasmids/in Vitro Translation

The mouse PPAR-α expression plasmid in pCMX (15) was provided by K. Umesono (Kyoto University, Kyoto, Japan). The mouse PPAR-δ (β) expression plasmid in pCMX (15) was provided by R.M. Evans (Salk Institute, La Jolla, USA). Mouse PPAR-γ1 and -γ2 expression plasmids in pSG5 (16) were provided by S.A. Kliewer (Glaxo Wellcome, Research Triangle Park, USA). These expression plasmids and the mouse RXR-α expression plasmid in pcDNA1/Amp (17) were in vitro translated using a TNT™ Kit (Promega, Madison, USA) either in the absence or presence of 35S-methionine according to the manufacturer’s instructions.

#### Preparation of Rat Kidney Nuclear Extracts/Western Immunoblot Analyses

Adult male Sprague-Dawley (SD) rats (200–250 g) were anesthetized with diethyl ether, and perfused with saline through cannulae inserted into the left ventricles. Their kidneys were removed and divided into the cortex and the medulla. Then their nuclear extracts were prepared as previously described (18). Twenty micrograms of rat kidney nuclear extracts or 15 µl of each in vitro translated PPAR isoform protein were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (9%...
acrylamide gel). After SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) (Immobilon P, Millipore, Bedford, USA), and immunoblot analyses were performed as previously described (14) using a 1:500 dilution of each anti-PPAR antibody as the primary antibody and 1:2000 diluted horseradish peroxidase-linked donkey anti-rabbit Ig (Amersham Biosciences, Buckinghamshire, UK) as the secondary antibody. In some experiments, each anti-PPAR antibody was incubated with the same volume of phosphate buffered saline (PBS) or the respective immunizing peptide in PBS (1 mg/ml) for 1 h at 4°C prior to immunoblotting (14). All procedures conformed to the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Tohoku University School of Medicine Animal Use and Care Committee.

**Immunoprecipitations**

Each in vitro translated 35S-labeled PPAR isoform protein (10 µl) was incubated with the respective anti-PPAR antibody for 2 h at 4°C, and was subsequently precipitated with 10% (v/v) Pansorbin (Calbiochem, San Diego, USA) as previously described (14). Precipitant was collected and electrophoresed with the same amount of input protein. In some experiments, each anti-PPAR antibody was incubated with the same volume of PBS or the respective immunizing peptide in PBS (1 mg/ml) for 1 h at 4°C prior to immunoprecipitation (14).

**Electrophoretic Mobility Shift Assay (EMSA)**

EMSA using 32P-labeled HMG-CoA synthase gene PPRE (HMGS-PPRE) as a probe was performed as previously described (19). Briefly, the probe was incubated either with 2 µl in vitro translated RXR-α and/or PPAR isoform, or 2 µg rat kidney nuclear extracts for 30 min at room temperature, and was subjected to electrophoresis on 4% polyacrylamide gels. For antibody supershift experiments, 1 µl anti-PPAR antibody or non-immune rabbit serum was added to the samples, which were then incubated for an additional 2 h at 4°C before being analyzed by electrophoresis (14, 19). For the competition experiment, the samples were co-incubated with a 100-fold excess of unlabeled oligonucleotides for HMGS-PPRE.

**RNA Preparation/Semi-Quantitative RT-PCR**

Total RNAs of the cortex or medulla were extracted from kidneys of adult male SD rats using an RNasey mini kit (Quiagen, Tokyo, Japan) according to the manufacturer’s instructions. The extracted RNAs (1 µg) were then subjected to RT-PCR using primers specific for either rat PPAR-γ (forward primer: 5’GGTTGATGCTTGAGGAATGC-3’; reverse primer: 5’ACTCTGGATACGGCTTGAC-3’ (19) or β-actin (forward primer: 5’GGGAAATCCTGTCGTC-3’; reverse primer: 5’GGTCTCTGCTGCGCT-3’ (20)) under the following conditions simultaneously: 30 min at 50°C and 2 min at 94°C for RT, followed by 28 cycles of 30 s at 94°C, 30 s at 60°C, and 1.5 min at 72°C for PCR. In the PCR condition, a linear correlation between the PCR cycles and the densitometry intensity of PCR products was confirmed.

**Immunohistochemistry**

Adult male SD rats were anesthetized with diethyl ether, and perfused with saline through cannulae inserted into the left ventricles. Their kidneys were removed and fixed for 24 h with 20% formaldehyde, and mounted onto paraffin blocks. Each 2 µm-thick section was deparaffined and rehydrated with PBS. The samples were then microwaved at 100°C for 10 min for the retrieval of antigenicity, and were incubated with 3% hydrogen peroxide in PBS for 10 min to block endogenous peroxidase activity. Each anti-PPAR antibody was used at a dilution of 1:1,500 (PPAR-α) or 1:3,000 (PPAR-γ). The samples were incubated with each antibody at 37°C for 60 min. Staining was performed with EnVision + (Dako, Carpinteria, USA) and a VIP Substrate Kit (Vector Laboratories, Burlingame, USA). The specificity of the immunohistochemical reaction was established by pre-absorption of each anti-PPAR antibody with 10 µmol/l of the respective immunization peptide overnight at 4°C before the immunohistochemical procedure (14).

**Results**

**Characterization of Anti-PPAR Antibodies**

To characterize the specificity of each antibody, we first performed Western immunoblot analyses (Fig. 2). Each PPAR isoform protein synthesized in vitro either in the presence or absence of 35S-methionine was electrophoresed simultaneously. Lanes 1–4 indicate 35S-labeled PPARs. In contrast to PPAR-γ1 in lane 3, PPAR-γ2 in lane 4 consists of two bands representing PPAR-γ2 (the upper band) and PPAR-γ1 (the lower band). The PPAR-γ1 was most likely translated from internal methionine at the 31st amino acid residue from PPAR-γ2 mRNA (Fig. 1). Lanes 5–12 indicate immunoblot analyses of unlabeled PPAR isoform proteins using anti-PPAR antibodies. As shown in lanes 5–8, anti-PPAR-α antibody specifically detected PPAR-α protein (lane 5). As shown in lanes 13–16, anti-PPAR-γ2 antibody specifically detected PPAR-γ2 protein (lane 16). Anti-PPAR-γ1 antibody (lanes 9–12) detected both PPAR-γ1 protein (lanes 11 and 12) and PPAR-γ2 protein (lane 12) as expected from its epitope location (Fig. 1).

We next performed immunoprecipitations using 35S-labeled PPARs (Fig. 3). Anti-PPAR-α antibody immunoprecipitated most of the input PPAR-α protein (lanes 1 and 2), and the immunoprecipitation was specifically inhibited by pre-incuba-
tion with its immunizing peptide (lane 3). Anti-PPAR-γ1 antibody also immunoprecipitated most of the input PPAR-γ1 protein (lanes 4 and 5), and the immunoprecipitation was specifically inhibited by pre-incubation with its immunizing peptide (lane 6). In case of anti-PPAR-γ2 antibody, it specifically immunoprecipitated most of the input PPAR-γ2 protein and did not affect PPAR-γ1 protein (lanes 7 and 8), and the immunoprecipitation was specifically inhibited by pre-incubation with its immunizing peptide (lane 9).

The specificity of each antibody was further characterized by antibody supershift experiments in EMSA using in vitro translated PPARs and RXR-α (Fig. 4). Unprogrammed reticulocyte lysate alone (lane 1), RXR-α alone (lane 2), or each PPAR isoform alone (lanes 3–5) did not show any binding to HMGS-PPRE DNA, while each PPAR isoform and RXR-α could form a heterodimer to bind to the DNA (lanes 6–19). PPAR-α/RXR-α heterodimer (lanes 6 and 13) was completely supershifted by anti-PPAR-α antibody (lane 7), and pre-incubation with its immunizing peptide completely inhibited the supershift (lane 8). Anti-PPAR-γ1 antibody did not affect the PPAR-α/RXR-α heterodimer (lane 14). PPAR-δ (β)/RXR-α heterodimer (lanes 9 and 15) was not affected by either anti-PPAR-α antibody (lane 10) or anti-PPAR-γ1 antibody (lane 16). PPAR-γ1/RXR-α heterodimer (lanes 11

**Fig. 2.** Western immunoblot analyses of in vitro translated PPAR isoform proteins using anti-PPAR antibodies. Lanes 1–4: electrophoresis of 35S-labeled PPAR isoform proteins. Lanes 5–8: immunoblot analyses of unlabeled PPAR isoform proteins using anti-PPAR-α antibody. Lanes 9–12: immunoblot analyses of unlabeled PPAR isoform proteins using anti-PPAR-γ1 antibody. Lanes 13–16: immunoblot analyses of unlabeled PPAR isoform proteins using anti-PPAR-γ2 antibody. α, PPAR-α protein; δ, PPAR-δ (β) protein; γ1, PPAR-γ1 protein; γ2, PPAR-γ2 protein.

**Fig. 3.** Immunoprecipitations of in vitro translated PPAR isoform proteins using anti-PPAR antibodies. Lanes 1, 4, and 7: electrophoresis of 35S-labeled PPARs (100% input). The same amounts of 35S-labeled PPAR isoform proteins were immunoprecipitated using anti-PPAR antibodies. Lanes 2 and 3: immunoprecipitation using anti-PPAR-α antibody. Lane 3: pre-incubation of anti-PPAR-α antibody with its immunizing peptide. Lanes 5 and 6: immunoprecipitation using anti-PPAR-γ1 antibody. Lane 6: pre-incubation of anti-PPAR-γ1 antibody with its immunizing peptide. Lanes 8 and 9: immunoprecipitation using anti-PPAR-γ2 antibody. Lane 9: pre-incubation of anti-PPAR-γ2 antibody with its immunizing peptide.
and 17) was completely supershifted by anti-PPAR-γ1 antibody (lane 18), and pre-incubation with its immunizing peptide completely inhibited the supershift (lane 19). Anti-PPAR-α antibody did not affect the PPAR-γ1/RXR-α heterodimer (lane 12).

These data indicate that each anti-PPAR antibody can specifically recognize the respective PPAR isoform protein, as demonstrated by Western immunoblot analyses, immunoprecipitations, and antibody supershift experiments in EMSA.

**PPAR Protein Expression in the Rat Kidney**

Western immunoblot analyses using rat kidney nuclear extracts were first performed using these antibodies (Fig. 5A). In the rat kidney cortex, expression of both PPAR-α protein (lane 1) and PPAR-γ1 protein (lane 5) was detected by the respective antibodies. Weak expression of PPAR-γ1 protein was also observed in the medulla (lane 6). Detection of these proteins was inhibited by pre-incubation of each antibody with the respective immunizing peptide (lanes 3, 7, and 8), suggesting that the detected proteins were specific. Expression of PPAR-γ2 protein was not observed either in the cortex (lane 9) or in the medulla (lane 10). These data suggest that PPAR-α and PPAR-γ1 proteins, but not PPAR-γ2 protein, are expressed in the rat kidney. We next performed antibody supershift experiments in EMSA (Fig. 5B). Interestingly, protein-DNA complexes formed with nuclear extracts of cortex were composed of two bands (lanes 2 and 11), while those formed with nuclear extracts of medulla were composed of one band (lanes 6 and 13). Although the reason for the difference in formation of protein-DNA complexes between the cortex and medulla remains uncertain, all these complexes were demonstrated to be specific, since their formation was completely inhibited by a competition experiment using excess amounts of unlabeled oligonucleotides for HMGS-PPRE (lanes 10–14). The protein-DNA complexes formed with nuclear extracts of cortex (lane 2) or medulla (lane 6) were partially supershifted by anti-PPAR-α antibody (lanes 4 and 8) or anti-PPAR-γ1 antibody (lanes 5 and 9), but not by non-immune rabbit serum (lanes 3 and 7). These data suggest that PPAR proteins are involved in the protein-DNA complexes. The discrepancy between the immunoblot analyses and antibody supershift experiments in EMSA with respect to PPAR-α expression in the medulla may have been due to the difference of detection threshold level between them. Since previous studies have demonstrated a predominant expression of PPAR-γ mRNA in the medulla (11–13), we next performed semi-quantitative RT-PCR to examine PPAR-γ1 expression at the mRNA level. In contrast to its protein expression level (Fig. 5A, lanes 5 and 6), PPAR-γ1 mRNA was more abundantly expressed in the medulla (Fig. 5C, lane 2) than in the cortex (Fig. 5C, lane 1), which was consistent with the previous studies (11–13).

In order to further localize the expression of each PPAR isoform protein in the nephron segments, immunohistochemical analyses were next performed. Figure 6A demonstrates the results of immunohistochemical studies using anti-PPAR-α antibody. In the cortex of the rat kidney (upper left and upper right), PPAR-α was expressed in the nuclei of glomeruli, proximal tubules (indicated by a large arrowhead), and distal tubules (indicated by a small arrowhead). In the glomeruli, PPAR-α was mainly expressed in mesangial
cells (indicated by an arrow) and some epithelial cells. The immunohistochemical staining was completely inhibited by peptide absorption (lower left), indicating that the staining was specific. In the medulla, PPAR-α was weakly expressed at the nuclei of the loop of Henle and medullary collecting ducts (lower right). PPAR-α expression was also observed in both the intima and media of renal vasculatures (inset at upper left). The discrepancy between the immunoblot analyses and immunohistochemical staining with respect to PPAR-α expression in the medulla may also have been due to the difference of detection threshold level between them. The results of the immunohistochemical studies using anti-PPAR-γ1 antibody are shown in Fig. 6B. In the cortex (upper left and upper right), PPAR-γ1 was also expressed in the nuclei of glomeruli, proximal tubules (indicated by a large arrowhead), and distal tubules (indicated by a small arrowhead). PPAR-γ1 was shown to be expressed in mesangial cells (indicated by an arrow) and epithelial cells in the glomeruli. The specificity of the staining was confirmed by peptide absorption (lower left). PPAR-γ1 was also expressed in the nuclei of the loop of Henle and medullary collecting ducts (lower right) in the medulla. PPAR-γ1 expression was also observed in both the intima and media of renal vasculatures (inset of upper left). These data suggest that both PPAR-α
and -γ1 proteins are widely expressed along the nephron segments.

**Discussion**

We have developed isoform-specific polyclonal antibodies against PPAR-α, -γ1, and -γ2 to study their protein expression. The specificity of these antibodies was demonstrated by their ability to recognize *in vitro* translated mouse PPAR-α, -γ1, and -γ2 proteins, respectively, as revealed by Western immunoblot analyses, immunoprecipitations, and supershift experiments in EMSA. Each antibody, therefore, can recog-
nize its respective PPAR isoform as a denatured protein as well as a native protein in solution or bound to DNA. The anti-PPAR antibodies are thus suggested to be useful in immunohistochemistry to identify their proteins.

Using these antibodies, we here demonstrated the expression of PPARs in the rat kidney at the protein level. Several studies have investigated the expression of PPARs in the kidney (11–13), although these expressions have mainly been demonstrated at the mRNA level. There exist several discrepancies between our study and previous reports. Regarding PPAR-α, we observed its expression widely along the nephron segments (glomeruli, proximal and distal tubules, the loop of Henle, medullary collecting ducts, and intima/media of renal vasculatures), whereas the previous studies reported that PPAR-α expression was mainly localized to the proximal tubules (11–13). Regarding PPAR-γ, we also observed that PPAR-γ1, but not PPAR-γ2, was expressed widely along the nephron segments (glomeruli, proximal and distal tubules, the loop of Henle, medullary collecting ducts, and intima/media of renal vasculatures). Since the expression of PPAR-γ in glomeruli has so far been reported only in rats undergoing 5/6 nephrectomy (10) and TZDs-treated rabbits (21), this is the first report describing PPAR-γ (γ1) expression in normal glomeruli. In particular, mesangial and epithelial cells in glomeruli were observed to express PPAR-γ1. In support of our data, PPAR-γ has been reported to be expressed in cultured mesangial cells (22–24). Our data thus demonstrated a broad expression of PPAR-γ1 protein in the kidney, predominantly in the cortex, which is discrepant with previous reports in which the PPAR-γ mRNA expression was mainly localized to the medulla (11–13). Since we also observed more abundant PPAR-γ1 mRNA expression in the medulla than in the cortex, the discrepancy between our data and previous reports may simply be due to the difference of PPAR-γ1 expression between the mRNA level and protein level. We therefore speculate that PPAR-γ1 mRNA in the cortex is more prone to be translated into protein than PPAR-γ1 mRNA in the medulla, or, alternatively, PPAR-γ1 protein in the medulla is more prone to be degraded than PPAR-γ1 protein in the cortex. Their precise mechanisms remain to be examined.

Recent studies have focused on the functions of PPARs in the kidney. Regarding PPAR-α, it is known to be involved in the control of fatty acid β-oxidation (4, 5). PPAR-α is also reported to regulate cytochrome P450 4A genes through their PPRE at proximal tubules in the kidney (4, 25), and may regulate sodium metabolism (4) and blood pressure (26). Moreover, Kamijo et al. recently reported that PPAR-α in the proximal tubules was involved in the maintenance of ATP homeostasis to degrade absorbed proteins, including albumin, as revealed by increased urinary albumin excretion in starved PPAR-α-null mice (6). Regarding PPAR-γ, its ligand TZDs have recently been reported to ameliorate albuminuria in diabetic rats (7–9), and to reduce proteinuria and glomerulosclerosis in 5/6 nephrectomized rats (10). Moreover, TZDs are known to cause fluid retention and edema as side effects (27). Our studies have demonstrated wide expression of PPAR-γ (γ1) along the nephron segments, including mesangial and epithelial cells in glomeruli, proximal and distal tubules, the loop of Henle, and medullary collecting ducts. Therefore, the above-described effects of TZDs may be mediated through PPAR-γ (γ1) activation. We have recently reported the inhibition of angiotensin II (AI1) type 1 receptor (AT1R) expression by TZDs through PPAR-γ activation in vascular smooth muscle cells (VSMCs) (19, 28). In the kidney, AT1R is also expressed in mesangial cells, proximal and distal tubules, and collecting ducts (29). Moreover, AT1R is reported to participate in cell proliferation and production of extracellular matrix in mesangial cells (30) and in reabsorption of water and sodium in renal tubules (31) through AT1R activation in the kidney. PPAR-γ activation may possibly inhibit AT1R expression in mesangial cells and tubules, and exert a beneficial effect against hypertension and nephrosclerosis. Moreover, we have recently demonstrated that PPAR-γ activation leads to the inhibition of both thromboxane (TX) receptor expression in VSMCs (17, 28) and TX synthase expression in macrophage (32). TX is known to increase tubuloglomerular feedback in the kidney, which may result in the retention of fluid and salt (33), and has been implicated in various renal diseases (34, 35). Inhibition of the TX system by PPAR-γ activation may protect against the progression of hypertension and renal damage.

Our studies have demonstrated that both PPAR-α and -γ1 proteins are more widely expressed along the nephron segments than previously reported. Although the activation of PPARs may possibly function against the progression of hypertension and nephrosclerosis in the kidney, further studies are needed to clarify the functional significance of PPARs in the kidney, including studies to identify their novel target genes along the nephron segments.

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